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CARACTÉRISATION BIOLOGIQUE ET MOLÉCULAIRE DE *BABESIA SPP.* INFECTANT LES PETITS RUMINANTS EN CHINE

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AbR	Antibody rate	mpi	Month post-infection
ADN = DNA	Acide désoxyribonucléique	MSA	merozoite surface antigen
ADNc = cDNA	Acide désoxyribonucléique complémentaire	MW	Molecular weight
ADNg = gDNA	Acide désoxyribonucléique génomique	NC	nitrocellulose
AMA	apical membrane antigen	NO	Nitric oxide
ARN = RNA	Acide ribonucléique	nPCR	Nested PCR
ARNm = mRNA	Acide ribonucléique messager	OD	Optical density
ARNr 18S	Acide ribonucléique ribosomal 18S	OIE	Organisation Mondiale de la Santé Animale
ATP	Adénosine Triphosphate	ORF	Open reading frame
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium	pb = bp	Paire de base
BdE	extract from *B. divergens* merozoites	PBMC	Peripheral blood mononuclear cells
BQ1DNA	*Babesia* sp. BQ1 (Lintan) DNA	PBS	Phosphate-buffered saline
BQ1E	extract from *Babesia* sp. BQ1 (Lintan) merozoites	PBST	PBS tween
BQHsp90	Hsp90 gene from *Babesia* sp. BQ1 (Lintan)	PCR	Polymerase chain reaction
BQMA	merozoite antigens of *Babesia* sp. BQ1 (Lintan)	PFU	Plaque forming unit
BQP35	protein of 35 kDa from *Babesia* sp. BQ1 (Lintan)	p.i.	Post infection
CAAS	Chinese Academy of Agricultural Science	pI	Point isoélectrique
cELISA	Competitive ELISA	PSAC	plasmodial surface anion channel
CFT	Complement fixation test	Q-TOF	Quadripole – Time of fly
CPM	Count per minute	RACE	rapid amplification of cDNA ends
dpi	Day post-infection	RAP	Rhoptry associated protein
ELISA	Enzyme-linked immunosorbent assay	RBC	Red blood cells
ENVN	Ecole Nationale Vétérinaire de nantes	rBQHsp90	Recombinant Hsp90 from *Babesia* sp. BQ1 (Lintan)
EST	expressed sequence tag	rBQP35	Recombinant protein of 35 kDa from *Babesia* sp. BQ1 (Lintan)
FBS	Fetal Bovine Serum	RLB	Reverse line blot
Hsp	Heat shock protein	rpm	Round per minute
IFAT	indirect fluorescence antibody test	RT	Room temperature
IFNγ	Gamma interferon	SBP	spherical body protein
IL10	Interleukin 10	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
IL12	Interleukin 12	SI	Stimulation index
iNOS	Inductible Nitric Oxide Synthase	SSU	small-subunit
IPTG	Isopropyl ß-D-1-thiogalactopyranoside	TBS	Tris buffered saline
iRBC	Infected red blood cells	TBST	TBS tween
ITPH	Interaction Tique-parasite-hôte	Th1	T helper 1
ITS	Internal transcribed spacer	Th2	T helper 2
IUP	intrinsically unstructured protein	TMB	3,3',5,5' tetramethylbenzidine
kDa	Kilo-dalton	TNFα	Tumor necrosis factor alpha
jpi	Jour post-infection	TRAP	thrombospondin-related anonymous protein
LAMP	Loop-mediated isothermal amplification	VMSA	variable merozoite surface antigens
LC-MS	Liquid chromatography-mass spectrometry	VVBD	Vector and vector Borne disease
LVRI	Lanzhou Veterinary Research Institute	WBgDNA	whole blood genomic DNA
MASP	Microaerophilus stationary phase culture	wpi	Week post-infection
ME	Microscopic examination		

INTRODUCTION – OBJECTIFS

Les parasites transmis par les tiques du genre *Babesia* (Phylum des *Apicomplexa*, Classe *Sporozoasida*, Ordre des *Piroplasmorina,* Famille des *Babesiidae*) sont les hémoparasites les plus communs après les trypanosomes chez les mammifères (Levine, 1985 ; Telford *et al*., 1993). Ces micro-organismes ont été découverts dans les érythrocytes de bovins par Victor Babes à la fin du 19ème siècle en Roumanie (Babes, 1888). Ils infectent uniquement les érythrocytes de vertébrés et différentes espèces de tiques (*Ixodidae*) au cours de leur cycle biologique. A ce jour, plus de 100 espèces de babésies sont décrites (Kuttler 1988b; Levine, 1988). Les espèces communément décrites chez les ruminants sont au nombre de 6 chez les bovins (*Babesia bovis*, *B. bigemina*, *B. divergens*, *B. major*, *B. ovata* and *B. orientalis*) et de 3 chez les ovins (*B. ovis*, *B. motasi* and *B. crassa*) (Levine, 1985 ; Kuttler, 1988b ; Friedhoff, 1988, 1997 ; Uilenberg 2006). La maladie provoquée par le développement des parasites dans les hématies des mammifères, appelée babésiose ou piroplasmose, est très répandue dans le Monde en Asie, Europe, Afrique, Australie et Amérique selon la distribution des vecteurs des différentes espèces de *Babesia*. Elle est particulièrement importante d'un point de vue économique dans les zones tropicales et subtropicales. La maladie est cliniquement caractérisée par de la fièvre, une anémie hémolytique et une hémoglobinurie et son évolution peut être mortelle (Kuttler 1988b). Plus de 1,5 milliards de bovins et de buffles et 1,9 milliards de moutons et de chèvres dans le monde (FAOSTAT, 2006) sont exposés à la babésiose. Toutefois ceci ne reflète pas le risque réel de maladie dans la mesure où il est difficile d'évaluer concrètement l'impact de la babésiose avec de simples outils d'évaluation de l'exposition et cela mériterait une évaluation plus systématique (McCosker, 1981).

Le diagnostic de laboratoire de la babésiose aiguë est aisé par observation au microscope optique d'étalements de sang ou de gouttes épaisses colorés au May-Grünwald Giemsa. En revanche, lors d'infection subclinique ou latente et pour la réalisation d'enquêtes épidémiologiques, ce type d'examen est difficilement utilisable en raison de sa faible sensibilité, du temps et de la technicité nécessaire à la lecture (Böse *et al*., 1995). Pour ces raisons, différents outils moléculaires ont été développés pour détecter l'ADN du parasite chez l'hôte vertébré ou chez la tique vectrice, notamment des sondes ADN, des outils PCR ou des techniques de « reverse line blot hybridization » (RLB) (Petchpoo *et al*., 1992 ; Schnittger *et al*., 2004 ; Aktas *et al*., 2005). Différentes techniques sérologiques sont également décrites : fixation du complément (CFT), immunofluorescence indirecte (IFAT) et ELISA (Tenter et Friedhoff, 1986 ; Papadopoulos, 1996 ; Ferrer, 1998 ; Molloy *et al*., 1998 ; Goff *et al*., 2003 ; Ogunremi *et al*., 2008;), bien que ce soit l'IFAT la plus utilisée actuellement.

En Chine, la babésiose est une hémoprotozoose commune chez les bovins, les buffles, les yaks, les "Pianniu" (un hybride de bovin et de yak), les moutons, les chèvres ainsi que chez les chevaux, les ânes et les chiens (Kong, 1981 ; Yin *et al*., 1997b; Liu ZL *et al*., 1997). En 1943, la babésiose équine a été identifiée pour la première fois dans la province du Heilongjiang et 5 ans plus tard, la babésiose bovine a été mise en évidence en Chine (Wang,

1993). Actuellement la babésiose bovine en Chine est attribuée à 6 espèces de *Babesia* : *B. bovis, B. bigemina, B. ovata, B. major, B. orientalis* et *Babesia* U sp. Kashi. *B. bovis*, *B. bigemina* et *B. orientalis* sont principalement distribués dans le centre et le sud-est de la Chine en lien avec la distribution de leurs vecteurs, *Rhipicephalus (Boophilus) microplus* ou *Rhipicephalus haemaphysaloides*. *B. ovata*, *B. major* et *Babesia* U sp. Kashi sont présents dans les régions du nord et du nord-ouest en lien avec la distribution des tiques vectrices *Haemaphysalis longicornis*, *Haemaphysalis punctata* and *Hyalomma* spp.. *B. caballi*, transmis par *Dermacentor nuttalli* et *Dermacentor silvarum,* est la seule espèce responsable de babésiose équine en Chine dans 9 provinces (Kong, 1981 ; Yin *et al*., 1997b ; Jiang, 2000 ; Liu et Zhao, 2001 ; Luo, 2004).

Les espèces de *Babesia* infectant les petits ruminants en Chine sont mal connues. Chen (1982) et Zhao *et al*. (1986) ont rapporté les premiers cas de babésiose ovine dans les provinces du Sichuan et du Heilongjiang respectivement et ils ont identifié le parasite comme *B. ovis* sur la base de caractères morphologiques du protozoaire observé sur des étalements sanguins et sur les manifestations cliniques de la maladie. En 1996, une explosion inattendue de babésiose ovine est apparue sur des moutons à queue courte (« Small-Tailed ») importés de la province du Shandong dans le comté de Ningxian à l'est de la province du Gansu ; le parasite, initialement considéré comme *B. ovis*, *B. motasi* ou une grande babésie inconnue, a été isolé du sang collecté sur des moutons malades au laboratoire de l'Institut vétérinaire de Lanzhou (Gansu, Chine) (Yin *et al*., 1997a ; Lian *et al*, 1997 ; Liu GY *et al*., 1997, 1999 ; Bai *et al*., 2002). Après ce premier isolement, différents isolats de grandes babésies ont été isolés soit à partir de sang de moutons soit à partir de tiques collectées sur le terrain et effectuant leur repas de sang sur des moutons splénectomisés. (Guan *et al*., 2001, 2002 ; Liu AH *et al*., 2007). Sur la base des séquences des gènes de l'ARNr 18S et ITS, ces isolats ont pu récemment être classés dans 2 groupes, d'une part un ensemble d'isolats « B. motasi-like » incluant *Babesia sp.* BQ1 (Lintan), *Babesia sp.* BQ1 (Ningxian), *Babesia sp.* Liaoning, *Babesia sp.* Tianzhu, *Babesia sp*. Madang et *Babesia sp.* Hebei, et d'autre part *Babesia sp.* Xinjiang (Liu AH *et al*., 2007 ; Niu *et al*., 2009a).

Peu de données biologiques pour ces babésies sont disponibles. Les seules existantes concernent *Babesia* sp. BQ1 (Ningxian) (isolé de sangs de moutons malades, caractères morphologiques similaires à *B. motasi*, parasite très virulent pour les moutons et pour les chèvres, la tique vectrice est *Haemaphysalis longicornis)*, *Babesia* sp. BQ1 (Lintan) (isolé de moutons parasités expérimentalement par *H. qinghaiensis* collecté dans des pâtures, parasite de faible virulence pour les moutons et les chèvres, tiques vectrices non identifiées expérimentalement) et *Babesia* sp. Xinjiang (parasite de faible virulence pour le Mouton, isolé de moutons infestés expérimentalement par des tiques *Rhipicephalus sanguineus* et *Hy. a. anatolicum* , caractéristiques morphologiques très différentes de *B. ovis*, *B. motasi* et *B. crassa)* (Bai *et al*., 2002 ; Guan *et al*., 2001, 2002). Pour les autres isolats, nous savons juste

qu'ils ont été isolés à partir de sang de moutons pâturant dans des zones où les tiques dominantes sont du genre *Haemaphysalis* (Liu AH *et al*., 2007). Pour les babésioses ovines chinoises, il n'existe pas d'outils moléculaires ou de méthodes sérologiques pour détecter les infections par *Babesia* spp et les seules méthodes disponibles sont l'examen microscopique et l'inoculation à un animal-hôte.

Objectifs de l'étude

Pour mieux comprendre la babésiose ovine en Chine, nous avons plus particulièrement étudié les 2 isolats *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang. Les travaux ont visé à mieux caractériser la taxonomie des isolats de *Babesia* ovines chinoises et à développer de nouveaux outils de diagnostic. Pour cela, nous avons étudié les caractères biologiques et moléculaires de ces babésies et leur interaction avec leur hôte vertébré.

Ce travail s'est divisé en 5 étapes :

A. Identification de la tique vectrice de *Babesia* sp. BQ1 (Lintan) et *Babesia* sp. Xinjiang ; les essais de transmission expérimentale ont été réalisés avec *Haemaphysalis* spp. (infecté par *Babesia* sp. BQ1 (Lintan)) et *Hyalomma anatolicum anatolicum* (infecté par *Babesia* sp. Xinjiang).
B. Développement d'un système de culture *in vitro* pour *Babesia* sp. BQ1 (Lintan) et évaluation de certains paramètres de croissance. Cette culture a été une source de parasites pour cette étude mais est aussi disponible pour de futurs travaux.
C. Évaluation chez le Mouton de la virulence de *Babesia* sp. BQ1 (Lintan) et de la réponse immunitaire cellulaire et humorale dirigée contre *Babesia* sp. BQ1 (Lintan). Cette étude a été réalisée en comparant l'infection de moutons par *Babesia* sp. BQ1 (Lintan) ou par *B. divergens.*
D. Mise au point d'une méthode de détection moléculaire (« loop-mediated isothermal amplification », LAMP) et d'outils de diagnostic sérologique (ELISA) pour la détection des infections par *Babesia* sp. BQ1 (Lintan) ou *Babesia* sp. Xinjiang.
E. Recherche de gènes-cibles potentiels pour de nouveaux outils de diagnostic sérologique ou moléculaire par immuno-screening d'une banque de cDNA de *Babesia* sp. BQ1 (Lintan) et par identification par western blot et spectrométrie de masse d'antigènes de mérozoïtes issus de culture *in vitro*.

PARTIE BIBLIOGRAPHIQUE

A. *Babesia* spp. et babésioses des ruminants domestiques

Les babésioses ovines et bovines sont des hémoprotozooses transmises par les tiques, provoquées par le développement de protozoaires du genre *Babesia* dans les érythrocytes.

1. Classification de *Babesia* spp.

Le genre *Babesia* est classé dans le phylum des *Apicomplexa*, la classe *Sporozoasida*, l'ordre des *Piroplasmorina* et la famille des *Babesiidae* (Levine, 1971, 1988). Dans l'ordre des *Piroplasmorina*, les membres des deux familles des *Babesiidae* et des *Theileriidae* sont parasites des érythrocytes, mais les *Theileriidae* ont une première phase de schizogonie dans les lymphocytes alors que les *Babesiidae* se multiplient exclusivement dans les hématies. Ainsi, *B. equi* a été reclassé parmi les *Theileria* sur la base de la mise en évidence de schizontes lymphocytaires.

Actuellement, plus de 100 espèces de *Babesia* ont été décrites (Kuttler 1988b ; Levine, 1988). Elles ont été historiquement classées en « grandes» babésies (plus de 3 µm de longueur, par exemple *B. bigemina*, *B. major*, *B. ovata*, *B. motasi*) et « petites» babésies (moins de 3 µm de longueur, par exemple., *B. bovis*, *B. ovis*, *B. divergens*) sur la base de leur taille et de leur hôte vertébré (Purnell, 1981 ; Levine, 1988 ; Kuttler 1988b). Toutefois, ce type de classification a été revu à la lumière des données issues d'études génétiques. Les avancées en biologie moléculaire et l'utilisation de séquences ADN permettent une nouvelle approche pour la classification phylogénétique du genre *Babesia*. Actuellement, au moins 32 espèces ou souches de babésies reconnues et 85 inconnues sont accessibles sur le site « NCBI-Taxonomy » et la plupart ont des gènes dont les séquences ont été déposées dans « GenBank » (http://www.ncbi.nlm.nih.gov/). La séquence la plus communément utilisée pour ces études phylogénétiques est celle du gène de la petite sous-unité ribosomale (ARNr 18S) (Allsopp *et al*., 1994, 2006 ; Ahmed *et al*., 2006 ; Altay *et al*., 2007 ; Cancrini *et al*., 2008) car elle contient des régions très conservées, des régions variables et des régions hypervariables et elle n'est pas traduite en protéines (Ludwig et Schleifer, 1994 ; Woese, 1987).

Les analyses phylogénétiques basées sur l'ARNr 18S ont abouti à une séparation du genre *Babesia* en au moins 4 groupes (Kjemtrup *et al*., 2000 ; Criado-Fornelia *et al*., 2003 ; Hunfeld *et al*., 2008) :

(1) un groupe de *Babesia* spp. sensu stricto composé principalement d'espèces de *Babesia* d'ongulés : *B. caballi*, *B. bigemina*, *B. ovis*, *B. bovis*, et d'autres espèces de *Babesia* du Bovin et du Mouton,

(2) un deuxième groupe de *Babesia* spp. sensu stricto incluant *B. canis* et *B. gibsoni* (asiatique) des canidés ainsi que *B. divergens* et *B. odocoilei*,

(3) un groupe d'organismes « *Theileria*-like » de l'ouest des Etats-Unis,

(4) un groupe « *B. microti*-like » contenant *B. rodhaini*, *B. felis*, *B. leo*, et *B. microti.*

Ainsi les espèces de *Babesia* infectant les ruminants sont probablement des « vraies » babésies aussi bien sur la base de la taxonomie usuelle que de la classification phylogénétique. Toutefois, *B. bovis* est difficile à grouper avec les autres clades dans les arbres phylogénétiques basés sur la séquence de l'ARNr 18S et est souvent classé à part comme une espèce monophylétique. Ainsi, pour la classification du genre *Babesia*, il apparaît nécessaire de tenir compte des données morphologiques et biologiques, telles que la spécificité d'hôte vertébré, la tique vectrice, la pathogénicité ou les caractères immunitaires en parallèle des études moléculaires et particulièrement pour les isolats inconnus du genre *Babesia.*

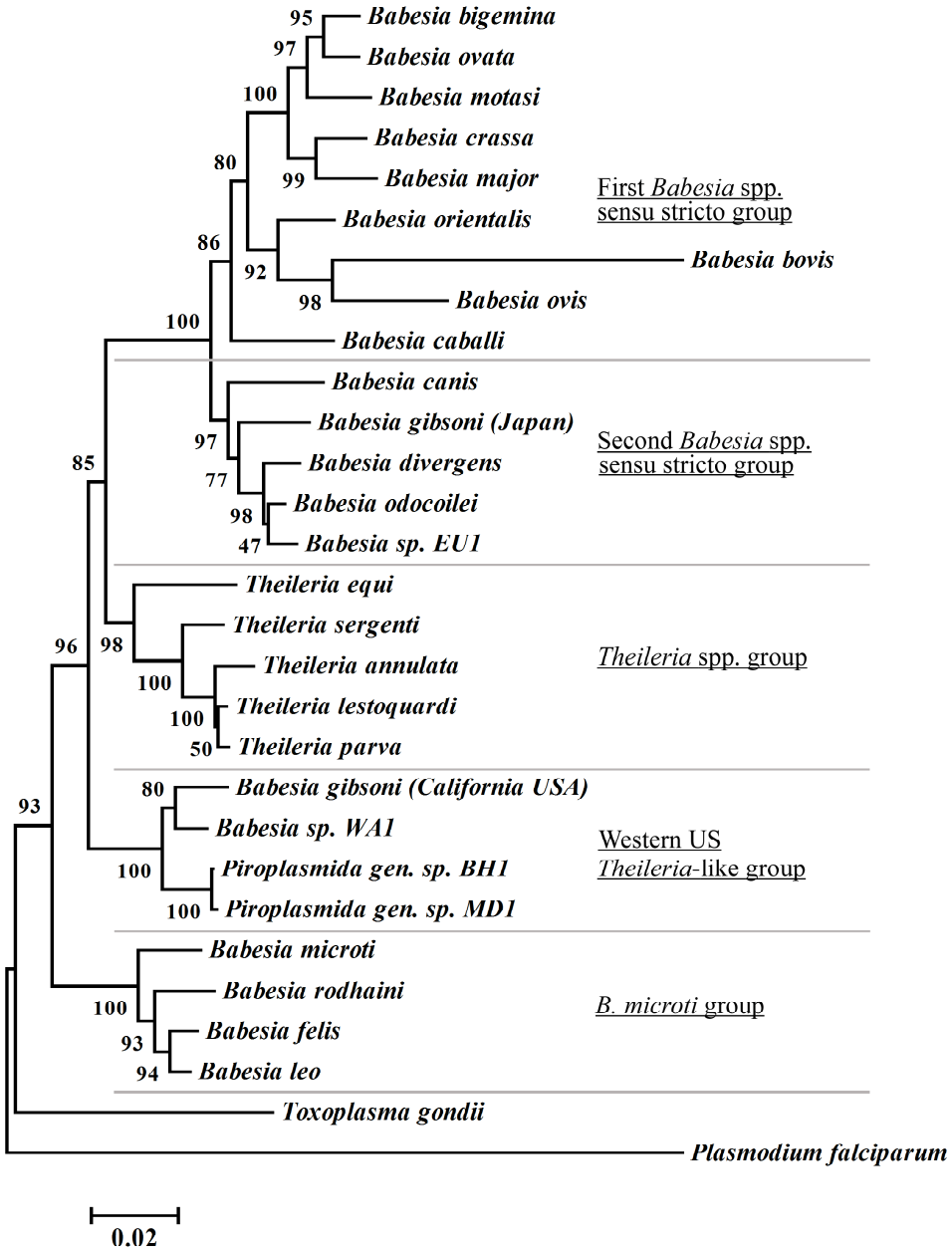

Figure 1. Classification phylogénique de *Babesia* spp. basée sur les séquences du gène ARNr 18S. L'histoire évolutive a été déterminée par la méthode du neighbour-joining. L'arbre optimal illustré a une somme des longueurs de branches de 0,77. Les valeurs de bootstrap mentionnées sont basées sur 1000 réplications. Le modèle de substitution nucléotidique utilisé correspond à la méthode du "maximum de vraisemblance composite" (prise en compte du biais entre transversions et transitions et de l'inégalité des fréquences des différents nucléotides; modèle de Tamura-Nei, équivalent au modèle HKY85). L'échelle utilisée pour représenter les longueurs de branche est exprimée dans la même unité que celle utilisée pour calculer les distances entre séquences. Tous les sites de l'alignement contenant des insertions - délétions ou des données manquantes ont été éliminées de l'analyse (option "complete deletion"). Le jeu de données final contient 1372 positions. Les analyses phylogénétiques ont été réalisées avec le logiciel MEGA4

2. Cycle biologique de *Babesia* spp.

Le cycle biologique des babésies est composé d'une phase de reproduction sexuée chez la tique vectrice et d'une phase de multiplication asexuée dans les érythrocytes du vertébré. Ce cycle a été décrit par Mehlhorn et Schein (1984), Young et Morzaria (1986), Friedhoff (1988) et Bock *et al*. (2004) pour les principales espèces d'importance économique telles que *B. bovis*, *B. bigemina*, *B. divergens* et *B. ovis*, mais la compréhension du cycle biologique des différentes espèces de *Babesia* reste très partielle malgré les études réalisées, notamment en raison de nombreuses inconnues sur le cycle sexué (Fig. 2). Les babésies ne parasitent que les érythrocytes de leur hôte vertébré (Friedhoff, 1988). Les sporozoïtes infectants sont inoculés dans le vertébré lors du repas sanguin de la tique ; ils pénètrent directement dans les érythrocytes grâce à leur complexe apical et commencent une phase de multiplication asexuée appelée mérogonie. Après une phase de croissance, le trophozoïte ou forme ronde se divise en deux mérozoïtes piriformes (cet aspect piriforme étant à l'origine de l'appelation « piroplasme ») par un processus de division binaire ; cette forme transitoire constituée par l'association des deux mérozoïtes en fin de division est communément appelée forme géminée. Plusieurs cycles de multiplication asexuée se succèdent dans les érythrocytes de l'hôte vertébré. La tique vectrice peut absorber des hématies parasitées lors de son repas de sang sur un hôte infecté. Une partie des parasites se développe alors dans le tube digestif de la tique en formes sexuées appelées corps rayonnants (« Strahlenkörper » ou « ray-bodies »). Après une phase multinuclée, les corps rayonnants uninuclés formés sont considérés comme les gamètes. Après fusion de deux gamètes (syngamie) se forme un zygote. Le zygote infecte les cellules épithéliales du tube digestif de la tique puis se divise en kinètes. Ceux-ci se disséminent par l'hémolymphe de la tique et infectent une grande variété de cellules et de tissus, y compris les oocytes de la tique où des cycles successifs de schizogonie se déroulent. Ainsi, une transmission transovarienne permet l'infection des œufs de la tique et le passage du parasite à la génération suivante de tiques. Les cycles de schizogonie peuvent aussi avoir lieu dans divers autres tissus, tels que les glandes salivaires, les corps gras ou les néphrocytes, assurant une transmission transstadiale du parasite de la larve à la nymphe et de la nymphe à l'adulte. Les kinètes pénétrant les glandes salivaires se transforment en stades multinuclés puis se différencient en nombreux sporozoïtes uninuclés haploïdes achevant la phase de sporogonie. Le développement des sporozoïtes ne débute que lorsque la tique infectée commence son repas sanguin, le complet développement en sporozoïtes infectants nécessitant quelques jours (dépendant des espèces de *Babesia*).

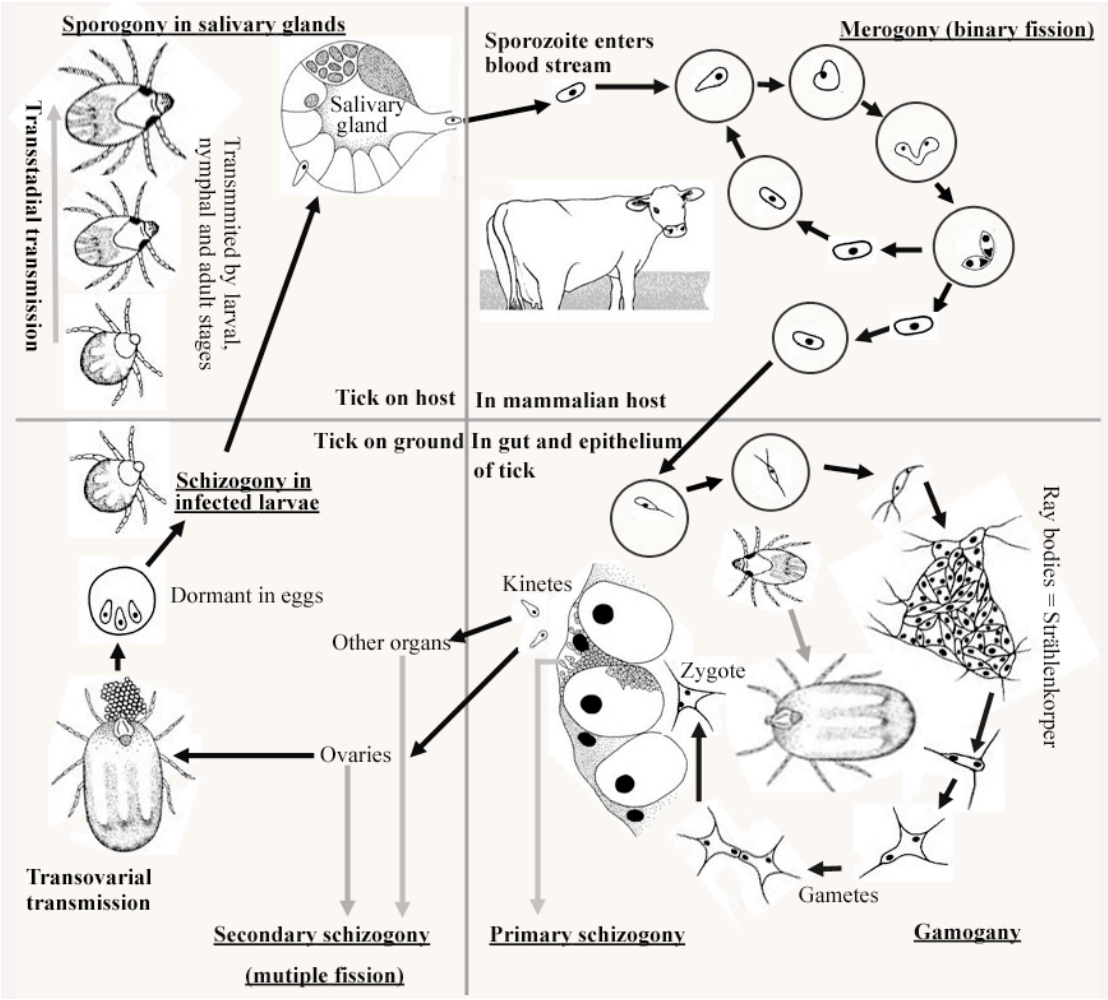

Figure 2. Cycle biologique de *Babesia* spp. infectant les ruminants (modifié d'après les descriptions de Young et Morzaria (1986) et de Bock *et al*. (2004)).

3. Principales espèces de *Babesia* infectant les ruminants

A la fin du 19ème siècle, Babes a découvert des micro-organismes dans les érythrocytes de bovins en Roumanie et les a associé à l'hémoglobinurie des bovins (« red water fever ») (Babes, 1888). Il a ensuite identifié des organismes similaires dans les globules rouges de moutons. Smith et Kilborne (1893) ont aussi trouvé ces micro-organismes aux USA et ont montré qu'ils étaient transmis par des tiques. La même année, Starcovici a donné à ces parasites les noms de *Babesia bovis*, *Babesia ovis* et *Babesia bigemina*, respectivement (Starcovici, 1893). Depuis ces organismes ont été isolés de ruminants dans différentes régions du monde et de nombreuses espèces ont été identifiées. Six espèces bovines et trois espèces ovines représentent les principales espèces de *Babesia* infectant les ruminants domestiques (table 1 et figure 3). Les principaux caractères de ces espèces sont listés ci-dessous.

Babesia bovis (Babes, 1888) est une petite babésie (« petite forme ») très virulente mesurant

jusqu'à 2,0 µm x 1,5 µm et distribuée mondialement. Cette espèce a été découverte en 1888 par Babes sur des bovins en Roumanie et a été nommée *Haematococcus bovis*. En 1893, Starcovici lui a donné le nom de *B. bovis*. Lignieres (1903) a identifié deux formes de « Tristeza » (babésiose) sur des bovins en Argentine, l'une provoquée par une grande babésie et l'autre par une petite forme. Il a donné à cette petite babésie le nom de *Piroplasma argentina*, renommée ultérieurement *B. argentina*. Les noms *B. argentina* et *B. bovis* sont actuellement considérés comme synonymes. Lors d'infection naturelle, la parasitémie est toujours inférieure à 1%. *Boophilus* (*Rhipicephalus*) *microplus* et *B. annulatus* sont deux vecteurs bien identifiés de *B. bovis*.

B. bigemina (Smith et Killorne, 1893) est une grande babésie mesurant 4,5 µm x 2 µm, qui a la même distribution que *B. bovis*. Smith *et al.* (1889) ont identifié un hémoprotozoaire chez des bovins lors d'étude sur la fièvre du Texas (Amérique du Nord), celui-ci étant transmis par *B. annulatus*. Ce parasite a été nommé *Pyrosoma bigeminum* (puis renommé *B. bigemina)* par Stiles (Kuttler, 1988b). Les animaux infectés naturellement présentent une parasitémie de 2 à 5 %. *B. decoloratus* est un vecteur de cette babésie en plus de *B.* (*R.*) *microplus* et *B. annulatus*.

B. divergens (M'Fadyean et Stockman, 1911) est une petite babésie mesurant 1,5-2,0 µm x 0,4 µm. Il a été découvert par M'Fadyear et Stockman en Angleterre en 1911 et ils l'ont nommé *Piroplasma divergens* (renommé ensuite *B. divergens*). Son aspect morphologique le plus typique est la forme géminée à deux mérozoïtes, ceux-ci formant un angle très obtus et étant localisés contre la paroi de l'hématie. *B. divergens* a pour hôte naturel le Bovin, mais il peut aussi parasiter l'Homme naturellement. De nombreux hôtes expérimentaux sont également décrits (gerbille de Mongolie, Mouton, Rat…) (Chauvin *et al*., 2009). *Ixodes ricinus* est la tique vectrice (Kuttler, 1988b).

B. major (Sergent *et al*, 1926) est une grande forme mesurant 2,6-3,7 µm x 1,5 µm distribué en Europe, Afrique du nord et Asie. En 1911, M'Fadyear et Stockman ont observé une grande forme de piroplasme sur des bovins en Angleterre (probablement *B. major*) mais ils ont considéré qu'il s'agissait de *B. bigemina*. Sergent (1926), observant une espèce de *Babesia* morphologiquement similaire à *B. bigemina* en Algérie, l'a nommé *B. major.* Zwart *et al*. (1968) ont montré qu'il s'agissait d'une espèce valide sur des critères morphologiques et sérologiques. *Haemaphysalis punctata* est le seul vecteur identifié de *B. major* (Brocklesby et Irvin ,1969 ; Joyner *et al*., 1972 ; Brocklesby et Sellwood, 1973 ; Kuttler, 1988b).

B. ovata (Minami et Ishihara, 1980) est une grande babésie : il mesure 3,2 µm x 1,7 µm et est localisé au centre des érythrocytes. Minami et Ishihara ont observé en 1980 chez des bovins japonais une grande babésie dont les caractères morphologiques, sérologiques et pathogènes étaient différents de ceux de *B. bovis*, *B. bigemina* et *B. divergens* mais dont les caractères morphologiques étaient similaires à *B. major.* Elle a été nommée *B. ovata* car sa forme classique dans les érythrocytes est ovale. Comme la tique vectrice, *Haemaphysalis*

longicornis, cette babésie est distribuée dans l'est de l'Asie (Minami *et al*., 1979 ; Minami et Ishihara, 1980; Uilenberg, 2006).

B. orientalis (Liu ZL *et al*., 1997), infectant uniquement le Buffle, a été observé en 1986 par Liu *et al*. (1986) sur des buffles dans le sud et le centre de la Chine. Il mesure 1,2-1,5 µm x 2,0-2,6 µm et le seul vecteur connu est *Rhipicephalus haemaphysaloides*. Sur des critères de spécificité d'hôtes (vertébré et vecteur) et des données moléculaires, *B. orientalis* est considéré comme une espèce valide (Liu *et al*., 1986, 1987, 1995, 1997 ; Liu *et al*., 2005).

B. ovis (Babes, 1888 ; Starcovici, 1893) est une petite babésie mesurant 1,0-2,0 µm x 0,5 µm considérée comme très pathogène pour le Mouton. Le taux de mortalité chez le Mouton atteint 30 à 50 % après infection naturelle ou expérimentale. En revanche, l'atteinte de la Chèvre est rare. Le vecteur communément reconnu est *Rhipicephalus bursa*, mais *Rhipicephalis turanicus* et certaines espèces de *Hyalomma* pourraient transmettre le parasite. La distribution de *B. ovis* est l'Asie, l'Europe et l'Afrique (Friedhoff, 1997 ; Uilenberg, 2006).

B. motasi (Wenyon, 1926) est une grande babésie mesurant 2,5-4,0 µm x 2,0 µm, isolé de diverses régions d'Asie, d'Europe et d'Afrique. Les études récentes montrent que cette espèce consiste en fait à plusieurs souches non identifiées dans les différentes zones géographiques ; en particulier, certains isolats sont très pathogènes alors que d'autres sont non pathogènes pour les animaux en bonne santé. *Haemaphysalis punctata* est communément considérée comme la tique vectrice de *B. motasi*. Mais dans certaines régions, il a été démontré que *Rhipicephalus bursa* pouvait également transmettre *B. motasi* (Friedhoff, 1997 ; Uilenberg, 2006).

B. crassa **(**Hashemi-Fesharki et Uilenberg, 1981**)** isolé du Mouton en Iran est une grande babésie mesurant 2,5 ± 0,43 µm de longueur. La forme la plus classique décrite dans les érythrocytes est une forme à 4 mérozoïtes. Ce parasite est peu pathogène pour le Mouton et la Chèvre. La tique vectrice n'est pas identifiée. (Friedhoff, 1997 ; Uilenberg, 2006).

Table 1. Espèces de *Babesia* des ruminants domestiques (Ristic, 1988 ; Bock et al., 2004 ; Uilenberg, 2006)

Espèces	Hôte domestique	Caractères morphologiques (longueur/largeur µm)	Virulence	Principales tiques vectrices	Distribution connue
B. bovis	Bovin, Buffle	Petite 2,0 x 1,5	Forte	*Boophilus(Rhipicephalus) microplus* *Boophilus annulatus* *Boophilus geigyi*	Afrique, Amérique centrale et du sud, Asie, Australie, Europe du sud ; (moins commune que *B. bigemina* en Afrique)
B. bigemina	Bovin, Buffle	Grande 4,5 x 2	Virulente	*Boophilus(Rhipicephalus) microplus* *Boophilus decoloratus* *Boophilus annulatus* *Boophilus geigyi* *Rhipicephalus evertsi*	Afrique, Amérique centrale et du sud, Asie, Australie, Europe du sud
B. divergens	Bovin	Petite 1,5-2,0 x 0,4	Modérée	*Ixodes ricinus* *Ixodes persulcatus*	Europe
B. major	Bovin	Grande 2,6-3,7x1,5	Faible	*Haemaphysalis punctata*	Europe, Asie, Afrique du nord
B. ovata	Bovin	Grande 3,2x1,7	Faible	*Haemaphysalis longicornis*	Est de l'Asie
B. orientalis	Buffle	Petite 2,2x1,3	Virulente	*Rhipicephalus haemaphysaloides*	Est de l'Asie
B. ovis	Mouton, Chèvre	Petite 1-2x0,5	Forte	*Rhipicephalus bursa*	Afrique du nord, Est de l'Asie, Sud-est de l'Europe
B. motasi	Mouton, Chèvre	Grande 2,5-4,0x2,0	Modérée	*Haemaphysalis punctata* *Rhipicephalus bursa*?	Afrique, Asie, Europe
B. crassa	Mouton, Chèvre	Grande 2,5±0,43	Faible	Inconnu	Asie centrale

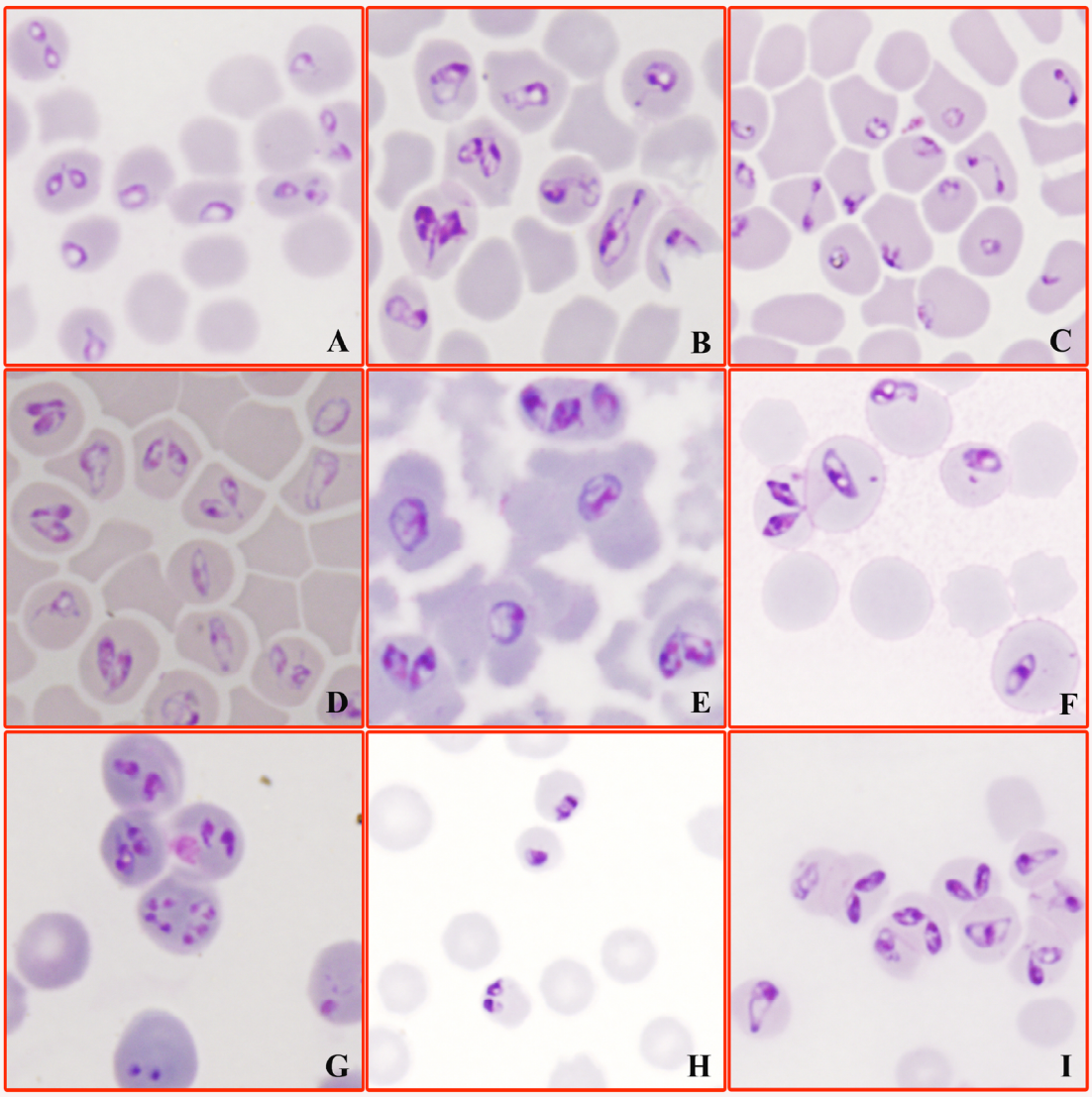

Figure 3. *Babesia* spp. intra-érythrocytaires chez les ruminants domestiques. A-I: *B. bovis* (Chine), *B. bigemina* (Chine). *B. divergens* (Europe), *B. ovata* (Chine), *B. major* (Chine), *B. major* (Europe), *B. orientalis* (Chine), *B. ovis* (Europe) et *B. motasi* (Europe). Clichés fournis par le Professeur Qi BAI du Laboratoire "Vector and Vector-borne disease" (VVBD, LVRI, Chine). Les lames colorées des espèces européennes ont été offertes par la Faculté de médecine vétérinaire de l'Université d'Utrecht (Pays-Bas), celles de *B. orientalis* ont été fournies par la faculté de médecine vétérinaire de l'Université d'agriculture de Huazhong (Chine), les autres proviennent du LVRI.

4. La babésiose

a. La maladie

La maladie provoquée par l'infection par les babésies est due à une combinaison d'effets directs liés à la destruction des hématies par le parasite et d'effets indirects médiés par la réponse immunitaire (Wright et Goodger, 1988 ; Homer *et al*., 2000). Les principaux signes cliniques sont une fièvre, une anémie, un ictère et une hémoglobinurie, notamment lors d'infection par les espèces de *Babesia* les plus virulentes. De plus, lors de la phase aiguë de l'infection par *B. bovis*, les animaux montrent une forte parasitémie dans les capillaires du cerveau, du muscle et du rein (Wright et Goodger, 1988 ; Habela *et al*., 1991 ; Yeruham *et al*., 1998;). Le plus souvent, les petites babésies sont plus virulentes que les grandes formes, par exemple *B. bovis*, *B. ovis* et *B. gibsoni,* bien qu'il puisse exister des variations selon les zones géographiques (*B. bigemina* d'Afrique du sud est très virulente alors que les autres isolats sont modérément virulents) (Wright et Goodger, 1988 ; Homer *et al*., 2000). La destruction des globules rouges apparaît généralement quand les mérozoïtes sont libérés après la mérogonie. Toutefois, la destruction des globules rouges par les petites babésies n'est qu'une composante mineure dans l'anémie induite, celle-ci n'étant pas nettement corrélée à la parasitémie. Une anémie hémolytique médiée par l'immunité est une des manifestations typiques de la babésiose. Le mécanisme communément implique des antigènes parasitaires solubles liés à la membrane de l'hématie, induisant une hémolyse par les anticorps ou le complément (De Vos *et al*., 1987 ; Homer *et al*., 2000;). La thrombocytopénie est une autre lésion de babésiose (Birkenheuer *et al*., 1999 ; Macintire *et al*., 2002). L'existence d'anticorps anti-plaquettes a été montrée par cytométrie en flux (Wilkerson *et al.,* 2001). Par ailleurs, les érythrocytes infectés par *Babesia* spp., notamment *B. bovis*, sont souvent séquestrées dans les capillaires du cerveau ou des poumons, induisant ainsi de sévères manifestations de babésiose cérébrale ou de détresse respiratoire parfois mortelles alors que la parasitémie périphérique est faible.

Les babésies sont bien adaptées pour survivre chez leur hôte vertébré et le plus souvent elles persistent longtemps à des parasitémies non détectables par les méthodes usuelles de recherche sur étalement de sang. Parallèlement, les animaux sont capables de développer une réponse immunitaire après infection et guérison ou après immunisation. La réponse immunitaire protectrice peut ainsi limiter la parasitémie, la morbidité et la mortalité quand les animaux sont ré-exposés au parasite (De Vos *et al*., 1987 ; Ristic, 1988 ; Homer *et al*., 2000). La réponse immunitaire de l'hôte à l'infection par *Babesia* sp. joue aussi un rôle important dans la pathogénie de la maladie (De Vos *et al*., 1987 ; Hemmer *et al*., 2000a, b). Celle-ci est complexe avec l'implication de multiples mécanismes si bien que les manifestations cliniques induites par l'infection dépendent beaucoup des statuts génétiques, physiologiques et immunitaires de l'animal. De plus, l'âge est un facteur particulièrement important pour les infections par *Babesia* sp chez les bovins puis les jeunes animaux sont plus résistants au

parasite que les adultes (Ristic 1988 ; Wright et Goodger, 1988).

b. Diagnostic

Les babésies peuvent être détectées par microscopie optique sur des étalements de sang correctement colorés, le plus souvent au May-Grünwald Giemsa. Cette méthode est la technique la plus communément utilisée pour le diagnostic de la babésiose clinique. Par ailleurs, sur les animaux morts, les babésies peuvent être détectées sur des calques d'organes colorés, notamment pour *B. bovis* sur des calques de cerveau. Lorsque la parasitémie est trop basse pour être détectée, l'historique de morsure de tiques, un syndrome fièvre-anémie-hémoglobinurie, des résultats sérologiques ou de PCR peuvent permettre d'orienter le diagnostic (Ristic 1988 ; Böse *et al*., 1995 ; Bock et al., 2004).

c. Traitement et prophylaxie

Il existe de nombreux babésiicides indiqués pour la chimiothérapie de la babésiose : la trypaflavine, le bleu trypan, le diacéturate de diminazène, l'iséthionate de phénamidine, le sulfate de quinuronium, le phosphate de primaquine et la chlortétracycline, mais le dipropionate d'imidocarb et le diacéturate de diminazène sont les molécules les plus utilisées (Kuttler, 1988a ; Vial et Gorenflot, 2006). L'imidocarb est de plus la seule substance utilisable pour la chimioprophylaxie, une injection protégeant contre les manifestations cliniques pendant une période de 3 à 6 semaines. Le diminazène est usuellement efficace contre *B. bovis* et *B. bigemina* chez les bovins à la dose de 3,5 mg/kg par voie intramusculaire. L'imidocarb s'utilise à la dose de 1,2 mg/kg en traitement et un dosage de 3 mg/kg permet une protection contre la babésiose pendant au moins un mois (Taylor et Mchardy, 1979). Toutefois, l'imidocarb est cher et il n'est pas toujours utilisable dans des troupeaux pour une large chimioprophylaxie, notamment dans les pays en développement. Le temps d'attente pour la viande est de 28 jours et le temps d'attente pour le lait est de de 2 jours chez la vache laitière en raison de résidus dans la viande et le lait. L'usage sur une large échelle d'une molécule est un facteur de risque de résistance médicamenteuse.

La vaccination serait une approche plus optimale pour protéger les animaux des infections par les babésies, en particulier pour les espèces les plus virulentes (De Waal et Combrink, 2006 ; Vial et Gorenflot, 2006). Les recherches sur la vaccination contre les babésioses ont débuté dès la fin du 19è siècle (Connoway et Francines, 1899). Bien que la production de vaccin anti-*Babesia* ait fait l'objet de nombreux essais, il n'existe actuellement que 2 vaccins commercialisés dans le Monde : d'une part, des vaccins atténués contre *B. bovis* et *B. bigemina* (érythrocytes infectés, produits sur animaux infectés ou par culture *in vitro*) principalement produits et utilisés en Australie, Argentine, Afrique du Sud, Israel et Uruguay, et, d'autre part, des vaccins inactivés contre *B. canis* à base d'antigènes solubles issus de surnageants de cultures *in vitro* (Shkap et Pipano 2000 ; Schetters, 2005 ; de Waal and

Combrink, 2006 ; Rojas *et al*., 2006 ; Shkap et al., 2007a, b; Fish et al., 2008). En plus de la complexité de production de vaccins vivants et des difficultés de production et de purification de grosses quantités d'antigènes parasitaires, les risques majeurs sont liés (i) à l'utilisation de produits dérivés de sang pour la production de ces vaccins et au risque de contamination par divers agents pathogènes et (ii) au risque de babésiose clinique causé par les souches atténuées vaccinales. Pour éviter ces risques, le développement de vaccins recombinants a été focalisé sur des antigènes de *Babesia* spp. reconnus par les anticorps ou les lymphocytes T chez des animaux naturellement infectés (Brown et Palmer, 1999). Une des protéines candidates bien identifiée pour plusieurs espèces de *Babesia* species est la protéine de rhoptrie RAP-1 (rhoptry associated protein) (Ridley et al. 1990 ; Dalrymple *et al.,* 1993 ; Palmer et McElwain, 1995 ; Skuce *et al*., 1996). RAP-1, MSA-1 (merozoite surface protein) et MSA-2 ont été identifiées chez *B. bovis* et les anticorps dirigés contre ces protéines ont montré une capacité d'inhibition de l'invasion des mérozoïtes dans la cellule-hôte (Suarez *et al*., 1998 ; Mosqueda *et al*., 2002a, b ; Norimine *et al*., 2003). Par ailleurs, SBP (spherical bodies protein), 12D3, VESA1 (variable erythrocyte surface antigens), ACS1, P0, AMA-1, TRAP (thrombospondin-related anonymous protein), gp45 et Bd37 identifiés chacune chez *B. bovis*, *B. bigemina* ou *B. divergens* par des approches empiriques, protéomiques, génomiques ou combinées génomique-protéomique peuvent aussi stimuler une réponse immune. Toutefois, certains bons candidats tels que MSA-1 et RAP-1, n'ont pas permis la production d'une immunité protectrice lors de tests d'immunisation suivis d'épreuves virulentes (Brown *et al.*, 2006a ; Musoke *et al*., 1997).

Le contôle des populations de tiques est une autre approche pour prévenir la babésiose car c'est le seul vecteur des babésies. Il y a actuellement 3 stratégies principales pour lutter contre les tiques, la chemothérapie, la lutte biologique et la vaccination contre les tiques (Jongejan et Uilenberg, 2004 ; Willadsen, 2006). Les acaricides chimiques sont efficaces s'ils sont bien appliqués. Par exemple, *Boophilus annulatus* et *B. (R) microplus* ont été éradiqués des Etats Unis grâce aux acaricides et à l'application stricte de mesures législatives (Programme national) (Pegram *et al*., 2000). Cependant, cette méthode a échoué au Pays de Galle et en Australie et a entraîné l'apparition de résistances aux acaricides (Jongejan et Uilenberg, 2004). De plus, l'application de ces molécules est accompagnée de rejet de résidus médicamenteux dans l'environnement et dans les produits animaux (lait et viande) (Graf *et al*., 2004). Le contrôle biologique des tiques est encore au stade de la recherche. Les principaux agents ciblés sont les champignons des genres *Beauveria* et *Metarhizium* (Samish *et al*., 2004). Si ce moyen de contrôle s'avère efficace sur le terrain, certains problèmes devront être résolus, notamment la formulation des agents biologiques vivants qui devra être stable dans l'environnement et sur les animux. La vaccination est une méthode considérée comme prometteuse pour lutter contre les tiques. Deux vaccins très efficaces contre *Boophilus*, commercialisés (TickGUARDND et GAVACND), ont été développés avec l'antigène

Bm86 dans les années 1990 (Rodríguez *et al*., 1995 ; Willadsen *et al*., 1995). Récemment il a été prouvé que l'utilisation du vaccin à base de la protéine Bm86 contre *B. annulatus* pouvait empêcher la transmission de *B. bigemina*, réduire celle de *B. bovis* (Pipano *et al*., 2003) et diminuer l'incidence de la babésiose (de la Fuente *et al*., 1999). Cela fait maintenant une 10^{aine} d'année que ces vaccins sont commercialisés mais actuellement aucun nouveau vaccin efficace contre d'autres espèces de tiques n'a été développé, malgré des essais expérimentaux à cause notamment de la difficulté à identifier des antigènes protecteurs de tiques, à formuler correctement le vaccin… Cependant, le développement de vaccins contre à la fois les tiques et la transmission de pathogènes est probablement une stratégie d'avenir pour contrôler ces acariens et les maladies transmises par les tiques. Ainsi, un contrôle intégré incluant 2 méthodes au moins tel que le plan "Integrated Pest Management" décrit par de Castro (1997) jouera probablement un rôle important dans le contrôle ou l'éradication des populations de tiques dans le futur.

B. La babésiose des Ruminants en Chine

1. Caractéristique géographique de la Chine

La République Populaire de Chine se situe à l'Est de l'Asie, sur le littoral Ouest de l'Océan Pacifique (figure 4). Sa surface représente 9,6 millions de kilomètres carrés. La distance entre le sud et le nord est de 5500 kilomètres et entre l'est et l'ouest de 5200 kilomètres. Le décalage horaire entre l'est et l'ouest est de 4 heures. Le paysage est divers, avec des régions montagneuses, des plateaux, des déserts, des collines, des plaines, des prairies, des bassins… La topologie de la Chine est également variée avec une altitude allant de 200 mètres à plus de 4000 mètres d'ouest en est. Les différentes zones climatiques tropicales, sub-tropicales, tempérées et froides sont rencontrées. Le climat est chaud et humide au sud et froid et sec au nord. La complexité écologique et géographique de la Chine entraîne une grande diversité d'habitats pour les plantes et les animaux. De ce fait, un grand nombre d'espèces de tiques y est rencontré et la majorité des maladies connues transmises par les tiques sont présentes en Chine.

Figure 4. Carte géographique de la Chine. (www.worldatlas.com)

2. Population et distribution des Ruminants domestiques en Chine

L'élevage d'animaux en Chine s'est développé rapidement après la réforme et l'ouverture du commerce extérieur au début des années 1980. Les productions animales représentent 33,7% des productions agricoles totales (2006, recueil annuel de l'agriculture chinoise http://www.agri.gov.cn/xxgktjxx). Les ruminants domestiques principaux sont les vaches, les buffles, les yaks, les piannius (hybride vache x yak), les moutons et les chèvres. La base de données FAOSTAT (http://faostat.fao.org) indique qu'en 2006 le nombre de vaches et de buffles est de 141,72 millions (9,09 % du cheptel mondial) et celui des moutons et de chèvres de 372,92 millions (19,07 % du cheptel mondial. Pour les yaks, les données statistiques montrent qu'il y avait 13 millions de têtes en 1996, représentant plus de 80 % de la population mondiale de yaks.

En Chine moyenne-orientale et du sud-est, les buffles et les bovins sont élevés sur des terres en association avec des cultures et sont utilisés comme animaux de trait. L'ouest et le nord-est de la Chine représente les principales aires de pâture pour les vaches, les yaks, les

moutons et les chèvres (figure 5) et la majorité des élevages d'animaux y sont présents. Dans ces régions, les élevages sont familiaux. Le nombre de têtes par élevage dépend de la surface des pâturages et du nombre de personnes pouvant s'en occuper. Dans la région du Tibet, une famille possède souvent de 50 à 300 yaks et/ou 100 à 1000 petits ruminants et ces animaux pâturent toute l'année (Yin *et al*., 2003). L'élevage est la principale source de revenus et de croissance économique dans ces régions. Le gouvernement a mis les priorités sur l'élevage de moutons et de chèvres dans les régions pastorales de la Chine de l'ouest car le climat y est particulièrement favorable pour la production de laine. Ainsi, 445510 tonnes de laine sont produites tous les ans. De plus, la viande de moutons et de chèvres représente 5,8 % (4,7 millions de tonnes par an) de la production totale de viande en Chine (80,5 millions de tonne par an) et 51,9 % est produite dans l'ouest de la Chine en 2006 (Ministère de l'agriculture de la RPC, http://www.agri.gov.cn). Cependant, du fait des modes d'élevage, ces animaux sont exposés toute l'année aux infestations par les tiques. Pour augmenter la qualité et la quantité de la production, il est donc nécessaire de contrôler les infestations par les tiques des petits ruminants et les infections par les agents pathogènes transmis par ces tiques dans ces régions.

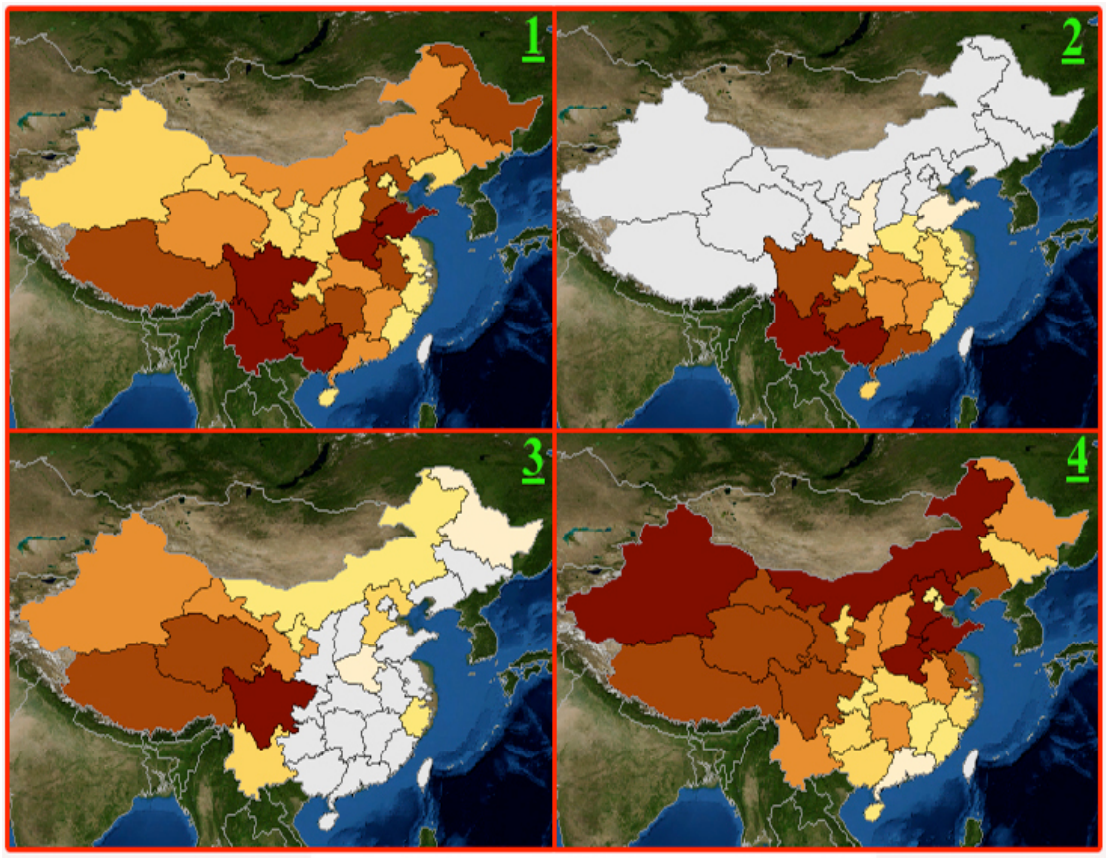

Figure 5. Distribution des ruminants domestiques en Chine. 1- distribution des vaches, 2- distribution des buffles, 3- distribution des yaks, 4- distribution des petits ruminants (moutons et chèvres). Plus la couleur est foncée, plus la densité est importante. (www.fao.org, données statistiques de: 1998 pour les vaches, 2002 pour les buffles, 1996 pour les Yaks, 2003 pour les petits ruminants)

3. Histoire et importance de la babésiose en Chine

En Chine, la babésiose a été décrite dès 1943 chez des chevaux dans la région de Heilongjiang (Lu *et al*., 1978) et la babésiose bovine 5 ans plus tard, en 1948 (Wang, 1993). La babésiose du buffle a été rapportée pour la 1ère fois en 1984 (Chen, 1984), dans 9 provinces de Chine. Cette même année, un cas de babésiose humaine a été enregistré dans la région du Yunnan (Li *et al*., 1984). Dans les années 1980, Chen (1982) et Zhao *et al.* (1986) ont décrit les 1er cas de babésiose ovine dans les régions du Sichuan et de Heilongjiang. Ces auteurs ont attribué ces cas à *B. ovis*, d'après la morphologie des parasites observés sur les étalements sanguins et les signes cliniques développés par les animaux malades. Enfin, plusieurs articles rapportent des cas de babésiose chez des animaux sauvages et notamment chez le cerf axis (*Axis axis*) (Zhang, 2007).

Il n'y a pas de données statistiques concernant la distribution et l'importance économique de la babésiose en Chine, bien que cela y soit une maladie importante. Les renseignements disponibles concernent seulement certaines régions ou parties de régions. Ainsi, 33387 cas de babésiose bovine clinique ont été enregistrées dans 19 régions en 1979 et 4946 bovins sont morts de cette maladie (Wang, 1993). Cependant, l'aspect le plus important est la forte mortalité due à la babésiose chez des animaux importés et sensibles. Afin d'augmenter les qualités de production des animaux, le gouvernement a fréquemment importé des animaux. Parfois, la mortalité a pu atteindre plus de 50% chez ces bovins importés (Zhang, 1959). Les pertes de production de viande et de lait chez les animaux guéris de babésiose aiguë ou chez les animaux porteurs asymptomatiques ne sont pas connues.

4. Les espèces de *Babesia* des Ruminants décrites en Chine et leurs distributions

Six espèces de *Babesia*, 5 identifiées et 1 non identifiée, sont décrites chez les bovins en Chine, incluant *B. bovis*, *B. bigemina*, *B. major*, *B. ovata*, *B. orientalis* et *Babesia* U sp. Kashi (Lu *et al*., 1988 ; Bai *et al*., 1990 ; Wang, 1993 ; Yin *et al*., 1997b ; Luo *et al*., 2002). Pour les ovins, les agents pathogènes de la babésiose sont peu connus : il y a au moins 2 espèces décrites, *B. motasi* et *Babesia sp*. Xinjiang, sur la base de données de phylogénie moléculaire (Liu AH *et al*., 2007; Niu *et al*., 2009a).

En Chine, il n'y a pas de données statistiques sur la babésiose des Ruminants. La distribution des espèces de *Babesia* provient principalement d'investigations épidémiologiques réalisées avec des moyens microscopiques, sérologiques ou moléculaires dans certains régions chinoises (Liu *et al*., 1986 ; Chen, 1989 ; Shen *et al*., 1997 ; Luo *et al*., 1998 ; Yao *et al*., 2002). En 1995, Lu *et al.* ont mené une étude à grande échelle sur les hémoprotozoaires bovins transmis par les tiques par des observations microscopiques

d'étalements sanguins provenant des 26 provinces chinoises, excepté les provinces de Hainan et de Taiwan. Pour la babésiose ovine, il n'y a pas de données disponibles exceptés les déclarations de cas et l'isolement de certains agents pathogènes à partir de tiques récoltées sur le terrain ou de sang de moutons paturant dans des zones où des tiques sont présentes. Dans cette revue bibliographique, nous faisons le bilan de la distribution connue des babésioses bovines et ovines, sur la base des cas et des *Babesia* recensées (figure 6 et 7).

a. Les *Babesia* des bovins

B. bovis et ***B. bigemina*** sont les principaux agents pathogènes de la babésiose bovine en Chine. Ces parasites ont été isolés et identifiés par Yang et Wang en 1964 dans la province du Guizhou et la tique vectrice a été confirmée comme étant *Boophilus (Rhipicephalus) microplus* (Lu *et al*., 1989). Les 2 sont transmis de façon transovarienne. Les expériences de transmission ont montré que les larves peuvent transmettre *B. bovis* et *B. bigemina* à des veaux splenectomisés, mais que les nymphes et les adultes peuvent transmettre uniquement *B. bigemina* (Lu *et al*., 1989). Les 2 espèces de babésies sont souvent trouvées en co-infection du fait de leur vecteur commun. Le taux d'infection des bovins par *B. bovis* et *B. bigemina* varie de 0,97 à 56,66%, mais peut atteindre dans certaines régions les 90% (Lu *et al*., 1995). La distribution de *B. bigemina* est corrélée à la distribution de *B.* (*R.) microplus*, dans les broussailles et les prairies, recouvrant 22 provinces. *B. bovis* a été rapportée dans seulement 16 provinces, ce qui ne coïncide pas tout à fait avec la distribution de *R. microplus* (Yin *et al*., 1997b). Les principales provinces atteintes sont celles de Hunan, Hubei, Yunnan, Guizhou et Sichuan mais on les trouve également dans les provinces de Guangdong, Anhui, Fujian, Jiangsu, Jiangxi, Zhejiang, Gansu, Shaanxi, Shandong, Henan, Hebei, Liaoning, Tibet (figure 7).

B. major et ***B. ovata*** ont été isolés, pour la 1ère, dans la province du Xinjiang et pour la 2ème, dans la province du Henan. En 1988, une babésie isolée de tiques *H. longicornis* collectées dans le comté du Lushi dans la province du Henan, a été suspectée comme étant *B. ovata* ou *B. major* (Lu *et al*., 1988 ; Bai *et al*., 1990). Elle a été appelée *B. ovata* Lushi. Afin d'étudier la distribution de ce parasite, plusieurs isolats ont été obtenus de différentes régions géographiques, Zhangjiachuan (*B. ovata* Zhangjiachuan) et Ningxian (*B. ovata* Ningxian), comtés de la province du Gansu, et Wenchuan (*B. ovata* Wenchuan), comté de la province du Sichuan (Bai *et al*., 1994 ; Yin *et al*., 2000 ; Liu *et al*., 2008). Entre temps, une espèce de babésie a été isolée de bovins infestés avec des tiques adultes *H. punctata* collectées dans le comté du Yili dans la province du Xinjiang. Ce parasite a été nommé *B. major* Yili, du fait de sa tique vectrice (Lu *et al*., 1992). En effet, traditionnellement, la discrimination entre *B. major* et *B. ovata* est principalement basée sur les tiques vectrices (Bai *et al*., 1990 ; Higuchi *et al*., 1991). *B. major* est transmis par *H. punctata* (Morzaria *et al*., 1977) alors que *B. ovata*

est transmis par *H. longicornis* (Minami et Ishihara, 1980). Cependant Yin *et al*. (1996) ont montré que *B. ovata* Lushi et *B. major* Yili pouvaient tous deux être transmis expérimentalement par *H. punctata* mais pas par *B.* (*R.) microplus*. Par conséquent les études se sont focalisées sur la classification de ces espèces car elles sont similaires d'un point de vue morphologique et pathogénique (Lu *et al*., 1988). Récemment, Luo *et al.* (2005a) et Liu *et al*. (2008) ont démontré, sur la base de données phylogénétiques des gènes ITS et de l'ARNr 18S, que les espèces transmises à la fois par *H. punctata* et *H. longicornis* appartiennent à l'espèce *B. ovata* (*B. ovata* Lushi, *B. ovata* Ningxian, *B. ovata* Wenchuan et *B. ovata* Zhangjiachuan) alors que les espèces transmises uniquement par *H. punctata* ont étaient identifiées comme étant *B. major* ((*B. major* Yili). Expérimentalement, ces 2 espèces ont un faible pouvoir pathogène pour les bovins non splenectomisés et ne sont généralement pas considérées comme des pathogènes aussi importants que *B. bovis* et *B. bigemina* (Lu *et al*., 1990 ; Bai *et al*., 1990 ; Yin *et al*., 1996). *B. major* est distribué comme sa tique vectrice et est présent seulement dans le nord de la province du Xinjiang alors que *B. ovata* est probablement largement répandu en Chine car *H. longicornis* est présente dans la plupart des régions en Chine (province du Gansu, Henan, Sichuan et Liaoning) (figure 7).

B. orientalis est une espèce présente dans 9 provinces du milieu et du sud de la Chine (Provinces du Hunan, Hubei, Jiangxi, Zhejiang, Jiangsu, Fujian, Guizhou, Anhui et Guangxi, figure 7) et infecte uniquement les buffles d'eau. Auparavant, ce parasite a été identifié comme *B. bovis* ou *B. bigemina* du fait de ses caractéristiques morphologiques dans les érythrocytes (Liu *et al*., 1986). Cependant *B.* (*R.) microplus*, le vecteur de *B. bovis* et *B. bigemina*, ne peut pas transmettre *B. orientalis* et il a été prouvé que *R. haemaphysaloides haemaphysaloides* était son vecteur (Ma *et al*., 1989a, b). Ce parasite a été nommé *B. orientalis* du fait de ces caractères biologiques et moléculaires (Liu *et al*., 1997 ; Liu *et al*., 2005).

En 2002, Luo *et al.* ont décrit une babésie bovine non identifiée de faible virulence, isolée de tiques *Hyalomma anatolicum anatolicum* collectées dans le Comté de Kashi dans le sud de la province du Xinjiang (figure 6-6 et figure 7). Elle a été nommée ***Babesia* U *sp.* Kashi** (Luo et al., 2002). Les expériences de transmission ont montré que ce protozoaire pouvait être transmis aux bovins par les stades larve, nymphe et adulte de *Hy. a. anatolicum*, *Hy. detritum* et *Hy. rufipes* Koch mais pas par *B.* (*R.) microplus* et *H. longicornis* (Luo *et al*., 2003). Une transmission transovarienne a été également observée (Luo et al., 2003). L'analyse moléculaire de la phylogénie sur la base des séquences du gène de l'ARNr 18S a montré que *Babesia* U *sp*. Kashi est différent des autres espèces de *Babesia*, mais jusqu'à présent il n'a pas pu être identifié comme nouvelle espèce du fait de l'indisponibilité de données moléculaires sur *B. beliceri* (décrite en Russie) et sur *B. ocultans* (décrite en Afrique du Sud), toutes 2 également transmises par *Hyalomma* spp. (Luo *et al*., 2005b).

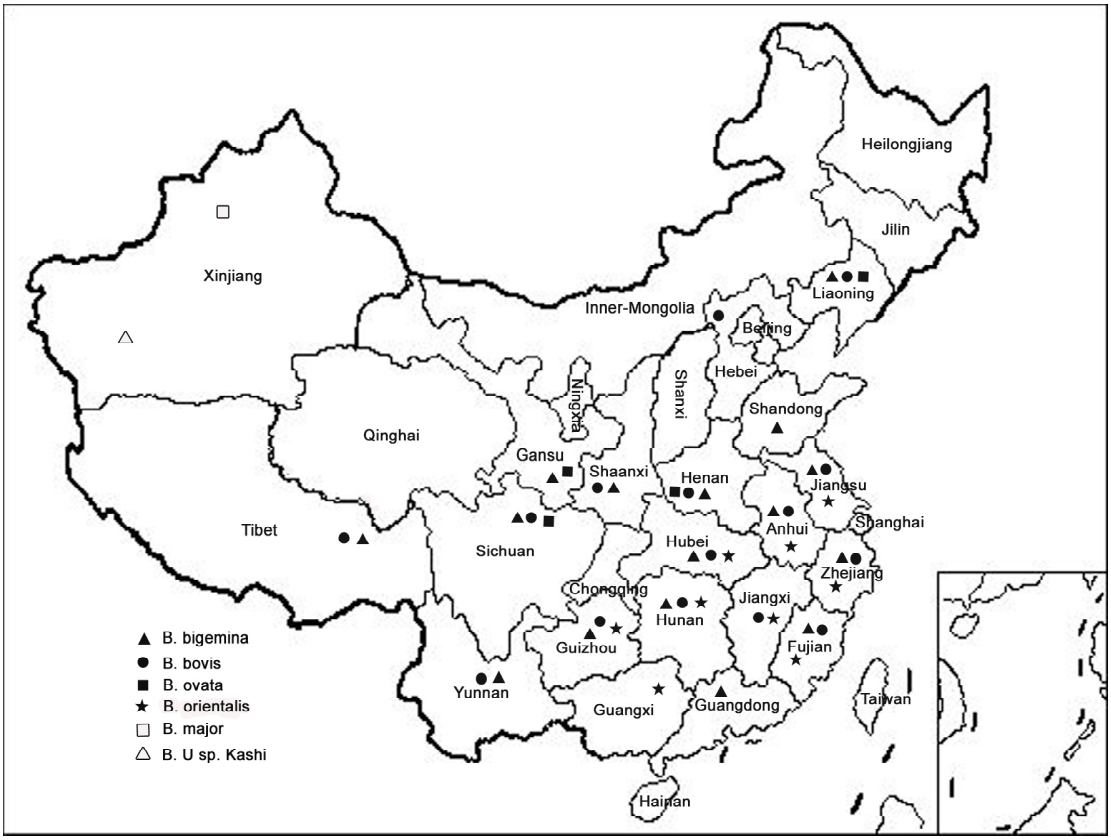

Figure 6. Carte de distribution géographique des *Babesia* spp. bovine enregistrées en Chine. (Lu *et al*., 1995 ; Yin *et al*., 1997 ; Luo *et al*., 1998 ; Liu *et al*., 1986)

b. Les *Babesia* des moutons

La situation des agents de la babésiose ovine est plus complexe et moins connue en Chine. Les 1er cas de babésiose ovine ont été décrits au cours des années 1980 dans les provinces du Sichuan et de Heilonjiang et le parasite responsable a été identifié comme étant *B. ovis* (Chen, 1982 ; Zhao *et al*., 1986). Cependant, aucun article ne rapporte de babésisose ovine dans ces régions depuis. Les essais pour isoler ces *Babesia* de sang de moutons ou de tiques collectées sur le terrain de ces 2 provinces ont toujours échoué (communication personnelle avec le professeur Bai). En 1996, une flambée de babésiose ovine a été observée dans plusieurs élevages de moutons à queue courte (« Small-Tailed ») importés de la province de Shandong dans le comté du Ningxian situé dans l'est de la province du Gansu. Deux souches de *Babesia* ont été isolées : l'une est une grande babésie fortement virulente pour les moutons et les chèvres (désignée tout d'abord comme *Babesia sp*. ou *B. motasi* puis actuellement *Babesia sp.* BQ1 (Ningxian)) et l'autre est une petite babésie faiblement virulente pour les petits Ruminants (désignée *B. ovis*) (Lian *et al*. 1997 ; Yin *et al*. 1997a). Néanmoins, les études suivantes ont montré que la petite forme de *Babesia* était probablement une *Theileria*. Guan *et al.* (2002) ont isolé une autre souche de *Babesia* ovine, de faible virulence, à partir de tiques *H. qinghaiensis* collecteés sur des pâtures du comté de Lintan de

la province du Gansu. (dans ce papier, il y a confusion entre *Babesia* ovine et *B. ovis*). Cette souche a été nommée *Babesia* sp. BQ1 (Lintan). Depuis, la babésiose ovine a été étudiée dans quelques provinces de Chine. Des sangs de moutons ou des tiques ont été prélevés sur le terrain dans différentes régions. Ces sangs ont été inoculés à des moutons splénectomisés ou ces tiques ont été mises à gorger également sur des moutons splénectomisés. Différentes souches de grandes babésies ont été ainsi isolées au laboratoire « Vector and Vector-borne disease (VVBD) à l'institut de recherche vétérinaire de Lanzhou (Lanzhou Veterinary Research Institute LVRI) (Guan *et al*., 2001 ; Liu AH *et al*., 2007). L'étude de la séquence des gènes de l'ARNr 18S et ITS a permis de diviser ces souches en 2 groupes : i- *B. motasi*-like incluant *Babesia sp*. BQ1 (Lintan), *Babesia sp*. BQ1 (Ningxian), *Babesia sp*. (Liaoning), *Babesia sp*. (Tianzhu), *Babesia sp*. (Madang), et *Babesia sp*. (Hebei) et ii- *Babesia sp*. Xinjiang (Liu *et al*., 2007 ; Niu *et al*., 2009a). *B. motasi*-like est décrite dans les provinces du Gansu, Hebei et Liaoning alors que *Babesia sp.* Xinjiang est décrite uniquement dans la province du Xinjiang. Enfin des cas de babesiose ovine ont été enregistrés dans les province de Heilongjiang, Henan, Shanxi, Yunnan et Sichuan mais les babésies responsables n'ont pas été identifiées (figure 7).

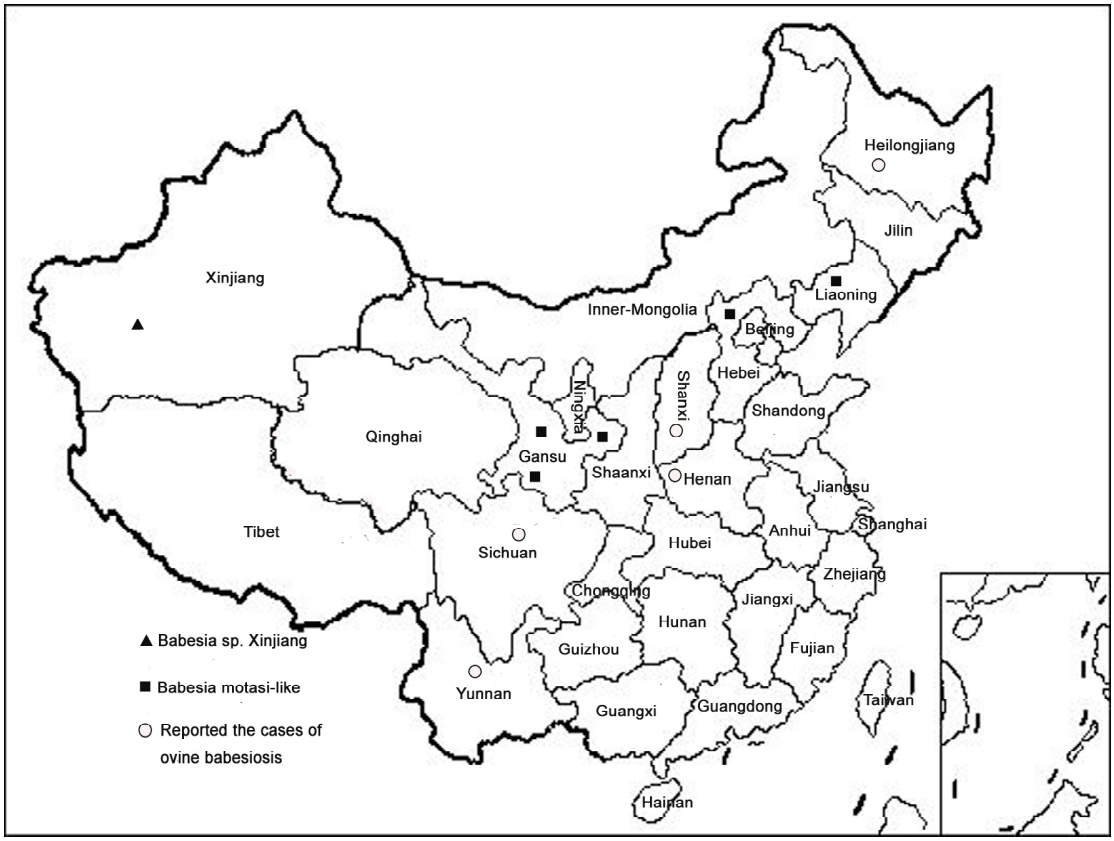

Figure 7. Carte de distribution géographique des *Babesia* spp. ovine enregistrées en Chine. (Zhao *et al*., 1986 ; Chen, 1982 ; Bai *et al*., 2002 ; Guan *et al*., 2001, 2002 ; Yang *et al*., 2003 ; Li *et al*., 2006 ; Liu AH *et al*., 2007 ; Yang *et al*., 2009)

Peu de choses sont connues sur la biologie de ces différentes souches (Tableau 2 et figure 8). *Babesia sp.* BQ1 (Ningxian), isolé de sang d'un mouton malade sur le terrain, est un parasite virulent pour les moutons et les chèvres et a une morphologie similaire à *B. motasi* Europe. Une transmission aux moutons et aux chèvres par les stades larvaires et nymphaux de *H. longicornis* et une transmission transovarienne ont été démontrées (Bai *et al*., 2002). *Babesia sp*. BQ1 (Lintan) a été isolé d'un mouton expérimentalement infesté par *H. qinghaiensis* collectées sur des pâtures. C'est un parasite faiblement virulent pour les moutons et les chèvres mais sa tique vectrice n'a jamais été pour l'instant identifiée expérimentalement (Guan *et al*., 2002). *Babesia sp*. Xinjiang a été isolé d'un mouton expérimentalement infesté par *R. sanguineus* et *Hy. a. anatolicum* collectées sur des pâtures. Il est faiblement virulent pour les moutons et ses caractéristiques morphologiques sont totalement différentes des autres souches isolées en Chine, de *B. ovis*, de *B. motasi* et de *B. crassa* (Guan *et al*., 2001). Pour les autres souches, nous savons juste qu'elles ont été isolées de sang de moutons pâturant dans des zones où des tiques du genre *Haemaphysalis* sont majoritairement présentes.

En résumé, il y a en Chine au moins 2 espèces de *Babesia* infectant les petits Ruminants domestiques, *B. motasi*-like transmise probablement par *Haemaphysalis* spp. et *Babesia sp.* Xinjiang transmise probablement par *R. sanguineus* ou *Hy. a. anatolicum*.

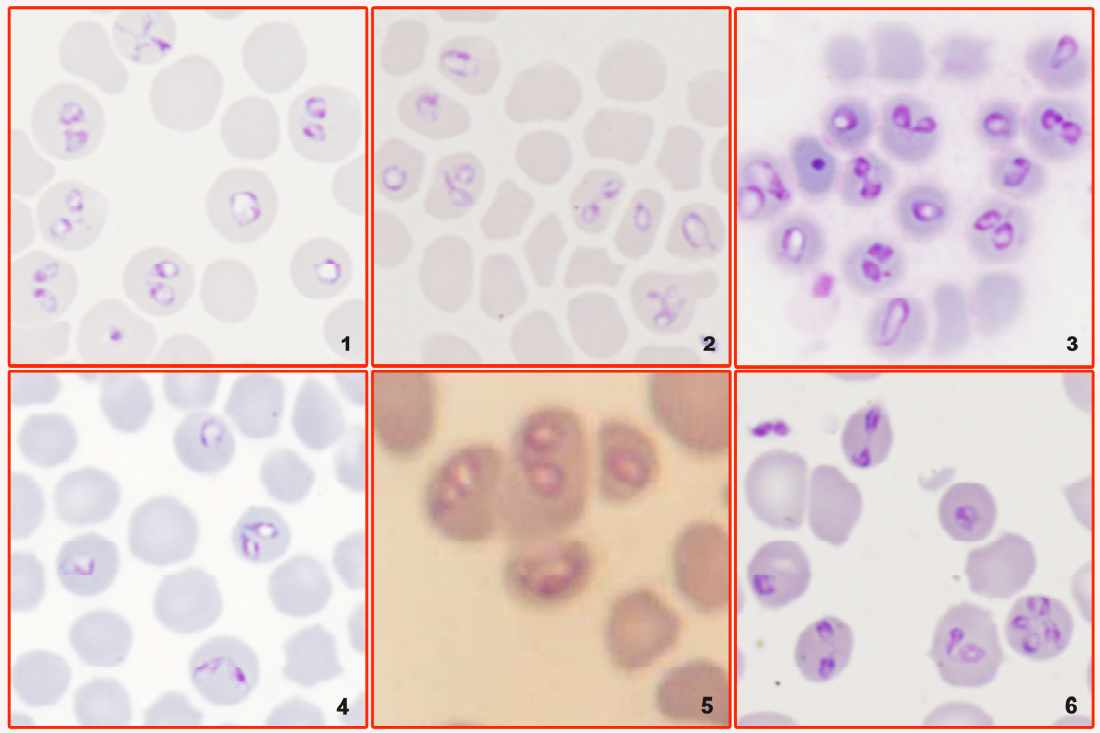

Figure 8. Caractères morphologiques de différentes souches de *Babesia* infectant les Ruminants en Chine.1: *Babesia sp*. BQ1 (Lintan), 2: *Babesia sp*. BQ1 (Ningxian), 3: *Babesia sp*. Hebei, 4 : *Babesia sp*. Xinjiang, 5: *Babesia sp*. Liaoning et 6: *Babesia* U *sp*. Kashi. 1 à 5 infectent les petits Ruminants, 6 est un parasite des bovins. Les étalements sanguins ont été fournis par le laboratoire VVBD, LVRI, CAAS, China.

Tableau 2. Souches de *Babesia* infectant les moutons et les chèvres en Chine, isolées durant les 20 dernières années.

Souches	Localisation (**Comté**, *province*)	1er isolement	Tique vectrice	Morphologie	Virulence pour les moutons et les chèvres	Phylogénie basée sur le gène ARNr 18S
Babesia sp. BQ1 (Lintan)	**Lintan**, *Gansu*	Adultes de *H. qinghaiensis* collectés sur pâture	Non identifiée	Grande, Similaire à *B. motasi*	Faible	*B. motasi*-like
Babesia sp. BQ1 (Ningxian)	**Ningxian**, *Gansu*	Sang de mouton malade en pâture	*H. longicornis*	Grande, Similaire à *B. motasi*	Modérée	*B. motasi*-like
Babesia sp. Hebei	Ville de Chende, *Hebei*	Sang de mouton non malade en pâture	Non identifiée	Grande, Similaire à *B. motasi*	Inconnue	*B. motasi*-like
Babesia sp. Tianzhu	**Tianzhu**, *Gansu*	Sang de mouton non malade en pâture	Non identifiée	Grande, Similaire à *B. motasi*	Inconnue	*B. motasi*-like
Babesia sp. Madang	**Madang**, *Gansu*	Sang de mouton non malade en pâture	Non identifiée	Grande, Similaire à *B. motasi*	Inconnue	*B. motasi*-like
Babesia sp. Liaoning	Ville de Liaoyang, *Liaoning*	Sang de mouton non malade en pâture	Non identifiée	Grande, Similaire à *B. motasi*	Inconnue	*B. motasi*-like
Babesia sp. Xinjiang	Ville de Kashi, *Xinjiang*	Adultes semi-gorgées de *Hy. a. anatolicum* et *R. sanguineus* collectés dur des bovins, moutons et chèvres en pâture	Non identifiée	Différente des autres *Babesia*	Faible	*Babesi*a sp. Xinjiang

Babesia sp. BQ1 (Ningxian): Yin *et al*. 1997a ; Lian *et al*., 1997 ; Bai *et al*., 2002
Babesia sp. BQ1 (Lintan) : Guan *et al*., 2002 ;
Babesia sp. Hebei, *Babesia* sp. Tianzhu, *Babesia* sp. Madang and *Babesia* sp. Liaoning: Liu *et al*., 2007
Babesia sp. Xinjiang: Guan *et al*., 2001

5. Les tiques des Ruminants en Chine

En Chine, 109 espèces de tiques appartenant à 8 genres de la famille des *Ixodidae* ont été enregistrées : *Dermacentor*, *Ixodes*, *Rhipicephalus*, *Haemaphysalis*, *Hyalomma*, *Amblyomma*, *Aponomma*, *Anomalohimalaya*. La plupart d'entre elles infestent les Ruminants (Guo et Xu, 1964 ; Zhang, 1964 ; Wang, 1980 ; Kong, 1981 ; Li, 1987 ; Teng et Jiang, 1991 ; Li et Chen, 1994). En 2007, Yin et Luo ont référencé au moins 45 espèces infestant les petits Ruminants, 10 *Dermacentor* spp., 5 *Ixodes* spp. 5 *Rhipicephalus* spp., 15 *Haemaphysalis* spp., 8 *Hyalomma* spp., *Amblyomma testudinarium* et *Boophilus (Rhipicephalus) microplus*. Elles peuvent transmettre l'anaplasmose, la theiléríose et la babésiose. En ce qui concerne les babésies infectant les Ruminants en Chine, les tiques vectrices connues sont récapitulées dans le tableau 3.

Tableau 3 : Tiques vectrices connues des *Babesia spp*. isolées en Chine

Tique vectrice	**Espèce de *Babesia* transmise**
Boophilus (Rhipicephalus) microplus	*B. bovis, B. bigemina*
Haemaphysalis punctata	*B. major, B. ovata*
Haemaphysalis longicornis	*B. ovata, Babesia sp.* BQ1 (Ningxian)
Rhipicephalus haemaphysaloides haemaphysaloides	*B. orientalis*
Hyalomma anatolicum anatolicum	*Babesia* U *sp.* Kashi
Hyalomma detritum	*Babesia* U *sp.* Kashi
Hyalomma rufipes koch	*Babesia* U *sp.* Kashi

C. Détection des infections à *Babesia* spp.

Actuellement, les techniques disponibles pour diagnostiquer ou dépister les infections à *Babesia* spp. sont principalement des tests directs permettant de détecter le protozoaire dans le sang des animaux et des tests indirects basés sur la recherche des anticorps anti-*Babesia* dans les sérums.

1. Recherche directe de *Babesia* spp.

a. Examen microscopique

Depuis la découverte par Babes de parasites dans les érythrocytes de bovin en utilisant l'examen microscopique d'étalement sanguin en 1888, cette technique est toujours utilisée pour détecter les infections à *Babesia* spp. du fait de sa facilité de réalisation. Les étalements sanguins ou les gouttes épaisses sont colorés au May-Grünwald-Giemsa et examinés au microscope optique avec l'objectif x100 afin de rechercher des hématies parasitées. En général, les étalements sont préférés aux gouttes épaisses. Les observations sont souvent longues mais du temps peut être gagné en utilisant l'objectif x 40 plutôt que l'objectif x100 (« une personne experimentée peut alors examiner 100 à 150 échantillons par jour ») (Böse *et al*., 1995). Ces mêmes auteurs estiment que la sensibilité peut atteindre une hématie parasitée pour 10^5 – 10^6 hématies (parasitémie de 10^{-5}- 10^{-6}). Cependant, lors d'infections subcliniques ou latentes, les parasites sont difficilement observables du fait des très faibles parasitémies (Böse *et al.*, 1995). Cette technique nécessite des techniciens qualifiés pour distinguer les *Babesia* d'autres hémoprotozoaires tels que les *Theileria* et pour différencier les différentes espèces de *Babesia.* Elle n'est pas adaptée à des études épidémiologiques à grande échelle.

b. Inoculation aux animaux

Cette méthode consiste à inoculer à des animaux du sang prélevé d'un animal suspecté d'être infecté. Elle est plutôt utilisée pour isoler des *Babesia* que pour le diagnostic clinique car elle est consommatrice en temps, elle est peu sensible et elle est compliquée du fait de la nécessité de splénectomiser les animaux le plus souvent. Pour les espèces de *Babesia* ayant une spécificité d'hôte stricte, excepté *B. divergens*, il est nécessaire d'utiliser des moutons ou des bovins pour identifier les infections à *Babesia* spp. des Ruminants. Pour *B. divergens*, l'infection peut être détectée avec des gerbilles de Mongolie (*Meriones unguiculatus*) inoculées avec au moins 10^3-10^4 parasites (Lewis *et al.* 1981a ; Hentrich *et al*., 1993). Cette technique tend à être remplacée actuellement par la culture *in vitro*.

c. Culture *in vitro*

Le développement de la culture *in vitro* en phase stationnaire (Microaerophilus stationary phase culture : MASP) de *Babesia* spp. par Levy et Ristic (1980), et notamment de *B. bovis* et *B. bigemina* (Vega *et al*., 1986 ; Rodriguez *et al*., 1983), a fourni un nouveau moyen pour isoler ce protozoaire et pour diagnostiquer la babésiose. Depuis, la culture *in vitro* de plusieurs autres espèces de *Babesia* a été mise au point (Thomford *et al*., 1993 ; Holman *et al*., 1994, 2005). Rodriguez *et al*. (1983) et Malandrin *et al*. (2004a) ont montré que *B. bovis* ou *B. divergens* peuvent être détectées avec cette méthode chez des bovins guéris de babésiose ou porteurs asymptomatiques alors que le parasite n'était pas observé sur des étalement sanguins. La sensibilité de la culture *in vitro* de *B. divergens* a été détaillée par Malandrin *et al*. (2004a). Une parasitémie de 10^{-9} (10 parasites par millilitre de sang) peut être détectée après 6 à 20 jours de culture, ce qui suggère que la sensibilité de la culture *in vitro* est similaire aux méthodes PCR. Cependant, cette technique nécessite un laboratoire de culture cellulaire et un délai long (jusqu'un mois) pour obtenir le résultat. Elle est donc rarement utilisée pour le diagnostic de la babésiose.

d. Détection de l'ADN de *Babesia* spp.

Les méthodes citées ci-dessus peuvent être utilisées pour détecter les *Babesia* chez leur hôte vertébré mais elles permettent difficilement de les observer chez leurs vecteurs et de distinguer les différentes espèces du fait de leurs similarités morphologiques. De plus, elles sont difficilement applicables à des enquêtes épidémiologiques. Les avancées en biologie moléculaire ont permis de développer des techniques de détection des *Babesia* chez leurs hôtes vertébrés et invertébrés et de différenciation des différentes espèces.

i. Sondes d'ADN

Cette technique consiste à appliquer des sondes d'ADN spécifiques des espèces de *Babesia* sur l'ADN isolé à partir d'échantillon de sang, de tissu ou de tiques. Sa sensibilité est limitée par la quantité d'ADN présent dans l'échantillon. A partir de sang, la sensiblité est similaire à celle de l'examen microscopique d'étalement sanguin (parasitémie de 10^{-5}-10^{-6}) (Posnett et Ambrosio, 1991 ; Petchpoo *et al*., 1992). Wu *et al*. (1998) ont développé cette technique pour *B. bovis* en Chine, permettant de détecter 32 pg d'ADN génomique de *B. bovis* sans aucune réaction croisée avec *B. bigemina*, *B. ovata*, *Theileria sergenti*, *Trypanosoma evansi* et *Anaplasma marginale*. Du fait de leurs grandes spécificités, les sondes ADN ont été utilisées pour distinguer des espèces indifférenciables morphologiquement telles que *B. bigemina* et *B. beliceri* ou *B. occultans* (Böse *et al*., 1995). Elles peuvent être également utilisées pour identifier le protozoaire dans les tiques vectrices pour des études de

transmission et des études épidémiologiques. Cependant cette technique est actuellement peu utilisée du fait de son coût important, son manque de sensibilité et de la nécessité d'un équipement spécialisé.

ii. Technique PCR

La PCR est un outil très puissant pour détecter les babésies. La mise en évidence des produits de PCR sur gel d'agarose avec coloration au bromure d'éthidium a une sensibilité de 10^{-7}-10^{-8}% (Aktas *et al.*, 2005 ; Fukumoto *et al*., 2001b). Lorsque les produits de PCR sont détectés avec des sondes nucléotidiques, elle permet d'observer les parasites dans le sang à une parasitémie de 10^{-9} et il n'y aurait aucune réaction croisée entre les différents piroplasmes et entre les différentes espèces de *Babesia* (Fahrimal *et al*., 1992 ; Figueroa *et al*., 1992 ; Persing *et al*., 1992). Récemment, toujours dans le but d'augmenter la sensibilité de la PCR, la PCR nichée a été développée : sa sensibilité est supérieure à 10^{-9} (Birkenheuer *et al*., 2003). La PCR multiplexe a été également mise au point pour déceler en un seul test les co-infections par *Babesia, Theileria* et *Anaplasma*, mais la sensibilité est alors diminuée (Figueroa *et al*., 1993 ; Alhassan *et al*., 2005). Grâce à la grande spécificité et sensibilité de la PCR, l'amplification de l'ADN parasitaire a donc été appliquée pour diagnostiquer l'infection et identifier les espèces de *Babesia* à partir d'échantillons sanguins et de tiques (Krause *et al*., 1996 ; Birkenheuer *et al.*, 2003 ; Song *et al*., 2004 ; Liu Q *et al*., 2007 ; Sun *et al*., 2008). Cependant, les procédures complexes et la nécessité d'un matériel sophistiqué tel qu'un thermocycleur restreignent son utilisation en routine pour le diagnostic.

iii. Reverse Line Blot (RLB)

Cette méthode est fondée sur la PCR et l'hybridation : les sondes spécifiques d'espèces sont liées de façon covalente à une membrane d'hybridation qui est ensuite hybridée avec un produit de PCR. Elle peut être utilisée pour détecter les co-infections par *Babesia* et *Theileria* chez l'hôte vertébré et la tique. De plus la différenciation de différentes souches géographiques de *Babesia* est possible grâce à sa grande spécificité apportée à la fois par la PCR et les sondes d'ADN. La sensibilité minimale est de 10^{-8} quand différents échantillons sont analysés simultanément (Gubbels *et al*., 1999 ; Schnittger *et al*., 2004 ; Garcia-Sanmartin *et al*., 2006 ; Niu *et al*., 2009b). Le RLB est donc un outil très efficace pour identifier des babésies ou pour mettre en évidence des co-infections mais il n'est pas utilisable pour le diagnostic ou pour des études épidémiologiques du fait de sa complexité.

iv. Loop-Mediated Isothermal Amplification (LAMP)

La LAMP est une technique développée par Notomi *et al*. en 2000. Elle permet l'amplification rapide d'ADN avec une grande sensibilité et une grande efficacité dans des conditions isothermiques dans un simple incubateur. Comparée à la PCR, elle ne nécessite pas

de cycles avec des étapes à différentes températures et a l'avantage d'être une réaction simple et sensible. Une réaction isothermique d'une heure est suffisante pour amplifier 10^9 fois la cible d'ADN, ce qui peut être évaluée simplement par une observation visuelle de la turbidité ou de la fluorescence dans le tube de réaction (Notomi *et al*., 2000). Récemment, elle a été utilisée pour détecter des espèces de *Babesia* (*B. caballi, B. orientalis*) avec une sensibilité 10 à 100 fois supérieure à celle de la PCR nichée (Ikadai *et al*., 2004 ; Iseki *et al*., 2007 ; Alhassan *et al*., 2007 ; He *et al*., 2009).

2. Recherche des anticorps anti-*Babesia* spp.

Différents tests sont actuellement décrits pour détecter les anticorps dirigés contre *Babesia* spp. (Weiland et Reiter, 1988; Wright, 1990). Trois d'entre eux sont utilisés en routine et sont des techniques de référence pour l'Organisation Mondiale de la Santé Animale (OIE) : le test de fixation du complément (CFT), le test d'immunofluorescence indirect (IFAT) et le test ELISA (enzyme linked immunosorbent assay).

a. Le test de fixation du complément (CFT)

Le test de fixation du complément permet de mettre en évidence des IgM au cours de l'infection précoce. Il a en revanche une faible sensibilité pour détecter l'infection pendant la phase chronique (Böse *et al*., 1995 ; Ogunremi *et al*., 2008). Les études comparant le test CFT avec d'autres techniques sérologiques ou avec la culture *in vitro* montrent qu'il manque de sensibilité (Tenter et Friedhoff, 1986 ; Weiland, 1986 ; Holman *et al*., 1993 ; Böse et Peymann, 1994 ; Kumar *et al*., 1997). Ce test est donc rarement utilisé sauf pour les infections à *Babesia sp*. chez les Équidés mais depuis 2004, l'OIE préconise l'utilisation de l'IFAT ou de l'ELISA compétitive plutôt que le test CFT pour détecter les infections à *B. caballi*.

b. Le test d'immunofluorescence indirect (IFAT)

Le test IFAT est le test le plus largement utilisé pour le diagnostic des infections à *Babesia* spp. car il nécessite peu de réactifs et une technicité modérée (Böse *et al*., 1995). En revanche, un nombre limité d'échantillons sont analysables à la fois et la lecture des lames est subjective et fatiguante pour l'opérateur, ce qui la rend difficile à standardiser et à utiliser pour des enquêtes épidémiologiques à grande échelle. Elle reste tout de même l'outil principal de diagnostic pour certaines espèces de *Babesia* telles que les babésies ovines et les babésies des animaux sauvages et elle est parfois utilisée comme test de référence lors de la mise au point d'autres techniques de séro-diagnostic (Ferrer *et al*., 1998 ; Aboge *et al*., 2007b ; Schmid *et al*., 2008).

c. Le test ELISA

L'ELISA est un test efficace et sensible pour détecter les infections à *Babesia*. Contrairement à l'IFAT, les données sont obtenues grâce à un spectrophotomètre et peuvent être évaluées par un ordinateur, ce qui rend cette technique facilement standardisable. Un grand nombre d'échantillons peuvent être traités en même temps. L'ELISA a donc les potentialités pour être un test standard de diagnostic de la babésiose et d'études épidémiologiques. En 1976, Purnell *et al*. ont développé pour la 1ère fois un test ELISA pour détecter des anticorps anti-*B. divergens* dans des sérums de bovins en utilisant un extrait brut de mérozoïtes. Depuis, plusieurs ELISA utilisant comme antigènes des extraits bruts ou des protéines purifiées de mérozoïtes ont été mises au point pour détecter les infections par diverses espèces de *Babesia* (Bidwell *et al*., 1978 ; Young et Purnell, 1980 ; Barry *et al*., 1982 ; O'Donoghue *et al*., 1985 ; Ruiz *et al*., 2001). Un test ELISA utilisant des antigènes de mérozoïtes préparés à partir d'érythrocytes infectés fortement enrichis a été validé comme technique de diagnostic de l'infection des bovins à *B. bovis* par l'OIE (Waltisbuhl *et al*., 1987 ; De Echaide *et al*., 1995 ; Molloy *et al*., 1998 ; OIE, 2009). Pour d'autres espèces de *Babesia*, il est difficile d'obtenir des préparations fortement enrichies en antigènes spécifiques : elles sont souvent contaminées avec des érythrocytes de l'hôte, ce qui peut entraîner des résultats faussement positifs à cause d'anticorps anti-érythrocytes. En parallèle avec le développement de la culture *in vitro* des babésies, des protéines dérivant de la culture ont été identifiées comme antigènes spécifiques pouvant être utilisés en ELISA, avec une forte sensibilité et spécificité (Montealegre *et al*., 1987 ; Chauvin *et al*., 1995).

La qualité de l'antigène est en général cruciale dans le développement de tests ELISA sensibles et spécifiques. Les protéines purifiées des extraits bruts sont difficiles à produire à grande échelle. Les proteines recombinantes sont considérées comme étant une alternative intéressante pour le développement de l'ELISA : elles ne contiennent pas de protéines d'hôte, elles sont relativement bon marché à produire lorsqu'elles sont disponibles, elles peuvent être produites à grande échelle et peu de variations entre lot de production sont observées (Böse et al., 1995). Les 1ères études ont montré que les antigènes recombinants fournissent une spécicité plus importante que les antigènes natifs (Verdida *et al*., 2004 ; Tamaki *et al*., 2004 ; Altangerel *et al*., 2009). Le prérequis le plus important pour mettre au point un ELISA est l'identification sans équivoque d'antigènes hautement spécifiques qui entraînent une réponse en anticorps intense et durable chez l'hôte naturel durant l'infection. Les protéines de rhoptries (RAP : Rhoptry Associated Protein) sont ainsi considérées comme des antigènes potentiels pour le sérodiagnostic de la babésiose et les réactions croisées entre *B. bovis* et *B. bigemina* peuvent être éliminées si l'antigène recombinant RAP-1 est privé de son extrémité C-terminale (Ikadai *et al*., 2000 ; Boonchit *et al*., 2002, 2004, 2006). Goff *et al* ont développé récemment 2 tests ELISA par compétition (cELISA) spécifiques d'espèce utilisant soit des épitopes B localisées dans la partie C-terminale de RAP-1 de *B. bovis*, soit une RAP de *B.*

bigemina. Les résultats montrent que les sensibilités de ces 2 méthodes sont supérieures à 91,1% et les spécificités supérieures à 98,3%. Après une application par différents laboratoires, il a été montré que ces 2 techniques cELISA sont très fiables et ont tous les caractéres nécessaires pour une validation internationale (Goff *et al*., 2003, 2006, 2008). Un autre antigène bien identifié est la protéine P50 de *B. gibsoni* (Fukumoto *et al*., 2001a). La protéine recombinante correspondante exprimée dans *Escherichia coli* ou dans des baculovirus a une bonne antigénicité et spécificité et n'entraîne pas de réaction croisée avec *B. canis* chez les chiens (Fukumoto *et al*., 2001a, 2003b, 2004 ; Verdida *et al*., 2004, 2005). L'antigène de mérozoïte P200 identifié chez *B. bigemina* est également un candidat pour permettre la distinction entre *B. bovis* et *B. bigemina* (Tebele *et al*., 2000 ; Altangerel *et al*., 2009). Beaucoup d'autres antigènes de sérodiagnotic ont été étudiés : MSA de *B. bovis*, TRAP, P12, 22, 29, 32, 57 de *B. gibsoni*, BoP29 de *B. orientalis* et BC134 de *B. caballi* (McElwain *et al*., 1998 ; Fukumoto *et al*., 2003a ; Tamaki *et al*., 2004 ; Bono *et al*., 2008 ; Konishi *et al*., 2008 ; Goo *et al*., 2008, 2009a, 2009b ; Aboge *et al*., 2007a, b ; Zhou *et al*., 2009). En ce qui concerne la babésiose ovine, aucune technique ELISA bien standardisée et utilisée dans des études à long terme n'est référencée, excepté celle décrite par Duzgun *et al*. (1991) utilisant un antigène synthétique de *B. bovis* pour détecter les infections à *B. ovis*.

3. Techniques utilisées pour le diagnostic de la babésiose en Chine

L'examen microscopique d'étalements sanguins colorés au May-Grünwald-Giemsa est la technique la plus couramment utilisée pour diagnostiquer la babésiose en phase aiguë en Chine. Différentes techniques sérologiques ont été également mises au point pour le diagnostic de la babésiose bovine et équine (IFAT, CFT, test d'inhibition de l'hémagglutination, ELISA) (Liu et Zhang, 1980 ; Wang et Liu, 1989 ; Deng et Liu. 1993 ; Luo *et al*., 1999 ; Shen *et al*., 2009) mais peu d'entre elles sont utilisées en routine. Des sondes ADN et la PCR ont été développées dans certains laboratoires mais il n'existe pas de kits disponibles sur le marché (Wu *et al*., 1998 ; Xue *et al*., 2007). Pour la babésiose ovine, il n'existe ni test sérologique ni méthode de biologie moléculaire excepté une PCR nichée pour détecter les babésies *B. motasi*-like (Sun *et al*., 2008).

PUBLICATIONS

Article N° 1 : A new ovine *Babesia* species transmitted by *Hyalomma anatolicum anatolicum*. (Une nouvelle espèces de *Babesia* de mouton transmise par *Hyalomma anatolicum anatolicum*)

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Ce travail a été réalisé au laboratoire de parasitologie vétérinaire de la province du Gansu, à Lanzhou.

Une *Babesia* « grande forme », appelée *Babesia sp.* Xinjiang, a été isolée d'un mouton splenectomisé sur lequel des tiques *Rhipicephalus sanguineus* et *Hyalomma anatolicum anatolicum* récoltées sur le terrain dans la région du Kashi, Région Autonome du Tibet Ganan dans le nord ouest de la Chine ont été mises à gorger. L'objectif de ce travail est de caractériser sa morphologie, son pouvoir pathogène chez le mouton et le bovin et sa phylogénie ainsi que d'identifier la tique vectrice.

Des moutons splénectomisés ou non et un veau splénectomisé ont été inoculés avec du sang d'un mouton infecté par *Babesia sp.* Xinjiang. Chez les moutons non splénectomisés, aucun signe clinique n'est enregistré et les parasites ne sont détectables qu'en utilisant la méthode LAMP. Chez les moutons splénectomisés, seule une réponse fébrile apparaît entre le 3ème et le 8ème jour après l'infection (jpi), durant 3 à 6 jours. Les signes cliniques de babésiose ne sont pas observés. Durant cette phase fébrile des parasites sur les étalements sanguins sont détectés. En revanche, les moutons splénectomisés et immunodéprimés par injection de corticoïde (dexaméthasone) sont morts du fait de l'infection. Chez le veau, ni symptômes ni parasites sur les étalements sanguins ne sont observés.

Des tiques adultes *Hyalomma anatolicum anatolicum* ont été mises à gorger sur des moutons splénectomisés et infectés par *Babesia sp.* Xinjiang. Les capacités de transmission de la descendance de ces tiques (larves, nymphes, adultes) ont été analysées en gorgeant chacun des stades sur ces moutons. Chez tous ces animaux, *Babesia sp.* Xinjiang a été détecté soit par examen microscopique d'étalement sanguin, soit par la méthode LAMP et certains ont exprimé des signes cliniques de babésiose.

Différentes formes morphologiques sont observées sur les étalement sanguins : forme ronde, forme en poire simple ou en paire, forme ovalaire…, forme classiquement observée au sein du genre *Babesia*. *Babesia sp.* Xinjiang est une *Babesia* de grande forme puisque les formes rondes ont un diamètre de 1,66 ± 0,3 µm et les formes en poire ont une dimension moyenne de 2,64 ± 0,37 µm x 1,43 ± 0,21 µm. En revanche, les caractères morphologiques de *Babesia sp.* Xinjiang sont très différents de *B. ovis*, *B. crassa* et *B. motasi*, les 3 *Babesia* décrites chez les moutons. Différentes formes parasitaires sont également décrites dans le tube digestif, dans les glandes salivaires, dans l'hémolymphe, dans les ovaires et dans les œufs des

tiques.

Enfin, l'analyse phylogénétique basée sur le gène de l'ARNr 18S montre une similarité inférieure à 82 % avec les autres espèces de *Babesia* décrites chez les ovins.

Au bilan, *Babesia sp*. Xinjiang est une nouvelle espèce de *Babesia* infectant les moutons. C'est une *Babesia* « grande forme », transmise par les différents stades de *Hyalomma anatolicum anatolicum*, faiblement pathogène chez le mouton et n'infectant probablement pas les bovins, quoique ceci doit être confirmé par la détection moléculaire du parasite chez des individus de cette espèce animale infectée expérimentalement.

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A new ovine *Babesia* species transmitted by *Hyalomma anatolicum anatolicum*

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ABSTRACT

The pathogenicity and morphology of a large *Babesia* species, *Babesia* sp. Xinjiang, are described here. The parasite has very low virulence for sheep, and caused no detectable clinical symptoms. Splenectomized sheep infected with the parasite showed mild fever and low parasitemia and would recover gradually. If splenectomized sheep were immuno-suppressed with dexamethasone, the parasitemia could reach 8.5%, and death occurred. A splenectomized calf could not be infected with the *Babesia* species. Paired parasites were the typical form of the *Babesia* species in erythrocytes and the average size of a pair of parasites was 2.42 (±0.35) μm × 1.06 (±0.22) μm. Merozoites were found in the gut, salivary gland, haemolymph, ovary and eggs of female *Hyalomma anatolicum anatolicum* engorged on sheep infected with the parasites. The results of experimental transmission showed that the larval, nymph and adult stages of *H. a. anatolicum* could transmit the *Babesia* species to sheep.

1. Introduction

A large *Babesia* species was isolated when a splenectomized sheep was infested with partially engorged *Rhipicephalus sanguineus* and *Hyalomma anatolicum anatolicum* collected on sheep, goats and cattle in Kashi region, Xinjiang Uygur Autonomous Region in northwestern China in 2001 as described by Guan et al. (2001). Microscopic examination of Giemsa stained blood films from the sheep suggested that the morphological characteristics of this new *Babesia* spp. were different from those of *B. motasi*, *B. ovis*, *B. crassa* and other *Babesia* spp. previously isolated infective for small ruminants in China. In addition, 18S rRNA gene sequences of the *Babesia* species were also different from the *B. ovis*, *B. motasi*, *B. crassa* and other strains isolated from China that had a close relationship with *B. motasi* (Liu et al., 2007).

In this study, we observed the pathogenicity of the *Babesia* species for splenectomized and intact sheep and the morphological characters of its erythrocytic stages. Parasite stages in the different organs of experimentally infected adult *H. a. anatolicum* are also described. In addition, the phylogeny of *Babesia* sp. Xinjiang and other bovine and ovine *Babesia* species were conducted based on the 18S rRNA gene sequences. Finally, experimental transmission studies were conducted using the offspring of *H. a. anatolicum* female ticks engorged on sheep infected with the *Babesia* species.

2. Materials and methods

2.1. Parasites

A novel *Babesia* sp. was identified in a splenectomized sheep infested by partially engorged *R. sanguineus* and *H. a. anatolicum* collected from sheep, goats and cattle in the Kashi region, Xinjiang Uygur Autonomous Region in northwestern People's Republic of China (Guan et al., 2001). Venous jugular blood was collected into Alsever's solution. Packed infected red blood cells were cryopreserved with 8% dimethyl sulphoxide in 5 ml aliquots as previously described by Dou et al. (1989). This batch of stabilates was named *Babesia* sp. Xinjiang and used for experimental studies described below after 18S rRNA gene sequencing.

2.2. Animals

Eighteen, 4–6 months old sheep and a 1 year-old calf were purchased from a *Babesia*-free region in Gansu Province and maintained at our laboratory in a quarantined stable. Tick free food and water were provided by technical staff. The calf and 16 sheep were splenectomized and all animals were monitored by microscopic examination of Giemsa stained blood smears 2–3 times

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per week for 1 month. Only animals confirmed negative for piroplasms were used in these experiments.

2.3. Ticks

Hyalomma anatolicum anatolicum ticks collected from field sheep, goats and cattle were maintained as a colony in our laboratory for three generations on rabbits, sheep and cattle. Splenectomized sheep and cattle were infested with fourth generation larvae, nymphs and adults and monitored for presence of parasites in Giemsa stained blood smears for 60 days to confirm the piroplasm-free status of the colony. Larval ticks from this generation were fed on rabbits and the engorged nymphs collected were maintained at 28 °C for development into unfed adults for use in the current study.

2.4. Infection of animals and observation of virulence

Sheep No. 2029 was inoculated intravenously with 10 ml of thawed cryopreserved blood containing the *Babesia* species. When the parasitemia reached 2%, jugular blood was collected from sheep No. 2029 into Alsever's solution and then cryopreserved with 8% dimethyl sulphoxide to produce a working cryostabilate. Sheep No. 3302, 3129, 1334 and 1211 were injected intravenously with 15 ml of the cryostabilate from No. 2029. Two spleen-intact sheep No. 3201 and 026 were each inoculated with 30 ml of cryostabilate. After inoculation, rectal temperatures, blood smears from the ear vein and clinical symptoms were taken and observed daily. The numbers of red blood cell and haemoglobin levels were examined three times at before experiment and three times from appearance to disappearance of parasite in blood smears, respectively. Blood smears were fixed with methanol, stained with Giemsa and examined microscopically for the presence of *Babesia*. The parasitemia were described as the number of infected cells per 1000 RBC (Luo et al., 2003). When the parasite was detected in blood smears, sheep No. 2029 and 3129 were injected intramuscularly with 10 mg/day dexamethasone for 3 days.

2.5. Transmission to cattle

Splenectomized calf No. 3134 was inoculated intravenously with 30 ml of cryostabilate from sheep No. 2029. The rectal temperature of the calf was measured daily. Blood smears were made daily from the ear vein, stained with Giemsa and monitored for the presence of haemoprotozoa for 60 days.

2.6. Infection of ticks

Haemoprotozoan-free adult ticks of *H. a. anatolicum* were put into a special cloth bags pasted on the shaved back of splenectomized sheep No. 1334, 1211 and 08102. On day three post-infestation, the sheep were each inoculated intravenously with 10 ml *Babesia* cryostabilate from sheep No. 2029. The rectal temperature of the sheep were measured daily. Blood smears were made daily from the ear vein stained with Giemsa and monitored for the presence of haemoprotozoa. Animals were injected with 10 mg/day dexamethasone for 3 days when the parasites were detected in the blood films (Fig. 1). Engorged female ticks were collected daily from infected sheep, placed in individual tubes and maintained at 28 °C and 70–90% relative humidity for oviposition.

2.7. Transmission of Babesia sp. Xinjiang to sheep by ticks

2.7.1. Screening for positive ticks

Subsamples of each egg batch from engorged female ticks dropped from sheep No. 1334, 1211 and 08102 were used to make smears. All smears were fixed with methanol, stained with Giemsa's stain and examined microscopically for the presence of *Babesia*. Only larval ticks derived from batches of eggs infected by the *Babesia* species were used in future transmission experiments.

2.7.2. Transmission by larvae

About 2000 infected larvae from sheep No. 1211 were placed on individual splenectomized sheep No. 1227 and 1243, respectively (Fig. 1). After infestation, rectal temperatures were measured and Giemsa stained blood smears from ear veins were examined for the presence of haemoprotozoa every day. During the patent period, parasitemias were calculated according to the method described by Luo et al. (2003). Engorged larvae were collected and maintained at 28 °C and 80–90% relative humidity for moulting.

2.7.3. Transmission by nymphs

Four splenectomized sheep were divided into two groups. In experiment 1, sheep No. 1230 and 2246 were each infested with about 2500 unfed nymphs derived from sheep No. 1227 and 1243 (see Fig. 1 and section above "Transmission by larvae"). In experiment 2, larvae from female ticks dropped from sheep No. 08102 (see Fig. 1) were engorged on rabbits and the unfed nymphs collected. About 3000 unfed nymphs were put on individual splenectomized sheep No. 08036 and 08062 (Fig. 1). Post infestation, 5 ml jugular blood was bled from sheep No. 08036 and 08062 every 2 days for genomic DNA extraction. Examination of animals and collection of engorged ticks were performed as described in "Transmission by larvae".

2.7.4. Transmission by adults

Two splenectomized sheep were used for each of two experiments. In experiment 3, infected larvae from females dropped from sheep No. 1334 were fed on rabbits (see Fig. 1). Engorged nymphs were collected and maintained in the laboratory for moulting as described in "Transmission by larvae". Sixty unfed adults were applied to splenectomized sheep No. 1321 and 1324. In experiment 4, sheep No. 2204 and 1216 were each infested with 150 adults similarly derived from females dropped from sheep No. 1211 (Fig. 1). Examination of animals was the same as above.

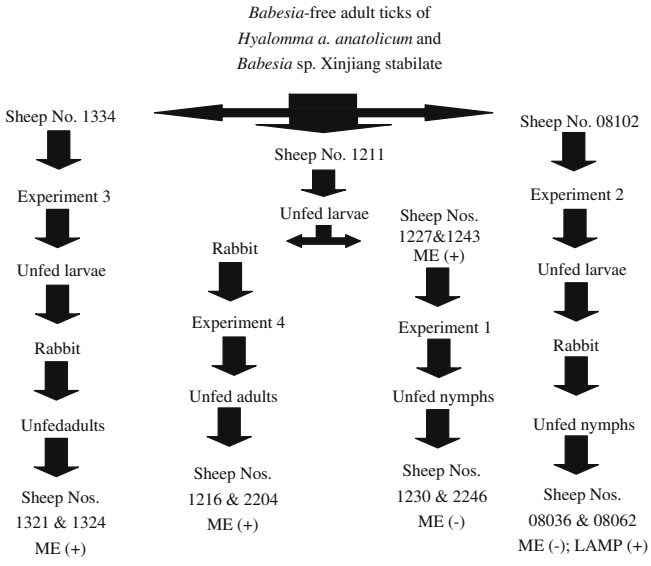

Fig. 1. A schematic diagram of experimental transmission to sheep. Note: ME (+) = Microscopic examination positive; ME (−) = Microscopic examination negative; LAMP (+) = LAMP examination positive.

2.7.5. Detection with LAMP

Genomic DNA was extracted from 300 μl packed erythrocyte pellet subsamples from blood collected from sheep No. 08036 and 08062 (see Section 2.7.3). DNA samples were amplified with the loop-mediated isothermal amplification (LAMP) method using specific primer BXJLAMP for 18S rRNA gene (Guan et al., 2008). The LAMP products were diluted appropriately with distilled water and amplified by PCR with primers that bind to the F2 region (5′-CGGG TTTCGTCTACTTCG-3′) and to the complementary strand of B1c region (5′-CCGTTCCTATTAACCATTACCA-3′). The PCR products were cloned into pGEM-T Easy vector (Promega, USA), and transformed into *Escherichia coli* JM109 competent cells for sequencing by the BigDye Terminator Mix of TakaRa Company (China).

2.8. Morphological observations of Babesia erythrocytic stages

While parasitaemias were patent, blood smears were collected daily from all animals, stained with Giemsa and parasites examined. The morphological characteristics were observed using methods described by Yin et al. (1996). Microscopic measurements were made of 500 pairs, 200 singles and 100 round parasites as suggested by Uilengberg et al. (1980) with an Olympus microscope BX51 and photographed with an Olympus DP71 Digital Camera System.

2.9. Morphological observations of Babesia in ticks

A batch of engorged females was collected from sheep No. 1334 and 1211 on 1 day and each day there after 2–3 of them were dissected and examined daily from the first day to end of oviposition. The legs of ticks were cut off and the excreted haemolymph was used to prepare smears. The salivary gland, midgut and ovary were separated under a dissecting microscope and smears made. Smears of eggs were also made when the ticks began laying eggs. All smears were fixed with methanol and stained with Giemsa. Parasite morphology and measurement was conducted (Bai et al., 1996) with Olympus microscopy BX51 and photographed with Olympus DP71 Digital Camera System.

2.10. Phylogeny

The sequences of 18S rRNA gene of Chinese *Babesia* species and other *Babesia* species are available in GenBank database (Liu et al., 2007; Luo et al., 2005). The original information of ovine and bovine *Babesia* spp. available in GenBank database included in this study is listed in Table 3. Alignment and analysis of 18S rRNA gene sequences were done with the MegAlign component of the DNAstar programme (Version 4.01 DNASTAR, Madison, WI). The MegAlign component performed multiple sequence alignments using the program algorithm of the Clustal V method and phylogenetic trees were inferred.

3. Results

3.1. Observation of virulence

Five splenectomized sheep (No. 2029, 3302, 3129, 1334 and 1211) and two intact sheep (No. 3201 and 026) were infected with *Babesia* sp. Xinjiang infected blood cryopreserved in liquid nitrogen. In splenectomized sheep (No. 2029, 3302, 3129, 1334 and 1211), the febrile responses appeared from third to eighth day post-infection and lasted for 3–6 days with the maximum temperature reaching 41.5 °C. During the febrile response, *Babesia* was detected in blood smears on days 3–9 after infection. The parasitemia of sheep No. 2029 and 3129 reached 50 and 85 parasites per 1000 RBC, respectively, because they were immuno-suppressed with dexamethasone. Not all animals showed typical clinical symptoms of babesiosis, e.g., haemoglobinuria and icterus. In sheep No. 2029 and 3129, the more serious symptoms of depression, lack of appetite, recumbency, serious anemia, and weight loss followed by death and were observed. No parasites or clinical symptoms were detected in the intact sheep No. 3201 and 026 after infection but parasites were detected in the second and third week after infection using the LAMP method (details in Guan et al., 2008). The numbers of red blood cell and haemoglobin levels of three splenectomized sheep No. 3302, 1334 and 1211 were monitored during 60 days and had decrease in different degree comparing with those of before experiment, which demonstrated the symptoms of anemia occurred in those sheep. The details are showed in Table 1.

3.2. Transmission to cattle

After the splenectomized calf was inoculated with stabilate of *Babesia* sp. Xinjiang, parasites were not detected in Giemsa stained thin blood smears during a 60-day monitoring period. Therefore, we considered that the calf was not infected by this *Babesia* species. The conclusion whether or not cattle could be infected by this *Babesia* species should be confirmed with the monitoring by molecular detection method.

3.3. Transmission of Babesia sp. Xinjiang to sheep by ticks

3.3.1. Transmission by larvae

Four to 8 days post-infestation, splenectomized sheep No. 1243 and 1227 produced approximately 2300 and 2500 engorged larval ticks, respectively. On day 11 and 14 post-infestation, the sheep

Table 1
The results of sheep infection with *Babesia* sp. Xinjiang.

Animal number	Condition	Latent period (days)	Maximum parasitemia (per1000RBC)	Duration of parasitemia (days)	Detection with LAMP	Death	Febrile response		Haematology			
							Duration of fever (days)	Maximum febrile response (°C)	RBC (10^4/μl)		Hb (g/dl)	
									AE	BE	AE	BE
2029	Splenectomized*	4	50	5	ND	+	3	40.8	ND	ND	ND	ND
3302	Splenectomized	5	2	3	ND	—	3	41.4	902	1270	7.3	9.5
3129	Splenectomized*	3	85	5	ND	+	5	41.3	ND	ND	ND	ND
1334	Splenectomized	9	2.5	5	ND	—	6	41.5	637	1238	5.7	10.3
1211	Splenectomized	3	2.5	12	ND	—	6	40.9	540	1179	6.3	9.7
3201	Intact	—	0	0	+	—	0	39.8	ND	ND	ND	ND
026	Intact	—	0	0	+	—	0	39.6	ND	ND	ND	ND

Note: ND, not done; —, negative; +, positive; *, immuno-suppressed with dexamethasone; BE, before experiment; AE, after experiment.

Table 2
The results of experimental transmission *Babesia* sp. Xinjiang to sheep by *H. a. anatolicum*.

Animal number		Tick		Outcome					
Source of infected ticks	Transmission	Stage	Number of engorged ticks collected	Detection of *Babesia* in blood smears	Detection of *Babesia* with LAMP	Prepatent period (days)	Maximum febrile response (°C)	Period of parasitemia (days)	Maximum parasitemia (per 1000RBC)
1334	1321	Adult	23	+	ND	18	41.5	4	0.17
	1324	Adult	25	+	ND	18	40.9	5	0.1
1211	1243*	Larvae	2300	+	ND	11	>42.0	10	10
	1227	Larvae	2500	+	ND	14	41.8	3	0.6
	2246	Nymph	1150	–	ND	–	<40.0	0	0
	1230	Nymph	1350	–	ND	–	<40.0	0	0
	2204	Adult	112	+	ND	12	41.9	7	27
	1216	Adult	56	+	ND	13	41.4	3	20
08102	08036	Nymph	2000	–	+	31	<40.0	–	–
	08062	Nymph	2000	–	+	5	<40.0	–	–

Note: ND, not done; –, negative; +, positive; <40.0, less than 40.0 °C; >42.0, more than 42.0 °C; *, died.

had febrile responses and *Babesia* was, respectively, detected in the blood films. Parasites disappeared in blood smears from sheep No. 1227 on 17th day post-infestation after parasitemia lasted 3 days. But sheep No. 1243 died on day 26 post-infestation (Table 2).

3.3.2. Transmission by nymphs

In experiment 1, about 2500 nymphs were put into a bag on the back of each splenectomized sheep No. 1230 and 2246. One thousand three hundred and fifty and 1150 engorged ticks were collected during days 5–8 post-infestation. In experiment 2, sheep No. 08036 and 08062 were each infested with 3000 nymphs and 1456 and 968 engorged ticks were dropped during 4–8 days post-infestation period. We did not detect *Babesia*-like parasites in blood smears or any clinical symptoms of babesiosis during the 60-day monitoring period for each experiment (Table 2).

3.3.3. Transmission by adults

In experiment 3, 23 and 25 engorged females were collected from sheep No. 1321 and 1324, respectively, during days 5–9 after infestation (see Fig. 1 and Table 2). On 18th day post-infestation, parasites were found in the blood smears of both animals and they had elevated temperatures. In experiment 4, sheep No. 2204 and 1216 that were each infested with 150 ticks produced 112 and 56 engorged female ticks, respectively. Parasites were detected in blood smears from the sheep on days 12 and 13, respectively (Table 2).

3.3.4. Detection with LAMP

Genomic DNA of the *Babesia* species was amplified successfully with the LAMP method on fifth and 20th day in blood from No. 08062 sheep and on 31st day in No. 08062 post infestation. The results of sequencing showed that the amplified DNA matched the target location of 18S rRNA gene of *Babesia* sp. Xinjiang (Table 2) demonstrating that the nymph ticks could also transmit the *Babesia* species to sheep.

3.4. Morphological characteristics of Babesia sp. Xinjiang in erythrocytes

The parasite described here is a large *Babesia* species infective to sheep. A wide variety of morphological forms was found within infected erythrocytes, including ring form, single piriform, paired piriform, trifoliate, rod shaped, budding forms and oval forms (Fig. 2). In most cases, the parasites were single and paired piriform.

The ring form: The whole parasites looked like the rings or circles when the parasite was single (Fig. 2, plates 1–4). The paired parasites were similar to the spectacle frames (Fig. 2, plates 5, 6). The diameters varied from 1.27 to 2.56 μm with a mean of 1.66 ± 0.3 μm. Nuclei were stained red–purple and located at the margin of the parasites.

Single piriform: The morphological shape seemed as a pear, one end was wide and obtuse and the other end was narrow. In most cases, the plasma of parasites was light blue and the nucleus located at the obtuse, acute end or on the margin (Fig. 2, plates 14–17). Some times, the whole parasite consisted mostly of nucleus, so they looked like the red–purple seed of a sliced pear (Fig. 2, plates 13, 18). The size of the piroplasms varied from 1.93 to 3.99 × 1.07 to 2.06 μm with mean dimensions of 2.64 (±0.37) μm × 1.43 (±0.21) μm.

Paired piriform: This paired form of the parasites resembled two sliced pears joined at the pointed ends. The majority of the pairs were at an acute angle (Fig. 2, plates 8, 9). Generally parasitized erythrocytes only contained one pair of parasites but occasionally more than one pair was observed in erythrocytes (Fig. 2, plates 23, 24). The plasma of parasites was mostly light blue and the red–purple nucleus located at both ends or on margins (Fig. 2,

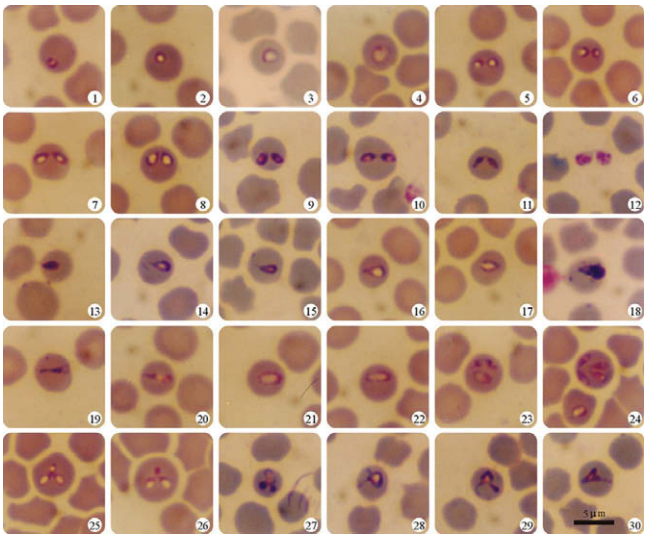

Fig. 2. Photomicrographs of *Babesia* sp. Xinjiang in ovine blood smears. Giemsa's stain. Bar = 5 μm.

plates 7–10). The nucleus of some parasites filled the whole cell (Fig. 2, plates 11, 12). The typical form was slender (Fig. 2, plates 7, 10, 24) but wide parasites were also presence in the smears (Fig. 2, plate 8). The parasites were sometimes observed outside of the erythrocytes some times (Fig. 2, plate 12). The size of parasites ranged from 1.66 to 3.44 × 0.49 to 1.92 μm, with the average being 2.42 (±0.35) μm × 1.06 (±0.22) μm.

Oval form: This parasite was similar to the single piriform except there were not the acute ends. The nucleus was located on the margin and the plasma was light blue (Fig. 2, plates 21, 22).

Three-leafed-shaped: A nucleus extended from the linking point of a paired piriform parasites (Fig. 2, plate 26) and became large gradually, and the plasma was formed around the nucleus, and finally developed into a single piriform parasite. The newly formed piriform and original pair piriform parasite formed a three-leafed-shaped parasite (Fig. 2, plate 25).

Budding forms: Same as the three-leafed-shaped parasites, the nucleus extruded out of the mother parasites (Fig. 2, plates 27–30).

Rod shaped: The parasites were slender. The length was almost same as the diameter of erythrocytes. Nuclei were located at two ends or whole parasite (Fig. 2, plates 19, 20).

3.5. Morphological observation in ticks

Parasites were detected in the gut, salivary gland, haemolymph, ovary and egg of *H. a. anatolicum* ticks after dissection. Unfortunately, the precise times the parasites were present in various tissues and organs of infected ticks could not be determined because the infection rate was relatively low.

3.5.1. In gut

In the first several days after engorgement, some parasite piriform and spherical stages without pellicle were observed in the gut lumen. (Fig. 3, plates 1–3). However, developing parasites, for example "strahlenkörper" and gametocytes were not found.

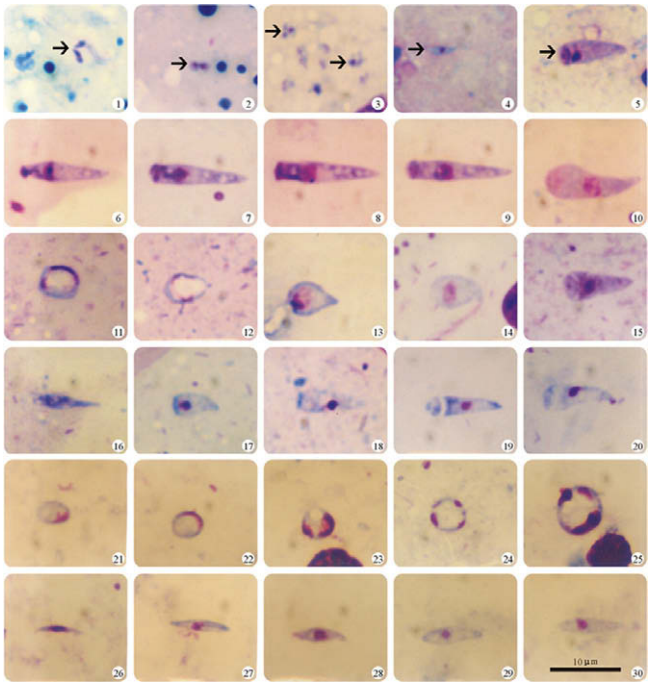

Fig. 3. Photomicrographs of Giemsa's stained smears of *Hyalomma anatolicum anatolicum* Gut, salivary gland, haemolymph, ovary to show *Babesia* sp. Xinjiang. Plates1–3: Gut contents; Plates 4, 5: In the salivary gland; Plates 6–10: Haemolymph; Plates 11–20: Ovary; Plates 21–30: Egg. Giemsa's staining. Bar = 10 μm.

3.5.2. In salivary gland

We found two types of merozoites (In some publications, the motile and club-shaped parasites are named sporokinetes while some authors call them merozoites. In this paper, we call them merozoites) in salivary gland smears. One type was small with two ends acute, being 4.0 × 1.0 μm in size. The nucleus was located medially in these merozoites (Fig. 3, plate 4). The other larger merozoites dimensions were 9.0 × 3.0 μm. One end was blunt and contained a single nucleus (Fig. 3, plate 5).

3.5.3. In haemolymph

Many large merozoites were observed in haemolymph and were morphologically similar to the large merozoites in the salivary gland. Most of them had one centrally located nucleus with a red–purple stained cap on the blunt end. The average size was 11.8 × 2.5 μm (Fig. 3, plates 6–10).

3.5.4. In ovary

Three types of merozoites were observed in the ovary. The first type was ring shaped, ranging from 4.0 to 5.0 μm in size. The merozoites central region was transparent and a single red–purple staining nucleus was located peripherally (Fig. 3, plate 11). The second was pear shaped and the average size was 5.5 × 4.0 μm. Dark staining single nuclei varied in location (Fig. 3, plates 12–14). The third was typical of merozoites described above being 6.0–10.0 × 2.0–3.0 μm with a single red–purple staining centrally located nucleus (Fig. 3, plates 15–20).

3.5.5. In egg

There were two types of merozoites found in eggs. The ring shaped merozoites had one, two or three nuclei in the margin and central parts were transparent. The size was 4.0–7.0 μm (Fig. 3, plates 21–25). Another type was tapering at each end, same as spindle. The fusiform merozoites always had one nucleus in the center. The average size was 7.0 × 2.0 μm (Fig. 3, plates 26–30).

3.6. Alignment and analysis based on 18S rRNA gene sequences

The percent identity among *Babesia* species infective for sheep and cattle was determined and a phylogenetic tree was constructed with the Chinese parasites and other valid *Babesia* species available in GenBank (Table 3 and Table 4 and Fig. 4). The phylogenetic tree showed that these parasites could be divided into five clades. The first clade just included the Chinese ovine *Babesia* isolates that were recently designed to be *B. motasi* (Liu et al., 2007; Niu et al., 2009). The second contained the European *B. motasi* while the third contained *B. crassa* alone. Isolates of *B. ovis* were deposited in the fourth clade. The fifth clade contained *B. orientalis*

Table 3
GenBank accession number, host and origins of the 18S rRNA gene sequences.

Species	Origin	Host	Accession number
Babesia sp. BQ1	Lintan (China)	Sheep	AY260181
Babesia sp. BQ1	Ningxian (China)	Sheep	AY260182
Babesia sp.	Madang (China)	Sheep	DQ159071
Babesia sp.	Tianzhu (China)	Sheep	DQ159072
B. motasi	Ameland (Netherlands)	Sheep	AY260179
B. motasi	Texel (Netherlands)	Sheep	AY260180
B. motasi	Spain	Sheep	AY533147
B. ovis	Spain	Goat	AY150058
B. ovis	Turkey	Sheep	AY260178
B. crassa	Iran	Sheep	AY260176
Babesia sp. Xinjiang	Xinjiang (China)	Sheep	DQ159073
Babesia U sp. Kashi 1	Xinjinag (China)	Cattle	AY726556
Babesia U sp. Kashi 2	Xinjiang (China)	Cattle	AY726557
B. orientalis	China	Buffalo	AY596279

Table 4
Percent identify of *Babesia* species from GenBank and Chinese *Babesia* spp. by Clustal V.

1	2	3	4	5	6	7	8	9	10	11	12	13	14		
	100	99.9	88.6	88.1	93.0	95.8	96.0	97.0	97.0	81.0	92.2	91.3	91.5	1	*B. motasi* Ameland (Netherlands)
		99.9	88.6	88.1	93.0	95.8	96.0	97.0	97.0	81.0	92.2	91.3	91.5	2	*B. motasi* Texel (Netherlands)
			88.2	88.0	92.8	95.6	96.0	96.8	96.8	81.7	92.3	91.9	92.2	3	*B. motasi* Spain
				99.0	88.6	87.8	87.4	88.5	88.4	81.2	90.3	91.0	91.3	4	*B. ovis* Turkey
					87.9	87.4	87.3	88.1	88.0	82.0	90.6	91.8	92.0	5	*B. ovis* Spain
						92.0	92.4	92.9	92.7	79.7	93.1	91.1	91.1	6	*B. crassa* Iran
							97.9	98.8	98.8	80.8	92.1	91.6	91.4	7	*Babesia* sp. BQ1 Lintan
								96.8	96.7	80.9	91.7	91.2	91.6	8	*Babesia* sp. BQ1 Ningxian
									99.9	81.8	93.2	92.7	92.4	9	*Babesia* sp. Madang
										81.6	93.1	92.6	92.3	10	*Babesia* sp. Tianzhu
											84.4	94.6	94.9	11	*Babesia* sp. Xinjiang
												98.0	97.2	12	*Babesia* U sp. Kashi 1
													98.9	13	*Babesia* U sp. Kashi 2
														14	*B. orientalis* China

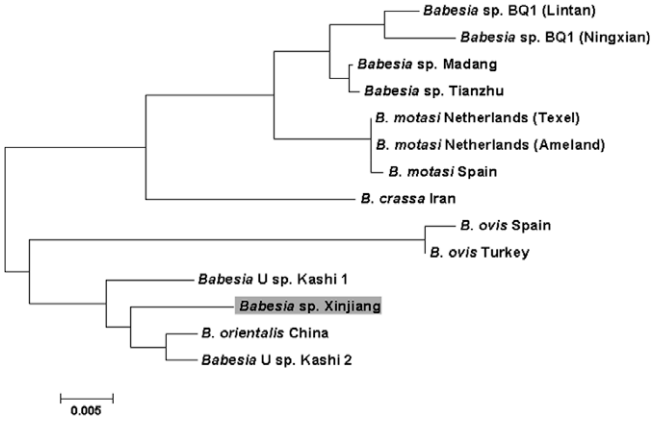

Fig. 4. Phylogenetic tree of *Babesia* species based on 18S rRNA gene.

infective to buffalo transmitted by *Rhipicephalus haemaphysaloides*, *Babesia* U sp. Kashi 1 and Kashi 2 isolated, respectively, from larval and adult *H. a. anatolicum* ticks, and *Babesia* sp. Xinjiang. The percent identities of *Babesia* sp. Xinjiang with *Babesia* U sp. Kashi 1 and Kashi 2 and *B. orientalis* were 84.4%, 94.6% and 94.9%, respectively.

4. Discussion

There are six *Babesia* species infective for cattle and buffalo, *B. bigemina*, *B. bovis*, *B. divergens*, *B. major*, *B. ovata* and *B. orientalis* that have been widely considered as valid species in the world (Friedhoff, 1988; Levine, 1985; Liu et al., 2005). In contrast, *Babesia* species infective for small ruminants have not had such rigorous taxonomic investigation. To date two *Babesia* species infective for small ruminants have been recognised as valid species, *B. motasi* and *B. ovis*, and the vector ticks are *H. punctata* and *R. bursa*, respectively (Alani and Herbert, 1980; Friedhoff, 1988, 1997; Levine, 1985; Uilengberg et al., 1980). In addition, Uilenberg (2006) considered that *B. motasi* may well consist of at least two species or subspecies, a low pathogenicity grouping in northern Europe and a high pathogenicity in southern Europe and the Mediterranean basin. *B. crassa* is another species isolated from Iran that infects sheep. This parasite is a large species; multiplying by quadruple division as well as binary fission (many red blood cells contain four parasites). It can be differentiated from *B. motasi* using serology. Its pathogenicity is low and it can also be differentiated from *B. motasi* and *B. ovis* using 18S rRNA gene sequences (Hashemi-Fesharki and Uilenburg, 1981; Hashemi-Fesharki, 1997; Schnittger et al., 2003). *Babesia crassa* is generally recognized as another valid *Babesia* species infective for small ruminants based on these data although its vector tick is still unknown. *Babesia taylori* and *B. foliata* were described 60 years ago in India (Ray and Raghavachari, 1941; Sarwar, 1935). Unfortunately, the parasites have not been reisolated and no further descriptions of the two *Babesia* species have been published.

In China, several *Babesia* isolates infective for small ruminants have been reported in the past two decades. Chen (1982) and Zhao et al. (1986) made the first reports of *Babesia* infecting small ruminants in China. They reported ovine babesiosis in Sichuan and Heilongjiang Province, respectively, and they identified the pathogens as *B. ovis*. However, as they neither isolated the pathogen nor kept the blood smears, we cannot be sure whether or not the pathogens were *B. ovis*. After that, several strains were isolated in China. These strains can be divided into two groups based on 18S rRNA gene sequences, one group includes *Babesia* sp. (Liaoning), *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. (Hebei), and the other just contains *Babesia* sp. Xinjiang (Liu et al., 2007). Recent analysis based on the ribosomal DNA internal transcribed spacer (ITS) sequences suggested that, except for *Babesia* sp. Xinjiang, all these strains should be considered to be *B. motasi* (Niu et al., 2009). This means that there are at least two *Babesia* species infective for small ruminants in the north of China, *B. motasi* and *Babesia* sp. Xinjiang. However, we know about the biology of these *Babesia* species. *Babesia* sp. BQ1 (Ningxian) is a large *Babesia*, and its vector is the tick *H. longicornis* and it is morphologically similar to *B. motasi*. *Babesia* sp. BQ1 (Lintan) was isolated from *H. qinghaiensis* and is a low virulent parasite strain. *Babesia* sp. Xinjiang was isolated from a sheep infested with the tick *R. sanguineus* and *H. a. anatolicum* collected from field (Bai et al., 2002; Guan et al., 2001; Guan et al., 2002).

In the present study, the preliminary biological characteristics of *Babesia* sp. Xinjiang are described. The results show that this *Babesia* species has almost no pathogenicity for intact sheep. No clinical symptoms presented in intact sheep after the animals were infected with the parasitized blood and even though the parasites were not detected in blood smears, they were detected using LAMP in the second and third week after infection (Guan et al., 2008). Infected splenectomized sheep showed clinical symptoms of fever, parasitemia and haemolytic anaemia but recovered naturally. The more serious symptoms, such as depression, lack of appetite, recumbency, serious anemia and weight loss, and even death, were presented if the animals were immuno-suppressed with injections of 10 mg dexamethasone per day. The highest parasitemia recorded was 8.5% and lasted between 3 and 12 days on the blood smears. If the parasitemia exceeded 5%, animals would die. The febrile responses lasted for 3–6 days with the maximum temperature recorded was 41.5 °C. The morphological characters in erythrocytic stages showed that the typical pair piriform was slender.

The size was 1.66–3.44 × 0.49–1.92 μm, and the averages were 2.42 (±0.35) μm × 1.06 (±0.22) μm. This species can be easily differentiated from the *B. motasi, B. ovis, B. crassa* and *Babesia* spp. that were reported by Bai et al. (2002) and Guan et al. (2002) in China based on virulence and morphological characteristics described here.

Babesia sp. Xinjiang could infect *H. a. anatolicum*. Parasites were observed in the gut, salivary gland, haemolymph, ovary and egg, which suggested that it could be transmitted transovarially and that *H. a. anatolicum* is vector. This was confirmed in experimental transmission studies which showed that larval and adult ticks could transmit *Babesia* sp. Xinjiang to the sheep to produce parasitaemias detectable by microscopic examination of Giemsa stained blood smears. In transmission studies with nymphs, we did not detect the parasites in blood smears within 60 days post-infestation, but genomic DNA could be detected by the LAMP method (Guan et al., 2008). The larval, nymph and adult ticks of *H. a. anatolicum* can experimentally transmit the parasite to sheep, although the transmission efficiency of the three stages is different. The adult and larval ticks efficiently transmit *Babesia* sp. Xinjiang to sheep, but the transmission rate of nymphal ticks is low. *H. a. anatolicum* can behave like two-host ticks on rodent hosts e.g., rabbits, under laboratory conditions. But normally the species has a three-host life cycle on sheep and cattle (Ghosh and Azhahianambi, 2007). We suppose that *H. a. anatolicum* has behavior of two-host ticks in field. The natural transmission pattern of this *Babesia* species in the field is from adults ticks to sheep. It is therefore not surprising that tick nymphs poorly transmit the *Babesia* species to sheep and may be an adaptation of this species of *Babesia* to ensure transmission to the correct vertebrate host. This hypothesis needs to be confirmed by further transmission studies with the different life stages of *H. a. anatolicum.*

The alignment of 18S rRNA gene sequences showed that *Babesia* sp. Xinjiang has less than 82.0% similarity with other ovine *Babesia*. It still has 84.4%, 94.6% and 94.9% percent identity with *Babesia* U sp. Kashi 1 and Kashi 2 and *B. orientalis*, although they were deposited in one clade in phylogenitic tree. Therefore, we could easily discriminate the *Babesia* species from the ovine *Babesia* spp. and *Babesia* U sp. Kashi and *B. orientalis* based on biological and molecular characteristics.

The results herein demonstrate that *Babesia* sp. Xinjiang is different from the other *Babesia* spp. infective for small ruminants previously reported in the world. In addition, the phylogenetic study based on the 18S rRNA gene sequence showed that the species is significantly different from other ovine *Babesia* spp. We report here a novel *Babesia* species infective for small ruminants in China.

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Article N° 2 : *Babesia* sp. BQ1 (Lintan): A molecular evidence of experimental transmission to sheep by *Haemaphysalis qinghaiensis* and *Haemaphysalis longicornis*. (*Babesia sp*. BQ1 (Lintan) : preuve moléculaire de la transmission expérimentale au mouton par *Haemaphysalis qinghaiensis* et *Haemaphysalis longicornis*)

Article accepté dans Parasitology International

Ce travail a été réalisé au laboratoire de parasitologie vétérinaire de la province du Gansu, à Lanzhou

Babesia sp. BQ1 (Lintan) est une *Babesia* isolée de sang de moutons naturellement infestés par *Haemaphysalis qinghaiensis*. L'objectif de ce travail est d'identifier les vecteurs de cet hémoprotozoaire. Des adultes de tiques des espèces *Haemaphysalis qinghaiensis* et *Haemaphysalis longicornis* ont été expérimentalement infectés par *Babesia sp*. BQ1 (Lintan) en les gorgeant sur un mouton parasité. Les capacités de transmission de la descendance de ces tiques (larves, nymphes, adultes) ont été analysées en gorgeant chacun des stades sur des moutons splénectomisés. Chez tous ces animaux, aucun parasite n'a été observé sur les étalements sanguins et aucuns n'ont exprimé des signes cliniques de babésiose. En revanche *Babesia sp*. BQ1 (Lintan) a été détecté par PCR nichée à certaines dates entre le 7ème et le 31ème jour après gorgement. Ce travail montre que *Babesia sp*. BQ1 (Lintan) peut être transmis par les larves, les nymphes et les adultes de *Haemaphysalis qinghaiensis* et de *Haemaphysalis longicornis*.

Babesia sp. BQ1 (Lintan): A molecular evidence of experimental transmission to sheep by *Haemaphysalis qinghaiensis* and *Haemaphysalis longicornis*

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Succinct title : Transmission of *Babesia sp*. BQ1 (Lintan) by H. *qinghaiensis* and *H. longicornis*,

Abstract

Ovine babesiosis is an economically important disease induced by tick transmitted haemoparasites throughout the world. In China, several ovine *Babesia* strains have been isolated from field-collected ticks or sheep blood during the last two decades but little is known about the vector ticks and transmission pattern. *Babesia* sp. BQ1 (Lintan) is a *Babesia* strain infective for sheep and goats, isolated from blood of sheep experimentally infested with *Haemaphysalis qinghaiensis* collected in field. In the present study, we explored the experimental transmission of *Babesia* sp. BQ1 (Lintan) to sheep by *H. qinghaiensis* and *H. longicornis*. Based on the evidence from microscopic examination and nested PCR, it suggested that *H. qinghaiensis* and *H. longicornis* could be the potential vector ticks of *Babesia* sp. BQ1 (Lintan) and larvae, nymphs and adults of both tick species were able to transmit *Babesia* sp. BQ1 (Lintan) to sheep. Parasites could be detected in the blood, by specific nested PCR, for one month post-infestation.

Key words: *Babesia* sp. BQ1 (Lintan), *Haemaphysalis qinghaiensis*, *Haemaphysalis longicornis*, experimental transmission, nested PCR

Ovine babesiosis is an economically important tick-borne disease, caused by *Babesia ovis*, *B. motasi*, *B. crassa* and several unrecognized *Babesia* spp.. Infected sheep and goats develop high fever, depression, and anemia, with mortality in severe cases, especially when infected by *B. ovis* [1-4]. Chen (1982) and Zhao et al. (1986) first reported ovine babesiosis in the Sichuan and Heilongjiang provinces of China in the 1980's and, on the basis of parasite morphology and clinical appearance of the sick animals, suspected that the pathogens were *B. ovis* [5, 6]. Since then, our laboratory has isolated several large *Babesia* strains from field-collected blood or ticks from different regions in China [7-11]. These strains can be divided into two groups, based on the 18S rRNA and ITS gene sequences, namely Chinese *B. motasi* (including *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. (Liaoning), *Babesia* sp. (Tianzhu), *Babesia* sp. (Madang), and *Babesia* sp. (Hebei)), and *Babesia* sp. Xinjiang [11, 12]. However, recent studies based on cross reaction analysis between the different strains and on the Hsp90 gene sequence have shown that Chinese *B. motasi* can be separated into two subgroups, one with *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. (Tianzhu), the other with *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. (Hebei) (unpublished data). This means that the Chinese *B. motasi* probably contains at least two species or subspecies, which is consistent with Uilenberg's hypothesis for *B. motasi* in Europe [13].

Little information is available about the biology of these Chinese *Babesia* strains. *Babesia* sp. BQ1 (Ningxian), isolated from the blood of diseased sheep in the field, is highly virulent both to sheep and goats and shows similar morphology to European *B. motasi*. The vector tick is *Haemaphysalis longicornis* and both larvae and nymphs can successfully transmit the parasite to sheep [10]. *Babesia* sp. Xinjiang isolated from a sheep infested with the ticks *Rhipicephalus sanguineus* and *Hy. a. anatolicum,* collected in the field, showed low virulence for sheep and very different morphological features from those of "Chinese *B. motasi*", European *B. motasi* and *B. crassa*. Experimental transmission studies confirmed that the larvae, nymphs and adults of *Hy. a. anatolicum* could transmit the parasite to sheep [9, 14-16]. For the other strains, it is just known that they were isolated from sheep blood collected in the field where *Haemaphysalis* are the dominant tick species [11].

In order to better understand ovine babesiosis in China, the taxonomy and biological characteristics of these *Babesia* strains must be clarified. Our study focused on *Babesia sp.* BQ1 (Lintan). This parasite has occasionally been isolated from splenectomized sheep infested by adult ticks of *H. qinghaiensis* collected on vegetation in Lintan County of the Gannan Tibetan Autonomous Region [8] and recently, *H. qinghaiensis* has been proved to be naturally infected by *Babesia* sp. BQ1 (Lintan) in this area [17]. It is of low virulence to sheep and goats [8]. We report our attempts to clarify the vector tick situation and transmission pattern by experimental transmission of *Babesia* sp. BQ1 (Lintan) to sheep with *H. qinghaiensis* and *H. longicornis*.

Fourteen 5-6 month-old lambs, purchased from Jingtai county of Gansu province which

is babesiosis-free, were splenectomized 1 month before the experiments and raised in a quarantined stable at the vector and vector-borne disease (VVBD) lab of Lanzhou Veterinary Research Institute (LVRI), China. Tick-free food and water were provided ad libitum. All animals were monitored by light microscopic examination (ME) of Giemsa stained blood smears and nested PCR (nPCR) specific for *B. motasi* [17], once a week for 1 month. All animals showed negative for piroplasms and were used in this study. Ticks infection and experimental transmissions were carried out using the methods previously described by Guan et al. (2009) [16]. Batches of unfed adults of *H. qinghaiensis* and *H. longicornis,* confirmed as *B. motasi*-free by nPCR, were each bred on sheep No. 08044 and 08114 (Fig 1). On the third day, each sheep was subcutaneously inoculated with 15 ml of blood from *Babesia* sp. BQ1 (Lintan)-infected sheep, cryopreserved in liquid nitrogen. The sheep were then monitored by ME for the presence of *Babesia* on blood smears and injected with 10 mg of dexamethasone per day for 3 days as soon as *Babesia* were detected on the blood smears. Engorged female ticks were collected into tubes when the parasites were observed and maintained at 28°C and 70–90% relative humidity for oviposition and hatching. The hatched larvae were kept at room temperature for two weeks. Unfed larvae were bred on healthy rabbits to obtain unfed nymphs and the unfed nymphs were then cultivated on healthy rabbits to obtain adults (Fig 1). Only those batches of ticks developed from the females engorged on sheep 08044 and 08114 when parasitemia was more than 4% were used in transmission experiments. Experimental transmissions were performed with unfed larvae, nymphs and adults of *H. qinghaiensis* and *H. longicornis* as shown in Figure 1. Each splenectomized sheep infested with about 10000 larvae, about 3000 nymphs or 1000 adult ticks was inoculated with 10 mg of dexamethsone per day at 7-9 days post-infestation (Table 1). The animals were examined daily for body temperature, clinical appearance (appetite, behaviour and diarrhea) and by ME of blood smears made from ear venous blood and jugular blood, twice a week for two months. Genomic DNA was extracted from each sample of whole blood (WBgDNA) and frozen at -20°C. Detection of *Babesia* sp. BQ1 (Lintan) DNA (BQ1DNA) from WBgDNA was performed by nested PCR as described by Sun et al. (2008) [17]. Briefly, primers TBall (universal for *Babesia* and *Theileria*) and Bm (universal for all *B. motasi*) (TBall-134-S: 5'-CATGGATAACCGTGCTAATT-3', TBall-892-AS: 5'-ATCGTCTTCGATCCCCTAACT-3'; Bm-202-S 5'- TAAACCAATTTGTTGGT -3', Bm-495-AS 5'-TCTGCCCAGGGTTTAAGTCGG -3') was used to detect *Babesia* sp. BQ1 (Lintan) DNA (BQ1DNA) from WBgDNA. The primary PCR cycle was practiced with initial step at 94 °C 3 min, 40 cycles at 94 °C for 30 s, 55 °C for 40 s and 72 °C for 1 min with a final extension step at 72 °C for 10 min. The nested PCR was used 1.0 μl of the primary product as template and was as follows: an initial step at 94 °C for 3 min, 40 cycles at 94 °C for 30 s, 52 °C for 40 s and 72 °C for 1 min with a final extension step of 72 °C for 10 min. Amplification was repeated and the PCR products were routinely cloned into pGEM-T Easy vector (Promega,

USA). The recombinant vectors were then transformed into *Escherichia coli* JM109 competent cells for sequencing (TakaRa Company, China). Alignments were made between the obtained sequences and the 18S rRNA gene sequence of *Babesia* sp. BQ1 (Lintan) (GenBank accession number no. AY260181).

Both *H. qinghaiensis* and *H. longicornis* can transmit *Babesia* sp. BQ1 (Lintan) under experimental conditions. Sheep were infested with the three tick stages of *H. qinghaiensis* and *H. longicornis* that developed from the engorged females on the *Babesia* sp. BQ1 (Lintan) infected sheep with more than 4% parasitemia (the maximum of parasitemia were 40% and 20% for each sheep No. 08044 and 08114 on 13^{th} and 5^{th} day post infection.). No *Babesia* parasites were observed on blood smears for 60 days p.i. (Table 1) but the nPCR results indicated that a nucleotide fragment of 294bp was amplified from certain samples (Figure 2). The extracted DNA fragments were sequenced. Only those sequences identical to the 18S rRNA gene sequence of *Babesia* sp. BQ1 (Lintan) (GenBank accession no. AY260181) were considered as positive amplifications. Genomic DNA of *Babesia* sp. BQ1 (Lintan) could be detected in the jugular blood of all experimental sheep from the 7^{th} to 32^{nd} day p.i.. Furthermore, all 3 stages (larvae, nymphs and adults) of these ticks had transmitted the protozoan to the sheep. As shown in Table 1, none of the infected sheep showed any clinical signs of babesiosis, as depression or anorexia, and their temperature was always below 40 °C, possibly because of the low virulence of *Babesia* sp. BQ1 (Lintan).

However, the animals experimentally infected by *H. qinghaiensis* or *H. longicornis* seemed to be infected by a low level of *Babesia* sp. BQ1 (Lintan). Indeed, gDNA of parasites was not observed regularly during the study period (table 1), probably because the concentration was below the level of detection and no parasites were detected on smears of blood from the ear vein. This could be explained by a low level of experimentally infected- *H. qinghaiensis* or *H. longicornis* and/or few *Babesia* sp. BQ1 (Lintan) in each infected tick. Sun et al. (2008) [17] reported that the level of naturally infected-*H. qinghaiensis* was low (0.74 %). In addition, Hodgson (1992) [18] demonstrated that temperature and the time length of quiescence could affect the transmission of *B. bigemina* in *Boophilus microplus*. The life cycle of *H. qinghaiensis* in natural conditions is not well known. Under laboratory conditions, *H. qinghaiensis* has always bred one generation per year. However, by the third generation, the engorged females lay insufficient eggs and these eggs are difficult to hatch, which suggests that the natural life cycle may have been remodelled under these conditions. Thus, it is still necessary to explore the natural life cycle of *H. qinghaiensis* and identify the effects, on future studies, of the ability of *H. qinghaiensis* to transmit *Babesia* when its life cycle has been artificially modified.

In conclusion, we provide some information about the vector tick of *Babesia* sp. BQ1 (Lintan). *Babesia* sp. BQ1 (Lintan) genomic DNA could be detected in blood of sheep infected with *H. qinghaiensis* or *H. longicornis*. This data suggests that *H. qinghaiensis* and

H. longicornis could be the potential vector ticks of *Babesia* sp. BQ1 (Lintan) and that all larvae, nymphs and adults are able to transmit the parasite to sheep.

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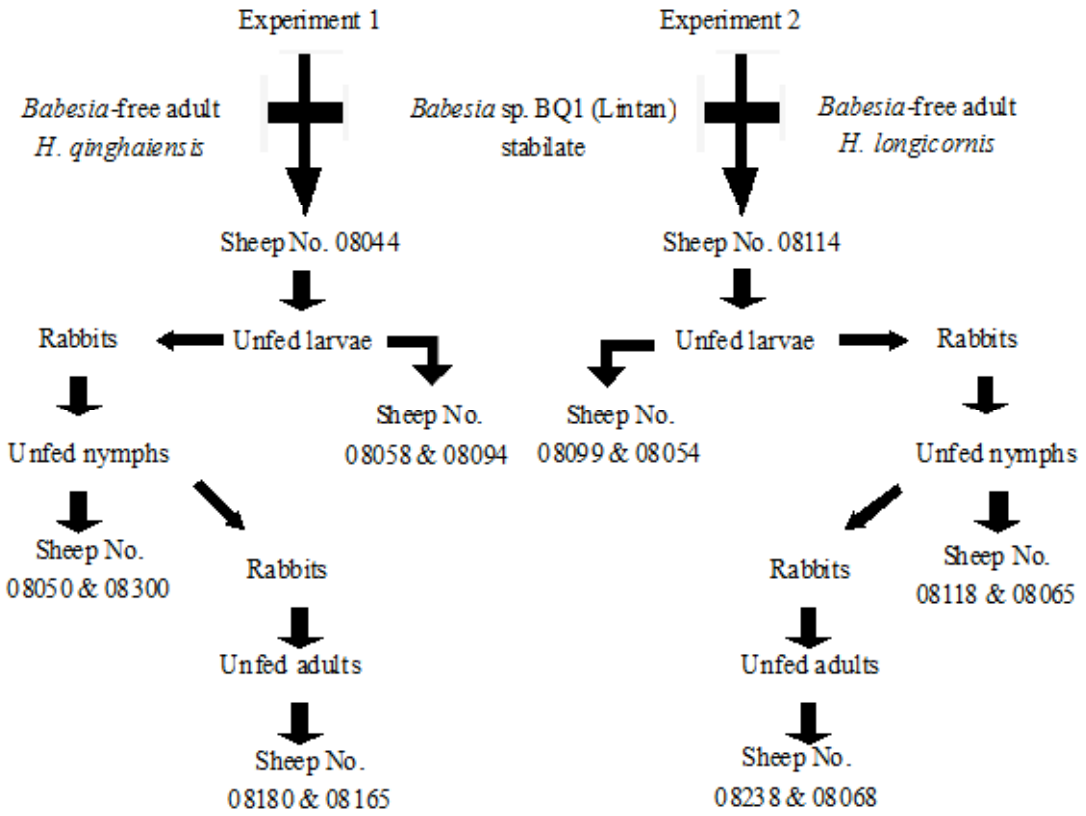

Figure 1. Diagram of the experimental transmission of *Babesia* sp. BQ1 (Lintan) to sheep with *H. qinghaiensis* and *H. longicornis*.

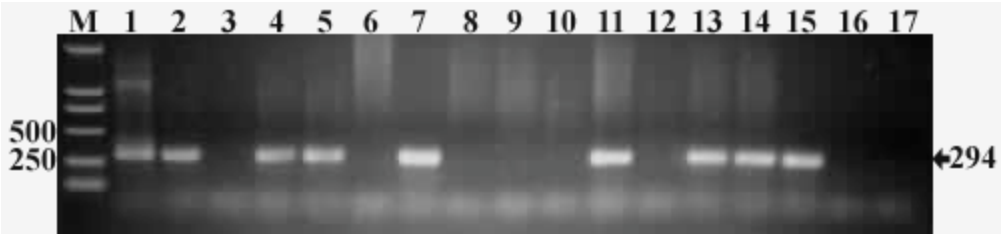

Figure 2. Agarose gel electrophoresis of PCR products amplified by nested PCR from gDNA of *Babesia* sp. BQ1 (Lintan) (Lane 1), WBgDNA of partial samples (lane 2-15), gDNA of healthy sheep (Lane 16) and distilled water (Lane 17).

Table 1. Results of experimental transmission of *Babesia* sp. BQ1 (Lintan) to sheep with *H. qinghaiensis* and *H. longicornis*

Ticks					Animals			
Species	Stage	Number infested	Number engorged	Time engorged (Day pi)	Sheep number	ME	Temperature	Times positive reaction present in nPCR (Day pi)
H. qinghaiensis	Larva	10000	1500	5-7	08058	_	Normal	11, 27
		10000	1500	5-7	08094	_	Normal	11, 19
	Nymph	3000	1150	3-8	08050	_	Normal	13, 15, and 31
		3000	1430	3-8	08300	_	Normal	15
	Adult	1000	420	11-14	08180	_	Normal	28
		1000	478	11-15	08165	_	Normal	7, 11, 25, 30
H. longicornis	Larva	10000	1500	5-7	08099	_	Normal	19, 29
		10000	3000	5-7	08054	_	Normal	13, 29
	Nymph	3000	1500	3-8	08118	_	Normal	12, 21, 32
		3000	1450	3-8	08065	_	Normal	25, 29
	Adult	1000	580	7-13	08238	_	Normal	11, 14, 16
		1000	509	7-13	08068	_	Normal	9, 35

Article N° 3 : Determination of erythrocyte susceptibility of Chinese sheep (Tan mutton breed) and French sheep (Vendéen breed) to *Babesia sp*. BQ1 (Lintan) by in vitro culture. (Détermination de la sensibilité des érythrocytes de moutons chinois (race Tan mutton) et de moutons français (race vendéenne) à *Babesia sp*. BQ1 (Lintan) par culture *in vitro*)

Article soumis à Veterinary parasitology.

Ce travail a été réalisé au Laboratoire de l'équipe ITPH à Nantes.

Dans cette étude, un système de culture *in vitro* en continue de *Babesia sp*. BQ1 (Lintan) a été obtenu en utilisant un milieu RPMI 1640 contenant des érythrocytes de moutons (7,5 %), du sérum de veau fœtal (20 %), de l'amphotéricine B et de la gentamicine. Les parasites sont cultivés dans un incubateur à CO2 (6 %) à 37°C. La culture est possible dans des plaques 6 ou 24 puits, permettant d'atteindre une parasitémie de 5 %, et dans des flasques de 75 cm^2 permettant d'atteindre une parasitémie de 10 %. Une lignée monoclonale (G7) a été obtenue par la méthode de dilution limite, qui est conservée dans de l'azote liquide au laboratoire ITPH, ENVN, Nantes et au laboratoire VVBD, LVRI en Chine. Le parasite mis en culture conserve la morphologie du parasite « sauvage » et garde son pouvoir infectant pour les moutons. La croissance de *Babesia* sp. BQ1 (Lintan) a été caractérisée grâce à une méthode spectrophotométrique mise au point lors de ce travail. La densité optique (DO) d'un surnageant de culture est corrélée à la parasitémie. Ainsi, le temps de génération de *Babesia sp*. BQ1 (Lintan) déterminé par la mesure de la DO des surnageants de culture est de 20,5 heures et de 26,41 heures lorsqu'il est déterminé par le calcul de la parasitémie. La mise en culture pourrait être une méthode sensible de diagnostic de l'infection à *Babesia sp.* BQ1 (Lintan) durant la phase précoce et la phase persistante de l'infection. Cependant, le délai d'obtention du résultat serait long du fait de la multiplication lente de *Babesia* sp. BQ1 (Lintan).

Cette technique de culture *in vitro* de *Babesia sp*. BQ1 (Lintan) a permis d'explorer la sensibilité (ie la capacité à permettre la multiplication du parasite) des érythrocytes de 60 moutons de race Tan (moutons « chinois ») et de 37 moutons de race vendéenne (moutons « français ») et de la comparer à la sensibilité de ces mêmes érythrocytes à *B. divergens*. Les érythrocytes des moutons « chinois » sont plus sensibles aux 2 parasites que les érythrocytes des moutons « français » (67% et 58% des moutons « chinois » ont une sensibilité de leurs érythrocytes à *Babesia* sp. BQ1 (Lintan) et à *B. divergens* respectivement supérieure à 20%, contre 32 et 5% des moutons « français »). Ces résultats montrent que la sensibilité des érythrocytes à *Babesia* spp. varie au sein d'une espèce animale et est lié probablement à une composante génétique. De plus, l'évolution de l'infection à *Babesia* spp. peut être influencé

par la sensibilité des érythrocytes de son hôte. En effet, la faible sensibilité des érythrocytes des moutons « français » pourrait expliquer que le mouton n'est pas un hôte naturel de *B. divergens* et qu'expérimentalement, l'infection des moutons est possible mais sans signes cliniques. Au contraire, le mouton « chinois » est un hôte naturel de *Babesia sp*. BQ1 (Lintan) et une proportion élevée de ces moutons ont des érythrocytes sensibles à ce parasite. Enfin, le processus d'invasion des érythrocytes de *Babesia sp*. BQ1 (Lintan) est probablement différent de celui de *B. divergens* puisqu'un mouton donné peut avoir des érythrocytes fortement sensibles à *Babesia sp*. BQ1 (Lintan) et faiblement sensible à *B. divergens* ou inversement.

En conclusion, la mise au point de la culture de *Babesia sp*. BQ1 (Lintan) est une étape indispensable pour explorer la biologie de ce parasite puisqu'elle permettra d'obtenir des mérozoïtes en grande quantité facilitant les études sur les interactions entre les mérozoïtes et les érythrocytes, sur les molécules des mérozoïtes par exemple, et pour développer des nouveaux moyens de contrôle de cette infection.

Determination of erythrocyte susceptibility of Chinese sheep (Tan mutton Breed) and French sheep (Vendéen breed) to *Babesia* sp. BQ1 (Lintan) by in vitro culture

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Abstract

The *Babesia* species "BQ1 (Lintan)" was isolated from *Haemaphysalis qinghaiensis* collected in Gannan Tibet Autonomous Region, China in April 2000 and is infective for sheep and goats. In this study, an in vitro culture system was developed for the propagation of *Babesia* sp. BQ1 (Lintan). Continuous cultivation and 5.0% parasitemia was obtained in vitro in RPMI 1640 medium with sheep erythrocytes (7.5%) supplemented with FBS (Fetal Bovine Serum) (20%), Amphotericin B (0.5 μg/ml) and Gentamicin (50 μg/ml) in an incubator at 37ºC and 6% CO_2 in 24-well and 6-well plates. Parasitemia could attain 10% in 75 cm^2 flasks with the same culture medium but with 2.5% erythrocytes. A clonal line of *Babesia* sp. BQ1 (Lintan) was screened using the limiting dilution method and designated G7. Growth of *Babesia* sp. BQ1 (Lintan) in vitro was measured by microtitre-based spectrophotometric method and from parasitemia counts. The generation time was between 20.5 and 26.41 hours. Three French sheep were successfully infected with the culture and the infectivity of the clonal line G7 was determined. Finally, this in vitro culture system was used to compare the susceptibility (capacity to sustain *Babesia* sp. growth in vitro) of erythrocytes from French sheep ("Vendéen" breed) and from Chinese sheep (Tan mutton) purchased from Jingtai county, Gansu province of China for *Babesia* sp. BQ1 (Lintan) and *B. divergens* were compared with this in vitro culture system. The lower susceptibility to *B. divergens* and *Babesia* sp. BQ1 (Lintan) of erythrocytes from French sheep, compared to Tan mutton sheep, is discussed.

Keywords: *Babesia* sp. BQ1 (Lintan), in vitro culture, growth characteristics, erythrocyte

Introduction

Babesiosis of domestic animals is an ubiquitous, tick-borne hemoprotozoan disease occurring in tropical and subtropical regions of the world. The disease is characterized by fever, anemia, hemoglobinuria and even death. Ovine babesiosis, caused by infection with *B. ovis*, *B. motasi*, *B. crassa* and several unrecognized *Babesia* species, is a disease of economic importance. At least two *Babesia* species are responsible for ovine babesiosis in China. One is closely related to *B. motasi* (and includes different geographic isolates such as *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesia* sp. Tianzhu, and *Babesia* sp. Liaoning). The other, based on phylogenetic analysis, is *Babesia* sp. Xinjiang (Liu et al., 2007; Niu et al., 2009). These parasites have caused problems in sheep and goats in the Gansu, Shandong and Shanxi provinces of China but little is known about them (Yin et al., 1997). *Babesia* sp. BQ1 (Lintan) was first isolated from a splenectomized sheep infected with adult *Haemaphysalis qinghaiensis* (Teng, 1980) collected in the field by Guan et al. (2002). In France, ovine babesiosis is mainly caused by *B. ovis* and *B. motasi* although Chauvin et al. (2002) and Malandrin et al. (2004b, 2009) have shown that sheep can serve as an experimental host of *B. divergens*: sheep erythrocytes can be used for in vitro culture of *B. divergens* and splenectomised or spleen-intact sheep can be experimentally infected if their erythrocytes are susceptible i.e., able to sustain growth of the parasites in vitro. Infection of sheep with *B. divergens* has never been demonstrated under field conditions.

In the present study, the in vitro erythrocyte susceptibility of French sheep (Vendéen breed), which can be an experimental but not a natural host of *B. divergens,* was compared with that of Chinese (Tan mutton) sheep which is the natural host of *Babesia* sp. BQ1 in China, to evaluate the possible role of the erythrocyte in the susceptibility of sheep to *Babesia* sp. in vivo. We then developed an in vitro culture system for the cultivation of *Babesia* sp. BQ1 (Lintan).

MATERIALS AND METHODS

Parasite

Blood infected with *Babesia* sp. BQ1 (Lintan), provided by the Lanzhou Veterinary Research Institute (LVRI), was inoculated into a splenectomized sheep immunosuppressed by injection of dexamethasone (20 mg everyday for 3 days). The animal was bled and the infected blood either cryopreserved in liquid nitrogen, when parasitemia reached 5% , or used to initiate in vitro culture.

B. divergens Rouen 1987 isolated from a patient admitted to Rouen CHU in 1987 (University Hospital Center, Rouen, France) was used in this study. It was re-isolated from experimentally infected sheep (Chauvin et al., 2002) and preserved in liquid nitrogen.

Sheep and preparation of erythrocyte suspension

All sheep used in this experiment were free of *Babesia* sp. and were kept in a sheep-fold with tick-free food and water available *ad libitum*. The French sheep ("Vendéen" breed) were provided by the "Lycée Nature" at La Roche sur Yon (France) and the Chinese (Tan mutton) sheep were purchased from Jingtai county, Gansu province of China.

Preparation of erythrocyte suspension

Blood from the sheep used in this experiment was taken by venipuncture into a 250ml sterile glass bottle containing 28ml Citrate Phosphate Dextrose Solution as anticoagulant (C7165-50ml, Sigma). The blood was centrifuged at 2400rpm for 10min at room temperature and the plasma and buffy coat were discarded. Red Blood Cells (RBC) were washed once with 30ml RPMI 1640 medium (12-115F, Lonza), and centrifuged as above. The pellets of packed erythrocytes were stored in 2 volumes RPMI 1640 and kept at 4ºC until use.

Peripheral blood was collected and erythrocyte suspension was prepared as previously described (Malandrin et al., 2004a, 2004b).

Initiation of culture

The blood collected from sheep infected with *Babesia* sp. BQ1 (Lintan) was used to initiate an in vitro culture in 24-well culture plates containing RPMI 1640 medium supplemented with 0.5μg/ml Amphotericin B (17-836E, Lonza), 50μg/ml Gentamicin (17-519Z, Lonza) and 20% FBS (DE 14-801F, Lonza) (fresh medium). The primary culture contained 150μl per well of RBC from the donor sheep used for *B. divergens* culture (i.e. which has erythrocytes that are able to sustain the in vitro multiplication of *B. divergens*) in 2ml fresh medium (7.5% RBC). The cultures were incubated at 37ºC in a humidified 6% CO_2 atmosphere. Every 2-3 days, the cultures were fed by removing 1.5ml of the supernatant medium, without disturbing the erythrocyte layer, and replacing it with an equal volume of fresh medium. Meanwhile, parasitemia was monitored by pipetting 5μl of RBC and preparing thin blood smears for Diff-Quik staining and microscopic examination. A subculture was prepared when parasitemia reached 2% by transferring 200μl of primary homogenized culture into another well containing 2ml fresh medium and 150μl of donor sheep RBC. The cultures were amplified in the 6-well-plates and in 75cm^2 flasks with the same ratio of RPMI 1640 (80%), erythrocytes (7.5%) and FBS (20%) as in the adaptation of the parasite to the culture system.

Screening of donor sheep for *Babesia* sp. BQ1 culture

We initiated the culture of *Babesia* sp. BQ1 by using RBC from a donor sheep which sustained a good culture of *B. divergens*. Sheep with RBC highly susceptible to *Babesia* sp. BQ1 were then selected so that the culture of *Babesia* sp. BQ1 could be maintained over a long period. Fifteen spleen-intact, 1-1.5 year old French sheep of "Vendéen" breed, kept in a

sheep-fold at the Veterinary school (Nantes, France), were screened to select the most suitable donor of normal erythrocytes. The RBC from 15 French "Vendéen" sheep were prepared and used to culture *Babesi*a sp. BQ1 as described above. Although seven of the sheep had RBCs that were able to sustain the growth of *Babesia* sp. BQ1, the best sheep was No.3342 which was then chosen as donor and maintained in a tick-free environment.

Cloning and cryopreservation of *Babesia* sp. BQ1 (Lintan)

One clonal line was obtained by limiting dilution, as previously described (Malandrin et al., 2004a). The clone was picked and transferred to a 24-well-plate for cultivation. After cloning, the parasite was identified by a PCR (Primers: BLT911-AS: AGGACTACGACGGTATCTGA, BLT623-S: ATCGCGTGCTTTTGGT) specific to *Babesia* sp. BQ1 (Lintan) based on the 18S rRNA gene sequence, and the PCR product was sequenced by the Takara company as previously described by Guan et al. (2008) when good growth had been obtained in the culture plates.

The resulting clone line was cryopreserved after amplification in 6-well-plate and 75cm^2 flasks. Infected RBC (iRBC) from culture were suspended with RPMI1640 (65%), FBS (25%), DMSO (10%) and then transferred into liquid nitrogen. For retrieval, samples were thawed rapidly at 37ºC, transferred into RPMI1640 (10 ml) and centrifuged at 1200g for 10min. The pellet was used for culture preparation (Chauvin et al., 2002).

Experimental infection of sheep

The infectivity of *Babesia* sp. BQ1 (Lintan) cultivated in vitro was verified by selecting 3 spleen-intact French sheep (No. 3390, 3533 and 3446) with erythrocytes able to sustain the growth of *Babesia* sp. BQ1 (Lintan) as described above, and subcutaneously injecting them with 10^7, 10^8 or 10^9 respectively of the iRBC obtained in culture. Infected sheep were bled every 2-3 days for 30 days. Presence of the parasite in the sheep blood stream was checked by in vitro culture as previously described (Malandrin et al., 2004a).

Evaluation of the growth dynamics and generation time of a monoclonal strain

In vitro growth dynamics were monitored, and the generation time was calculated as previously described by Malandrin et al. (2004b) with a few modifications. Three 75 cm^2 flasks were each inoculated with 4 x10^7 iRBC and one was left as control. Four hundred microliters of homogenized culture were regularly sampled and centrifuged at 1200g for 10min. The supernatants were frozen at -20 ºC and the optical density was determined on an ELISA reader (Stat Fax 2600, USA) at 405nm (A_{405}). The A_{405} of the control culture was subtracted from that of each infected culture to eliminate the effect of RBC self-lysis. Meanwhile, thin blood smears were prepared with the iRBC pellets and stained with Diff-Quik. Parasitemia was assessed by counting the infected cells in 2500 to 4000 RBCs. The mean parasitemia values and the A_{405} of three cultures were calculated. The generation times were calculated with Microsoft Excel 2000. The correlation analysis of A_{405} and parasitemia

was carried out with statistic software SPSS 11.5.

Comparisons of erythrocyte susceptibilities of "Vendéen" and Tan mutton sheep for each *Babesia* sp. BQ1 (Lintan) and *B. divergens* in vitro

A population of 37 French "Vendéen" sheep and 60 Chinese Tan mutton sheep was tested for in vitro susceptibility of their RBC to *Babesia* sp. BQ1 (Lintan) and to *B. divergens*. Parasite growth in RBC from each sheep was monitored using a spectrophotometric hemoglobin measurement in the culture supernatant as described by Malandrin et al. (2004b, 2009). Briefly, the experiment was performed in triplicate in 96-well plates with a culture prepared from 10^9 RBC/ml for each tested sheep and 5 x10^5 iRBC/ml for *Babesia* sp. BQ1 or 2 x10^5 iRBC/ml for *B. divergens*. After incubation for 120 hours (*Babesia* sp. BQ1) or 96 hours (*B. divergens)*, the plates were centrifuged and 100 μl of supernatant were recovered from each well. The absorbance of the supernatant was read at 405nm (A_{405}). Erythrocyte self-lysis was measured in a parasite-free control culture included in triplicate and the mean absorbance was subtracted from the corresponding test value (ΔA_{405}). Parasite growth was monitored in control sheep erythrocytes (No. 3342 for *Babesia* sp. BQ1 and No. 3264 for *B. divergens*) in all experiments. The percentage susceptibility of each sheep was calculated by comparing the ΔA_{405} of test sheep with the ΔA_{405} of control sheep using the following formula.

$$\text{Susceptibility (\%)} = \frac{\Delta A_{405} \text{ of test sheep}}{\Delta A_{405} \text{ of control sheep}} \text{ x100}$$

This experiment was repeated twice on the same sheep population and the mean percentage susceptibility was calculated from the 2 experiments (m% susceptibility).

RESULTS

In vitro culture

A continuous culture system was set up for the in vitro propagation of *Babesia* sp. BQ1 (Lintan). Parasitemia in RPMI 1640 medium with 7.5% of sheep erythrocytes supplemented with 20% FBS, peaked at 5% in 24- or 6-well-plates and was increased to 10% with 2.5% erythrocytes in 75 cm^2 flasks. The morphological features of the parasites grown in vivo or in vitro were conserved (Figure 1). A clonal line of *Babesia* sp. BQ1 (Lintan) was obtained by limiting dilution method, identified by PCR and sequenced, then designated G7. It was then cryopreserved in liquid nitrogen at the ITPH laboratory (ENVN, Nantes, France) and at the Vector and Vector-borne Disease (VVBD) lab (LVRI, CAAS, Lanzhou, China).

Evaluation of growth dynamics and generation time of the monoclonal line

A curve of the growth dynamics of *Babesia* sp. BQ1 (Lintan) was constructed based on the mean values of parasitemia and optimal density (A405) of 3 separate cultures corresponding to same time point (Figure 2). Statistical analysis revealed an excellent relationship between parasitemia and the OD value of the culture supernatant. (Correlation coefficient $r = 0.99$, $p<0.01$). The generation time was between 20.57 hours (based on OD of the A405 supernatant) and 26.41 hours (based on parasitemia).

Experimental infection of sheep

The viability and infectivity of in vitro cultured parasites were estimated by experimental infection of sheep. *Babesia* sp. BQ1 (Lintan) was detected from the 2nd day post-infection (dpi) (sheep 3446) and from the 4th dpi (sheep 3390 and 3533) by in vitro culture (Table 1). Sheep 3446 and 3533 were persistently infected by *Babesia* sp. BQ1 (Lintan): parasitemia remained stable from the 7th day post-infection to the end of the experiment. Although Sheep 3390 was well-infected, no parasites were detected after 16 dpi. The dates when 1% parasitemia was measured were used to determine the level of infection for each sheep. 1% parasitemia was obtained after 4 to 26 (9.55 ± 6.17) days of culture from sheep 3446 between the 2nd and 25thdpi, after 4 to 19 (9.3 ± 5.87) days of culture from sheep 3533 between 4th and 25th dpi, and after 4 to 29 (16.5 ± 8.69) days of culture from sheep 3390 between 4th and 16th dpi. Thus sheep 3390 seemed to be less infected than sheep 3446 and 3533.

Susceptibility of Chinese and French sheep erythrocytes to *Babesia* sp. BQ1 (Lintan) and *B. divergens* in vitro

The susceptibility of erythrocytes from 37 French sheep and 60 Chinese sheep to *Babesia* sp. BQ1 (Lintan) (i.e., ability of the erythrocyte to sustain growth of *Babesia* sp. BQ1 (Lintan)) were tested in this experiment. It was similarly investigated for *B. divergens* as control (Figure 3). The mean susceptibilities of Chinese sheep erythrocytes to *Babesia* sp. BQ1 (Lintan) and *B. divergens* were 36±27.04% and 40±33.77%, respectively and those of French sheep were 20±19.67% and 6±4.01%. The erythrocytes of Chinese sheep seemed to be more susceptible to both *B. divergens* and *Babesia* sp. BQ1 than French sheep. Indeed, 67% and 58% of the Chinese sheep exhibited an erythrocyte susceptibility of more than 20% to *Babesia* sp. BQ1 (Lintan) and *B. divergens*, respectively, compared to 32 % and 5% of French sheep (Figure 3). Furthermore, a given sheep could show very different erythrocyte susceptibilities to *B. divergens* and *Babesia* sp. BQ1. For example, in Chinese sheep No. 27, erythrocyte susceptibility to *B. divergens* was 6% and to *Babesia* sp. BQ1 was 31%. In contrast, in Chinese sheep No. 31, erythrocyte susceptibility to *B. divergens* was 77% and to *Babesia* sp. BQ1 was 38%. Similar results were observed for French sheep (e.g. French sheep No. 16 and 36, Figure 4). The variability in susceptibility of a given breed of sheep to

different *Babesia* spp., and the correlation between susceptibility to *Babesia* sp. BQ1 (Lintan) and *B. divergens* in Chinese and French sheep were determined using the statistical software SPSS11.5 and Excel 2000. The correlation coefficients for Chinese and French sheep were 0.88 and 0.11 respectively, indicating that sheep have different susceptibilities to different *Babesia* species.

DISCUSSION

A continuous culture system for the cultivation of *Babesia* sp. BQ1 (Lintan), using RPMI 1640 medium with 2.5% sheep erythrocytes and 20% FBS, was developed in this study. Parasites obtained by in vitro culture did not show any morphological changes when compared with parasites from infected sheep blood. A monoclonal strain of *Babesia* sp. BQ1 (Lintan) was obtained, (G7), which was identified by specific PCR and sequenced, then deposited at the ITPH lab (ENVN, Nantes, France), and the Vector and Vector-borne Disease (VVBD) lab (LVRI, CAAS Lanzhou, China). The G7 monoclonal strain retained its in vivo infectivity. The infections obtained with G7 in spleen-intact sheep were similar to those obtained with the original isolate. Monitoring with in vitro culture revealed that the parasite persisted in sheep blood from the 2nd or 4th day post infection. It was demonstrated by Guan et al. (2008) that *Babesia* sp. BQ1 (Lintan) could be detected by loop-mediated isothermal amplification method (LAMP) on the 3rd day post-infection, in spleen-intact sheep infected with the original isolate. Thus the characteristics of *Babesia* sp. BQ1 (Lintan) were not modified after in vitro culture and the G7 clonal line could be used as a standard strain in future studies, e.g. molecular investigations, studies of interaction between merozoites and erythrocyte, obtaining merozoites for host infection.

In vitro culture could also be used to confirm the existence of *Babesia* infection. *Babesia* sp. infections are usually confirmed by examination of a blood smear or inoculation of experimental animals. The drawback of these techniques is their low sensitivity (Lewis, 1981; Holman et al., 1988; Hentrich, 1993). Rodriguez et al. (1983) and Malandrin et al. (2004a) showed that *B. bovis* or *B. divergens* could be detected by in vitro culture in cows that had recovered from babesiosis and in carrier-cows even though the parasite was not detected in the blood smears. The sensitivity of *B. divergens* in in vitro culture was described in detail by Malandrin et al. (2004a). Parasitemia of 10^{-7}% (10 parasites per ml of blood) could be detected for 6 to 20 days post-cultivation, which suggests that the sensitivity of in vitro culture is the same as some PCR-based methods. For example, the sensitivity of a PCR method for *B. ovis* developed by Aktas et al. (2005) was high enough to detect 10^{-5}% parasitemia in infected sheep blood; Gubbels et al. (1999) developed a reverse line blot for

simultaneous detection of bovine *Theileria* and *Babesia* and the lowest detectable parasitemia was 10^{-6}%. Hence, our culture method could also be used to diagnose, isolate and identify *Babesia* species from sheep or goats during the early and persistent phases of the infection. However this method would be time-consuming because 2 to 29 days of culture are needed to obtain 1% parasitemia. This can be explained by the long in vitro generation time of *Babesia* sp. BQ1 (Lintan) (20.57- 26.41 hours) compared with that of *B. divergens* (8.6 to 8.9 hours; Malandrin et al., 2004b).

The in vitro susceptibility of erythrocytes from French sheep to *B. divergens* was demonstrated by Malandrin et al. (2009). These authors claimed that erythrocyte susceptibility to *B. divergens* varied not only between different sheep breeds but also between individuals of the same breed and could represent the in vivo susceptibility of sheep to the parasite. They concluded that sheep were difficult to infect with *B. divergens* if their erythrocytes were refractory to this parasite in vivo. In our study, we compared the in vitro erythrocyte susceptibility of French (Vendéen) sheep and Chinese (Tan mutton) sheep to each *Babesia* sp. BQ1 (Lintan) and *B. divergens*. Several conclusions could be drawn from this experiment. Firstly, as in Malandrin's experiment, the susceptibility of erythrocytes to *Babesia* spp. varied between individuals. Secondly, erythrocytes from Chinese sheep were more susceptible to *Babesia* sp. BQ1 (Lintan) and to *B. divergens* than erythrocytes from the Vendeen breed of French sheep, which means that susceptibility to *Babesia* spp. varies within a particular vertebrate host. Host genetic resistance against hemoparasite infections has been studied for *Plasmodium* spp. in mice (Foote et al., 1997; Fortin et al., 2001; Burt et al., 2002). Differences in resistance to *Babesia* have also been described between different breeds of cattle (Bock et al., 1997; Benavides and Sacco, 2007). Bock et al. (1999) demonstrated that pure-bred *Bos indicus* cattle showed a high level of resistance to babesiosis. Hence, the vertebrate genotype also plays an important role in *Babesia* infection. However, genetic resistance to *Babesia* in sheep has rarely been mentioned in the literature. Thirdly, the course of infection with this parasite can be influenced by the in vitro erythrocyte susceptibility of hosts to *Babesia* spp. i.e. the possibility of the protozoan infecting or not a species or a breed of vertebrate, and the development or not of clinical signs by infected hosts. Indeed sheep is not considered to be a natural vertebrate host of *B. divergens* in France. We have shown here that few Vendéen sheep have erythrocytes that are able to sustain the growth of *B. divergens*. This could explain why infection with *B. divergens* could not be demonstrated in such sheep under field conditions, and why these sheep could only be infected with *B. divergens* under experimental conditions, and without clinical signs, if an in vitro culture of their erythrocytes was susceptible (Malandrin et al., 2009). In contrast Chinese sheep are natural hosts of *Babesia* sp. BQ1 (Lintan) and the erythrocytes of many Chinese sheep show high

susceptibility to *Babesia* sp. BQ1 (Lintan). Similarly, the erythrocytes of most cattle, which are natural hosts of *B. divergens*, are highly susceptible to *B. divergens* (Malandrin et al., personal communications). Finally, comparisons of the susceptibilities of erythrocytes from a given individual to *Babesia* sp. BQ1 (Lintan) and *B. divergens* revealed that erythrocytes from the same individual could show different susceptibilities to different *Babesia* species (correlation coefficient of 0.88 for Chinese sheep to *Babesia* sp. BQ1 (Lintan) and *B. divergens*, and 0.11 for French sheep). This suggests that different hemoparasites may require different molecules during their invasion or development in red blood cells. For example Alkhalil et al. (2006) reported that *Plasmodium falciparum* and *B. divergens* use diffent mechanisms to increase the permeability of infected human erythrocytes. *P. falciparum* induces changes in permeability of the host RBC membrane through the plasmodial surface anion channel (PSAC), whereas this channel is of negligible importance in RBC infected with *B. divergens*.

In conclusion, a continuous culture system for cultivation of *Babesia* sp. BQ1 (Lintan) has been developed for the first time. A monoclonal strain of *Babesia* sp. BQ1 (Lintan) was obtained which had the same morphological and infectivity characteristics as the field isolate. Maintenance of an in vitro culture of *Babesia* sp. BQ1 (Lintan) in the laboratory will open up many possibilities for studying *Babesia* of small ruminants, e.g. molecular investigations, studies of interactions between merozoites and erythrocytes, and between merozoites and the vertebrate host. This should lead to better understanding of the biology of *Babesia* sp. BQ1 (Lintan) and to the development of new means of controlling *Babesia* infection.

Conflict of interest

None declared

Acknowledgements

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Figure legends

Figure1. Morphologic characteristics of *Babesia* sp. BQ1 (Lintan) in smears made with blood collected from an infected sheep (A) and from culture (B), bar = 5μm

Figure 2. Growth dynamics of *Babesia* sp. BQ1 (Lintan) monitored by parasitemia (■) and optical density of the culture supernatant (A_{405}) (▲).

Figure 3. Comparison of susceptibilities of erythrocytes from Chinese sheep (A) and French sheep (B) to each *Babesia* sp. BQ1 (Lintan) and *B. divergens*

Figure 1

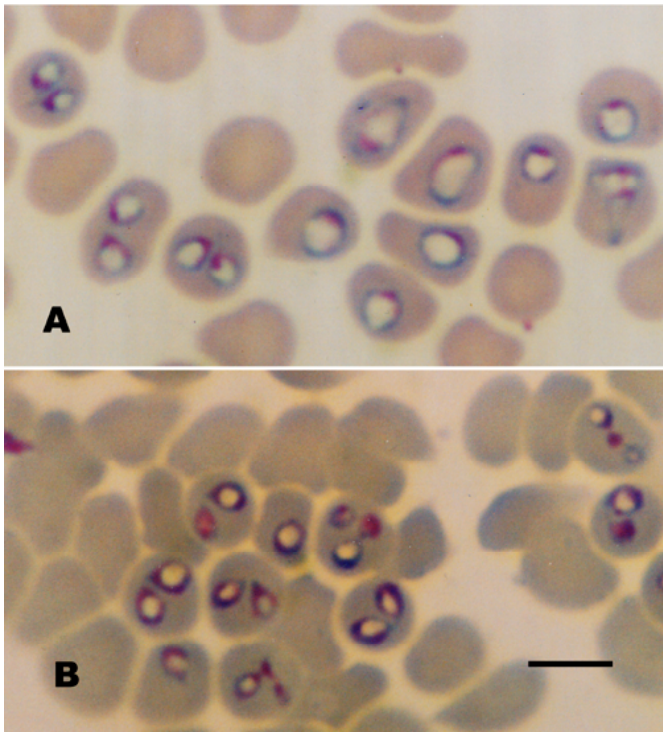


Figure 2

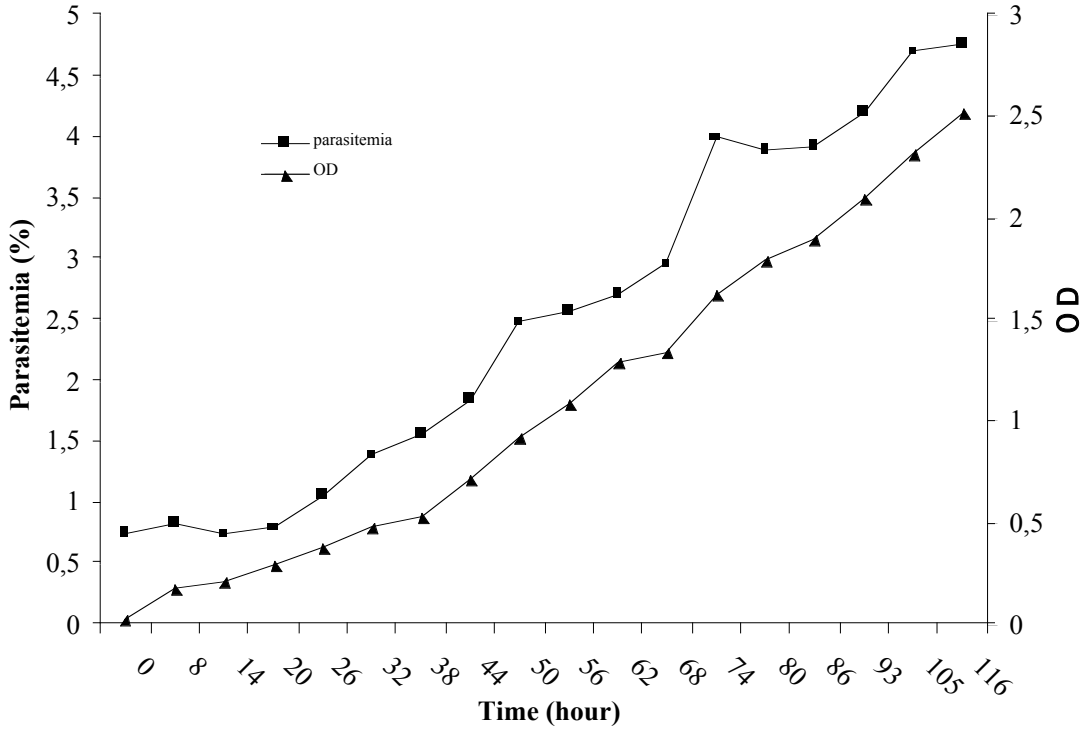
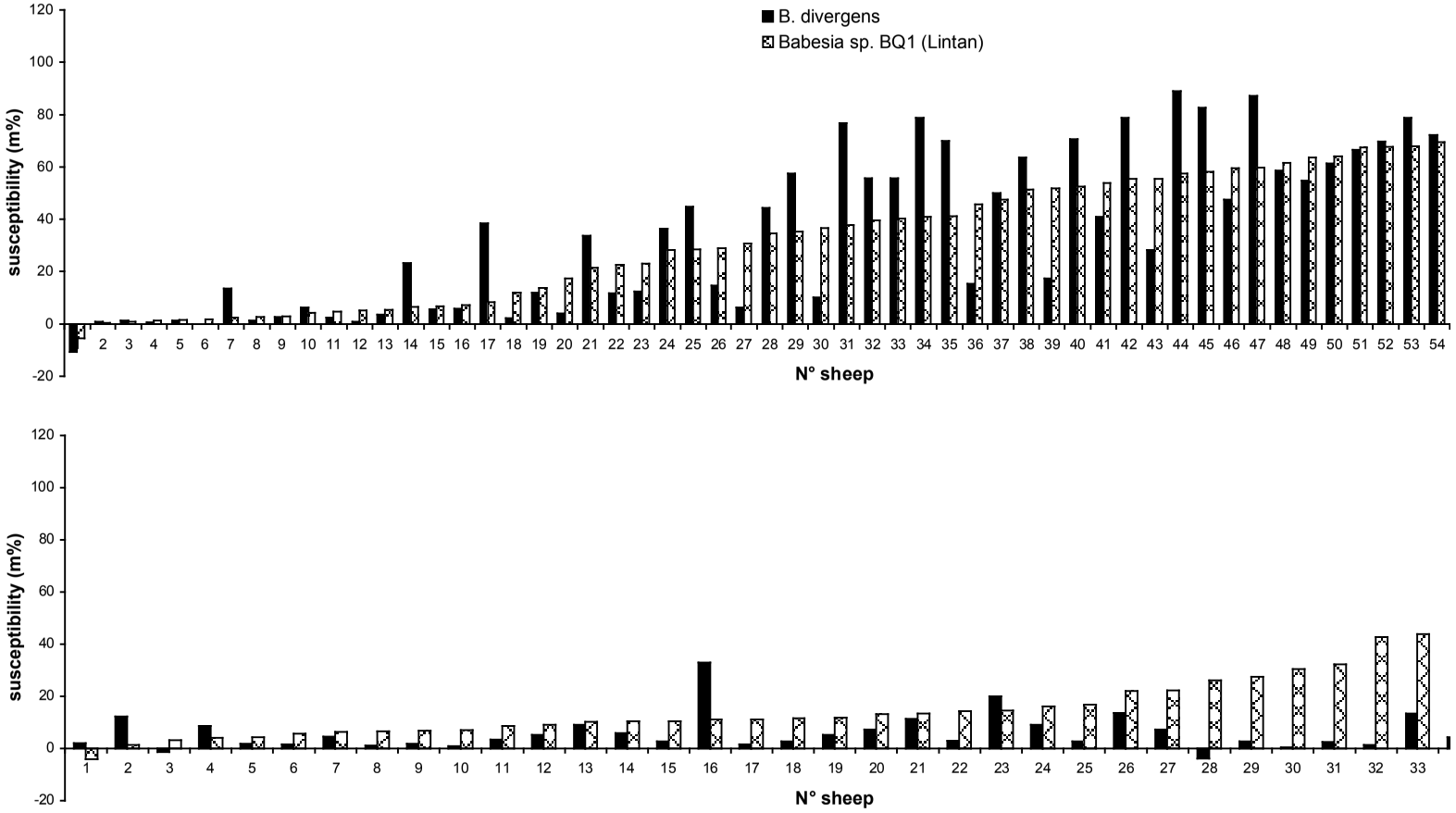

parasitemia
OD
Parasitemia (%)
OD
Time (hour)
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
5
0
8
14
20
26
32
38
44
50
56
62
68
74
80
86
93
105
116

Figure 3


B. divergens
Babesia sp. BQ1 (Lintan)
susceptibility (m%)
N° sheep
susceptibility (m%)
N° sheep

Article N° 4 : Course of infection by *Babesia sp.* BQ1 (Lintan) and by *B. divergens* in sheep depends on the production of IFNγ and IL10. (L'évolution de l'infection à *Babesia sp.* BQ1 (Lintan) et à *B. divergens* chez le mouton dépend de la production d' IFNγ et d'IL10)

Article accepté dans Parasite immunology.

Ce travail a été réalisé au Laboratoire de l'équipe ITPH à Nantes.

Babesia sp. BQ1 (Lintan) est un parasite des érythrocytes, infectant naturellement les moutons dans la province du Gansu en Chine. Expérimentalement, *Babesia sp.* BQ1 (Lintan) infecte des moutons non splénectomisés avec une parasitémie patente (parasites observés sur étalement sanguin coloré au May Grünwald Giemsa) pouvant atteindre 10 % et chez des chèvres splénectomisées infectées, des signes cliniques de babésiose sont observés avec une parasitémie de 85%. Au contraire, *B. divergens* est un hémoparasite des bovins mais il a été montré qu'il pouvait infecter expérimentalement des moutons non splénectomisés, sans entraîner de signes cliniques ni de parasitémie patente. L'objectif de cette étude était de comparer le pouvoir pathogène de *Babesia sp.* BQ1 (Lintan) et de *B. divergens* chez le mouton ainsi que la réponse immunitaire induite par ces 2 parasites.

Six moutons ont été inoculés par voie sous-cutanée avec des globules rouges infectés (iRBC) soit par *Babesia sp*. BQ1 (Lintan) soit par *B. divergens* ($2,5 \times 10^7$ iRBC/kg) et 3 moutons non infectés ont servi de d'animaux témoins. Le suivi de l'infection a été réalisé par observation clinique des animaux, prise de la température rectale, détermination de l'hématocrite et recherche de parasite sur des étalements de sang pris au niveau des capillaires de l'oreille et par mise en culture du sang prélévé à la veine jugulaire. Tous les animaux ont été infectés de façon persistente par *Babesia sp.* BQ1 (Lintan) ou par *B. divergens* : des parasites sont détectés après mise en culture du sang dès le 1er ou 2ème jours après l'infection (jpi) jusque la fin de l'expérimentation (78 jpi). En revanche, contrairement au moutons infectés par *B. divergens,* une parasitémie patente est irrégulièrement observée entre 4 et 17 jpi chez les moutons infectés par *Babesia sp.* BQ1 (Lintan). Chez ces mêmes animaux, la température rectale a augmenté significativement entre le 3ème et le 6ème jpi. Cependant, aucun signe clinique de babésiose ni aucune diminution de l'hématocrite ne sont observés chez les moutons infectés par *Babesia sp.* BQ1 (Lintan) ou par *B. divergens*. Ces résultats montrent qu'il existe une variation de pouvoir pathogène entre *Babesia sp.* BQ1 (Lintan) et *B. divergens*, le 1er étant plus pathogène chez le mouton que le 2ème.

Chez les animaux infectés par *Babesia sp.* BQ1 (Lintan) ou par *B. divergens*, nous avons comparé la réponse immunitaire induite par ces 2 parasites en explorant d'une part la réponse immunitaire humorale (cinétique de production d'IgM, IgG, IgG1 et IgG2) et d'autre part la réponse immunitaire cellulaire (cinétique de prolifération des cellules mononucléées

sanguines stimulées spécifiquement et production d'IFNγ et d'IL12 par ces cellules). Les résultats de cette étude sont résumés dans le tableau suivant :

		BQ1 / T	Bd / T	BQ1 / Bd
Réponse humorale	IgM	BQ1 > T entre la 1ère et la 7ème spi (p<0,05)	Bd = T	BQ1 > Bd (p<0,05 à différentes dates)
	IgG	BQ1 > T à partir de la 1ère spi (p<0,05)	Bd > T à partir de la 2ème spi (p<0,05)	BQ1 > Bd entre la 1ère et la 4ème spi (p<0,05)
	IgG1	BQ1 > T à partir de la 2ème spi (p<0,05)	Bd > T à partir de la 2ème spi (p<0,05)	BQ1 > Bd entre la 2ème et la 3ème spi (p<0,05)
	IgG2	BQ1 > T à partir de la 1ère spi (p<0,05)	Bd > T à partir de la 1ère spi (p<0,05)	BQ1 > Bd entre la 2ème et la 3ème spi (p<0,05)
Réponse cellulaire	Prolifération des CMS	BQ1 = T	Bd > T (p<0,05 à la 1ère spi et entre la 3ème et la 5ème spi)	Bd > BQ1 (p<0,05 à la 2ème spi et entre la 4ème et la 7ème spi)
	IFNγ	BQ1 = T	Bd > T (p<0,05 à la 4ème et 5ème spi)	Bd > BQ1 (p<0,05 à la 4ème spi)
	IL10	BQ1 > T (p<0 ,05 à la 3ème spi)	Bd = T	BQ1 > Bd (p<0,05 à la 3ème spi et entre la 5ème et la 8ème spi)

CMS : cellules mononucléées sanguines, BQ1/T : comparaison entre les animaux infectés par *Babesia sp*. BQ1 et les animaux témoins, Bd/T : comparaison entre les animaux infectés par *B. divergens* et les animaux témoins ; BQ1/Bd : comparaison entre les animaux infectés par *Babesia sp.* BQ1 et les animaux infectés par *B. divergens*

En résumé, la réponse immunitaire dirigée contre *Babesia sp*. BQ1 (Lintan) se caractérise par une réponse immunitaire humorale intense, précoce et persistente, avec production d'IgM, IgG, IgG1 et IgG2, et par une réponse cellulaire faible dominée par une production d'IL10. La réponse immunitaire humorale anti-*B. divergens* est plus faible, tout du moins en début d'infection, avec production d'IgG, IgG1 et IgG2 mais sans production d'IgM. La réponse cellulaire anti-*B. divergens* est plus intense que celle induite par *Babesia sp*. BQ1, avec production d'IFNγ mais pas d'IL10.

Ainsi, le pouvoir pathogène *de Babesia sp*. BQ1 (Lintan) et de *B. divergens* chez le mouton ne semble pas lié à la production d'anticorps. En revanche la réponse cellulaire semble être très importante : la production d'IFNγ serait liée à une réponse protectrice (au moins partiellement) et expliquerait le faible pouvoir pathogène de *B. divergens* alors que la production d'IL10 ne serait pas protectrice et expliquerait le plus fort pouvoir pathogène de *Babesia sp*. BQ1 (Lintan) chez le mouton.

Course of infection by *Babesia sp.* BQ1 (Lintan) and to *B. divergens* in sheep depends on the production of IFNγ and IL10

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Disclosures : none

ABSTRACT

Ovine babesiosis is an important disease in China and responsible for economic losses. Several *Babesia* strains are involved but *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. BQ1 (Ningxian) are particularly prevalent in the Guansu region. *B. divergens,* in contrast, can experimentally infect spleen-intact sheep but does not induce clinical signs. The immune response of spleen-intact sheep to *Babesia* sp. BQ1 (Lintan) and to *B. divergens* was therefore compared to identify the immune mechanisms involved in pathogenicity. The greater pathogenicity of *Babesia* sp. BQ1 (Lintan) than to *B. divergens* was confirmed: sheep infected with *Babesia* sp. BQ1 (Lintan), but not *B. divergens,* developed hyperthermia and showed patent parasitemia in Giemsa-stained blood smears from the ear vein. Furthermore, more parasites were also detected in blood from the jugular vein of *Babesia* sp. BQ1 (Lintan)-infected sheep. Pathogenicity of *Babesia* spp. involved cellular responses but not humoral responses. Interferon-gamma was produced only by specifically-activated PBMC from *B. divergens*-infected sheep and interleukin-10 only by specifically-activated PBMC from *Babesia* sp. BQ1 (Lintan)-infected sheep. The role of these cytokines in the course of infection by *Babesia* spp. is discussed.

KEY WORDS : *Babesia*, sheep, pathogenicity, Interferon-γ , interleukin-10, humoral response, cellular response

INTRODUCTION

Ovine babesiosis, caused mainly by *Babesia ovis* and *Babesia motasi,* is an important hemoprotozoan disease responsible for considerable economic losses in sheep. Several distinct geographic strains of *Babesia* spp. naturally infective to sheep have been collected in China: *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. (Tianzhu), *Babesia* sp. (Hebei), *Babesia* sp. (Madang), *Babesia* sp. (Liaoning) and *Babesia* sp. Xinjiang-2005 (1). *Babesia* sp. BQ1 (Lintan) is particularly prevalent in some provinces of China: in a recent study, 14.3 % of sheep blood collected from Gansu province were detected positive by the LAMP method (1). *Babesia* sp. BQ1 (Lintan) is similar to *B. motasi* in terms of morphological size, tick vectors and 18S rRNA gene sequence (per cent identity = 92.9%) (2). *B. motasi* consists of several strains that exhibit different features in separate geographical regions (3). In northern Europe, *B. motasi* is considered non-pathogenic for intact sheep and can be responsible for anemia but very rarely for icterus and hemoglobinuria (4, 5). In India, *B. motasi* causes disease with fever, dyspnea, hemoglobinuria in sheep and goats while in Morocco, it is responsible for mortality (10%) (6). The pathogenicity of *Babesia* sp. BQ1 (Lintan) in China has been little investigated. Under experimental conditions, *Babesia* sp. BQ1 (Lintan) infected intact sheep with an observed patent parasitemia of up to 10 % in blood smears and immunosuppressed goat showed typical symptoms of babesiosis with up to 85% parasitemia (7). In contrast, the European *Babesia divergens* seems to be less pathogenic for sheep. This protozoan is naturally responsible for a mild to severe disease in cattle, reindeer (8) and splenectomized humans (9) but no case has been reported under field conditions for sheep. Spleen-intact sheep could be infected by *B. divergens* under experimental conditions, but without clinical signs of babesiosis or patent parasitemia (10). Splenectomised sheep showed mild hyperthermia and transitory patent parasitemia of up to 3% (11).

Host-related factors such as age, species or breed have been associated with resistance to *Babesia.* Indeed an inverse age-resistance to *Babesia* spp. infection has been described with young animals being less susceptible to *Babesia* spp than adult cattle (12, 13). *Bos indicus* were less susceptible to *Babesia bovis* than *Bos taurus* (14) and C57BL/6 mice were less susceptible than C3H mice to *Babesia* sp. WA1 (15). Immune mechanisms are involved in this inverse age-resistance. One babesicidal molecule, produced by mononuclear phagocytes, has been identified as nitric oxide (NO). NO production was found to be up-regulated by interferon-gamma (IFNγ) and interleukin-12 (IL12) and down-regulated by interleukin-10 (IL10). Goff et al (2001) (16) confirmed that a transient inducible nitric oxide synthetase (iNOS) was induced in the spleen of calves infected with *B. bovis* followed by early production of IFNγ and IL12 whereas in adult cattle the expression levels of IL10 were greater. In adult cattle, IL10 could cause down-regulation of IL12 and IFNγ production and

then down-regulation of NO in the spleen.

The characteristics of the immune response to *Babesia* sp. BQ1 (Lintan) and to *B. divergens* need further investigation in order to explain the differences of pathogenicity between the 2 parasites. In this paper, the immune mechanisms involved in sheep infected by *Babesia* sp. BQ1 (Lintan) and to *B. divergens* were identified by comparing, for the first time, the kinetics of antibody production (IgM, IgG, IgG1 and IgG2) and cytokine (IFNγ and IL10) produced by peripheral bood mononuclear cells (PBMC) in sheep infected by *B. divergens* or *Babesia* sp. BQ1 (Lintan).

MATERIALS AND METHODS

B. divergens and *Babesia sp*. BQ1 (Lintan) in vitro culture

B. divergens Rouen 1987 isolated from a patient admitted to Rouen CHU in 1987 (University Hospital Center, Rouen, France) was used in this study. It was re-isolated from experimentally infected sheep (11) and preserved in liquid nitrogen. *Babesia* sp. BQ1 (Lintan) was isolated from a spleen-intact sheep infested by *Haemaphysalis qinghaiensis* collected in the field (7). Infected blood was inoculated intravenously into a splenectomized sheep and when parasitemia reached 20%, blood samples were collected and deposited in the centre for parasite strain collection and preservation at the Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, China. These strains were used for the experimental infection of sheep.

B. divergens and *Babesia* sp. BQ1 (Lintan) were cultivated in sheep erythrocytes as previously described (11, 17, 18). RPMI 1640 (Lonza, Levallois-Perret, France) was supplemented with gentamycin (50 μg/ml), heat-inactivated (30 min at 56°C), fetal calf serum at 10% v/v (Lonza, Levallois-Perret, France), and sheep erythrocytes collected from the blood donor and used at a concentration of 5 to 8 x 10^8 red blood cells (RBC)/ml. Cultures were established at 37 °C under a humidified 6% CO_2 atmosphere, in 75 cm^2 flasks or 24-well plates with culture volumes of 40 ml and 2 ml respectively. Cultures were inoculated at about 0.1% parasitemia and subcultured every two or three days. Parasitemia in the 75 cm^2 flasks was increased by changing the medium (RPMI + serum) every day.

Experimental infections of spleen-intact sheep

Six sheep (9 month-old) with erythrocytes able to sustain the growth of *B. divergens* or *Babesia sp*. BQ1 (Lintan) in vitro were used (sheep Bd1, Bd2, Bd3 and sheep BQ1-1, BQ1-2, BQ1-3 respectively), as described by Malandrin et al, 2009 (10). They were kept in a sheep-fold with food and water available *ad libitum*. The sheep were infected subcutaneously with autogenous infected red blood cells washed with PBS (2.5 x 10^7 iRBC/kg, i.e., 8 to 13 x 10^8 iRBC/sheep). The in vitro erythrocyte susceptibility of each sheep and the inoculation dose are indicated in Table 1. Three control sheep (sheep C1, C2 and C3) were injected

subcutaneously with their own PBS-washed red blood cells.

Infection was monitored daily for the first three weeks and then weekly for 8 weeks. Rectal temperatures were taken whenever blood samples were drawn. Packed cell volume (PCV) was measured with a microhaematocentrifuge. Presence and/or persistence of the parasite in the sheep blood stream was checked by in vitro culture as previously described (17). The evolution of in vivo parasitemia was estimated by measuring the number of days needed to obtain 1% parasitemia in standardised cultures ($d_{1\%}$). The obtained value $d_{1\%}$ was subtracted from the maximum duration (32 days) allowed for the culture ($32\text{-}d_{1\%}$) (table 2). Giemsa-stained blood smears from the ear and jugular veins were also examined microscopically for the presence of haemoprotozoa.

Preparation of extracts from *B. divergens* or *Babesia* sp. BQ1 (Lintan) merozoites (BdE or BQ1E)

B. divergens or *Babesia sp*. BQ1 (Lintan) merozoites were isolated by centrifugation (15 min, 2500 *g*) on a Percoll solution adjusted to a density of 1.08 g/ml, as described by Malandrin et al, 2009 (10). The merozoites were disrupted by 4 cycles of freeze-thawing, diluted in PBS and then subjected to ultrasonication for 30 sec at 4°C (Sonifier cell disruptor B15, Branson), repeated five times at 30 sec intervals. After centrifugation, the supernatant was recovered and filtered through a 0.2 µm cellulose acetate filter. Protein content was determined by the Bicinchoninic Acid (BCA) method (Interchim, Montluçon, France).

Determination of sheep antibody levels during infection by ELISA

The antibody levels of each animal serum to BdE or BQ1E were determined weekly using an ELISA. Microplates (Microwell Nunc) were coated with BdE or BQ1E (2.5 µg/ml) in 0.1 M carbonate buffer pH 9.6 at 37°C for 1 h, then at 4°C overnight. After 5 washes with PBS/Tween 20 0.1% (PBST), the unoccupied sites in each well were blocked by adding 150 µl of gelatin (Bio-rad, Marne la Coquette, France) solution (2% w/v in 0.1 M carbonate buffer pH 9.6) and incubated for 30 min at 37°C. Sera diluted at 1/200 for IgG, IgG1 and IgM and at 1/20 for IgG2 in PBST (test sera) or PBST alone (blank test) were added to the wells (100 µl per well) in duplicate and incubated for 1 h at 37°C. After 5 washes in PBST, 100 µl of mouse monoclonal antibodies directed against sheep IgG1 or IgG2, diluted to 1/500 and 1/100 respectively, were added. These monoclonal antibodies were kindly provided by A. Pernthaner (AgResearch Ltd, Hopkirk Research Institute, Grasslands Research Centre, Palmerston North, New Zealand). After incubation for 1 hour, the plates were washed and then incubated for another 1 hour with horseradish peroxidase conjugated to sheep anti-mouse IgG (Sigma, Saint Quentin-Fallavier, France) diluted to 1/10000 for IgG1 and to 1/2500 for IgG2 in PBST. Total sheep IgG and IgM were assayed directly with horseradish peroxidase (HRP) conjugated mouse anti-sheep IgG (Sigma, Saint Quentin-Fallavier, France) diluted to 1/1000 and with HRP conjugated rabbit anti-sheep IgM (Serotec) diluted to 1/10000. After 5 washes in PBST, the plates were incubated for 10 min with 50 µl TMB (3,3',5,5'

tetramethylbenzidine) liquid substrate (Sigma, Saint Quentin-Fallavier, France) and 50 µl of 2M H_2SO_4 solution was added to stop the reaction. The optical densities (OD) were read at 450 nm using an ELISA automate (MRX Microplate reader, USA).

The mean OD of each duplicate test was first subtracted from the mean OD of the blank test (mOD) and then from the corresponding value measured at 0 week post-infection (wpi) (ΔOD = m OD at n wpi – mOD at 0 wpi).

Peripheral Blood Mononuclear cells (PBMC) proliferation assay

The response of peripheral blood mononuclear cells (PBMC) to BdE or BQ1E was studied weekly for 9 weeks. Blood was collected in sterile lithium heparin vacutainers (Venoject, France). PBMCs were isolated by centrifuging over Lymphocytes separation medium (d = 1.077) (Eurobio). The cells were then resuspended in culture medium (RPMI 1640 containing 10% autologous plasma, 50 µg/ml gentamicin, 2.5 µg/ml amphotericin B, 2 mM L glutamine, 1 mM pyruvate and 50 µM β mercaptoethanol). Autologous plasma were harvested before infection to avoid the presence of specific antibodies against *B. divergens* or *Babesia sp*. BQ1 (Lintan).

Cells (2 x 10^5 cells/well) from each sheep were cultivated in triplicate in 96-well plates with BdE or BQ1E (10 µg/well) or without BdE or BQ1E (6 wells as control culture) for 5 days at 37 °C in a humidified atmosphere with 6% CO_2. PBMC proliferation was determined by tritiated thymidine incorporation.

Stimulation indices (SI) were calculated for each sheep as follows:

$$SI = \frac{\text{Mean counts per minute (CPM) of triplicate BdE or BQ1E stimulated culture}}{\text{Mean CPM of 6 wells control culture}}$$

For each sheep, the SI obtained at 0 week post-infection (wpi) was subtracted from the SI measured at each wpi (ΔSI = SI at n wpi – SI at 0 wpi).

Cytokine production by PBMC

Cytokine production (IFNγ and IL10) in culture supernatants of PBMC stimulated by BdE or BQ1E (10 µg/well) as described above was measured weekly by ELISA for 9 weeks. Supernatants were harvested after 5 days of culture. IFNγ was measured with an ELISA kit for ovine IFNγ (Mabtech, Sweden), as described by the manufacturer. IL10 secreted by PBMC from infected sheep stimulated by BQ1E or BdE (10 µg/well) in culture supernatants was measured with an enzyme-linked immunosorbent assay (ELISA), as previously described by Kwong et al., (2002) and Tourais-Esteves *et al*., (2008) (19, 20). Maxisorp plates (NUNC, France) were coated with mouse anti-bovine IL10 (clone CC318, Serotec) diluted at 6 µg/ml in 0.1 M carbonate buffer pH 9.6 and incubated overnight at room temperature (RT). The plates were washed with 0.05% Tween 20 in PBS (PBS-T) between steps. The blocking buffer was sodium casein (1 mg/ml) in PBS. Test samples (PBMC culture supernatants) were added to the wells (100 µl/well) and incubated for 1 h at RT. Cytokines were detected by adding biotinylated mouse antibodies (clone CC320, Serotec) diluted at 2 µg/ml in blocking

buffer to each well, and incubating for 1 h at RT. Extravidin-horseradish peroxidase (Sigma-Aldrich, France) was then added. Finally, liquid substrate was added as described above.

The IFNγ concentration of the sample was calculated in reference to a standard curve using recombinant bovine IFNγ (rBoIFNγ, 3116-1H-6, MABTECH AB, France) or recombinant bovine IL10 kindly provided by Jayne C. Hope (Institute for Animal Health, Compton, United Kingdom). For each sheep, each cytokine concentration in the wells measured at 0 wpi were subtracted from the corresponding value measured at various wpi (Δ[IFNγ] = [IFNγ] at n wpi – [IFNγ] at 0 wpi and Δ[IL10] = [IL10] at n wpi – [IL10] at 0 wpi).

Statistical analysis

The non parametric Mann-Whitney tests were used to compare the results between control sheep group and *B. divergens*- or *Babesia* sp. BQ1 (Lintan)-infected sheep group and the non parametric Wilcoxon tests were used to compare the results between the 2 infected-sheep group.

RESULTS

Infection of sheep by *B. divergens* or *Babesia* sp. BQ1 (Lintan)

All spleen-intact sheep were persistently infected by *B. divergens* or *Babesia* sp. BQ1 (Lintan): parasites were detected in blood by culture methods from 1 or 2 day post-infection (dpi) until the end of the experiment (78 dpi = 11 wpi). Differences between sheep infection by *Babesia* sp. BQ1 (Lintan) and *B. divergens* were nevertheless observed. Infection of the 3 sheep with *Babesia* sp. BQ1 (Lintan) was very homogeneous: parasites appeared in the blood from 1 or 2 dpi and the number of parasites reached a plateau at 4 dpi (31-$d_{1\%}$ between 20 and 24) which continued until the end of the experiment (Table 2). Merozoites of *Babesia* sp. BQ1 (Lintan) were observed irregularly from 4 to 17 dpi on blood smears from the ear vein, but not from the jugular vein. *B. divergens* was apparent in the blood stream from 1 or 2 until 7 dpi but variation between animals was observed. The presence of parasites in the blood stream then fluctuated from 8 dpi onwards. Sometimes none were detected at all probably, because the concentrations fell below the level of detection (between 8 and 15 dpi and at 17 dpi for sheep Bd1; at 8, 9, 11, 14 and 15 dpi for sheep Bd2; between 8 and 29 dpi for sheep Bd3). After 18 dpi, the number of *B. divergens* in the blood stream of sheep Bd1 and Bd2 seemed to reach a plateau at a similar level to that of *Babesia sp*. BQ1 (Lintan) (31-$d_{1\%}$ around 20) but again with some fluctuations. In sheep Bd3, the number of parasites after 4 wpi increased to reach a similar plateau to the other infected sheep at 9 wpi (31-$d_{1\%}$ = 18 – 19). *B. divergens,* unlike *Babesia* sp. BQ1 (Lintan), was never observed in blood smears from the jugular and ear veins.

Clinical signs of babesiosis, rectal temperature and PVC were monitored daily during the

first 3 weeks of infection. No clinical signs and no decrease of PVC were observed in either infected group. Rectal temperature in the 3 sheep infected with *Babesia* sp. BQ1 (Lintan), and even from 1 dpi in sheep BQ1-1 and BQ1-2, significantly increased by 0.8 to 1.2 °C between 3 and 6 dpi (fig. 1). Rectal temperature was modified in one (Bd3) of the three *B. divergens*-infected sheep during the same period (between 1 and 7 dpi). Rectal temperature increased during the second week of infection in some sheep (C1, BQ1-1, BQ1-2, Bd1 and Bd3) but the results could not be interpreted.

Humoral immune response of infected sheep

The kinetics of IgM, IgG, IgG1 and IgG2 production by sheep against *Babesia* sp. BQ1 (Lintan) and *B. divergens* were studied weekly by ELISA for 11 weeks. The production of all classes and subclasses of immunoglobulins in sheep infected by *Babesia* sp. BQ1 (Lintan), increased significantly soon after infection, i.e., from 1 or 2 wpi onwards (fig. 2). IgM reached a peak at 4 or 5 wpi and then decreased to the control level at 8 wpi (fig. 2A). The production of IgG against BQ1E increased very quickly, attaining a plateau at 2 wpi which continued until the end of the experiment (11 wpi) (fig. 2C). The humoral responses in sheep infected with *B. divergens* and *Babesia* sp. BQ1 (Lintan) were slightly different. Antibodies against *B. divergens* were produced later (notably from 4 wpi) and few were observed at the beginning of infection (until 3 or 4 wpi). No production of IgM against *B. divergens* was detected by ELISA. IgG production in the 3 *B. divergens*-infected animals was highly heterogeneous (fig. 2D). It increased regularly in sheep Bd1 and Bd2, and attained a plateau at 8 or 9 wpi but in sheep Bd3, it was weak for 7 wpi and then increased to reach a similar level to sheep Bd1 and Bd2 at 10 wpi (fig. 2D). Both IgG1 and IgG2 subtypes of IgG were produced by the 2 groups of infected-sheep and the kinetics of IgG1 and IgG2 production were similar to those of IgG (fig. 2E, F, G, H).

IFNγ and IL10 produced by PBMC during infection with *Babesia sp.* BQ1 (Lintan) or *B. divergens*

The cellular immune responses against *Babesia sp*. BQ1 (Lintan) and *B. divergens* were estimated from the proliferation and cytokine productions (IFNγ and IL10) of PBMC stimulated by BdE or BQ1E during the first 9 weeks of infection and constantly differed between the 2 infected groups. The proliferation of PBMC from *Babesia sp*. BQ1 (Lintan)-infected sheep was significantly lower than the proliferation of PBMC from B. divergens-infected sheep between 2 and 7 wpi. PBMC proliferation induced by BQ1E in Babesia sp. BQ1 (Lintan)-infected sheep was very low ($\Delta SI_{max} < 2.5$) and varied between sheep: no proliferation was observed for BQ1-2, a small peak of proliferation was detected at 1 wpi for BQ1-1 ($\Delta SI = 2.2$) and at 8 wpi for BQ1-3 ($\Delta SI = 2$) (fig3A). In contrast, the proliferation of PBMC from *B. divergens*-infected sheep, compared to the control sheep, was higher (ΔSI_{max} between 4 and 4.5) and increased between 1 and 7 wpi (increase significant at 1, 3, 4 and 5 wpi) (fig 3B). Nevertheless the kinetics of PBMC stimulation by BdE differed between the *B.*

divergens-infected sheep with peak proliferation occurring at 2 wpi in Bd1, 7 wpi in Bd2 and 5 wpi in Bd3). Thereafter PBMC proliferation fell to the control level. No proliferation was detected after 8 wpi in any B. divergens-infected sheep.

Similarly, the IFNγ and IL10 produced in the supernatant of activated PBMC differed considerably between the 2 infected groups. At time points studied (weekly), no IFNγ was detected in the supernatant of PBMC from two Babesia sp. BQ1 (Lintan)-infected sheep (BQ1-1 and BQ1-3), and only a slight increase (Δ[IFNγ] = 0.57 ng/ml), was observed at 6 wpi in sheep BQ1-2 (fig. 3C) whereas IL10 was never detected in the supernatant of PBMC from *B. divergens*-infected sheep (fig. 3F). No IFNγ production was detected in supernatant from sheep Bd1 whereas in sheep Bd2 and Bd3, IFNγ secretion was significantly higher than control sheep and *Babesia sp*. BQ1 (Lintan)-infected sheep at 4 wpi and then decreased to the control level (fig. 3D). The production of IL10 by BQ1E-stimulated PBMC from *Babesia* sp. BQ1 (Lintan)-infected sheep was significantly higher than the production of IL10 by BdE-stimulated PBMC from *B. divergens*-infected sheep at several wpi (3, 5, 6, 7, 8 wpi, fig 3E and F). The kinetics of IL10 secretion varied from one sheep to another (fig. 3E). No production of IL10 was detected in sheep BQ1-2 whereas IL10 production peaked at 1 wpi and 3 wpi respectively in sheep BQ1-1and BQ1-3 ($\Delta[IL10]_{max}$ = 1.7 units/ml for sheep BQ1-1 and $\Delta[IL10]_{max}$ = 2.6 units/ml for sheep BQ1-3)., and then fluctuated until the end of the experiment.

DISCUSSION

This report is the first to demonstrate the involvement of cellular immune responses, especially those of cytokines IFNγ and IL10, in the course of infection by *Babesia* sp. BQ1 (Lintan) and *B. divergens* in spleen-intact sheep. Indeed, several features described in this paper confirmed the lower pathogenicity of *B. divergens* than with *Babesia* sp. BQ1 (Lintan) in sheep.

(i) No clinical sign of babesiosis was observed in either infected group, except for a significant increase of body temperature in *Babesia* sp. BQ1 (Lintan)-infected sheep during the first week of infection

(ii) Patent parasitemia was detected in Giemsa-stained blood smears from the ear vein of sheep infected with *Babesia* sp. BQ1 (Lintan). The presence of parasite in the ear vein but not the jugular vein could be explained by the high concentration of this parasite in the capillaries and perhaps by the adhesion of iRBC to endothelial cells, as described for *B. bovis*-infected erythrocytes (21). This adhesion has not been reported for *B. divergens*.

(iii) Although the delay to obtain 1% parasitemia in standardised cultures was approximately the same at the end of infection with both parasites (32-$d_{1\%}$

between 19 and 24 hours), the generation time of *Babesia sp*. BQ1 (Lintan) is much higher (20 hours, unpublished data) than that of *B. divergens* (8 hours, 18). Thus, for the same $d_{1\%}$, the number of *Babesia* sp. BQ1 (Lintan) in sheep blood is probably higher than that of *B. divergens*.

(iv) The course of parasitemia was identical in the three *Babesia sp*. BQ1 (Lintan)-infected sheep, but between-animal variation was observed in the sheep infected with *B. divergens*. Furthermore, the time taken for parasitemia to plateau was longer in *B. divergens*-infected sheep than in *Babesia sp.* BQ1 (Lintan)-infected sheep. This could not be due to a need for *Babesia sp.* BQ1 (Lintan) and *B. divergens* to adjust to new erythrocytes after the injection of iRBC because the sheep were infected with their own infected red blood cells.

Malandrin et al (2009) (10) demonstrated that the in vivo susceptibility of sheep to *B. divergens* is dependent on the capacity of the erythrocytes to sustain growth of the parasite. Indeed, sheep with non susceptible erythrocytes could not be infected by *B. divergens*. In our study, the level of *B. divergens* or *Babesia* sp. BQ1 (Lintan) pathogenicity could not be explained by the in vitro susceptibility of the erythrocytes from each sheep (Table 1). Indeed, the in vitro susceptibility of erythrocytes was approximately the same for sheep BQ1-2 and Bd2 (60-70%) and for sheep BQ1-3 and Bd3 (80-90%). Erythrocytes from sheep BQ1-1 were less able to sustain multiplication of the parasite than erythrocytes from Bd1 (32 and 102 % respectively) but sheep BQ1-1 seemed to be more susceptible than sheep Bd1 in vivo (table 1). Several studies of host vertebrate susceptibility to *Babesia* spp. have suggested the important roles of genetic and immune responses (13, 15, 16, 22)

As expected, the humoral immune responses confirmed that the immune protection of sheep infected by *Babesia* spp. did not involve antibodies. Productions of IgG, IgG1 and IgG2 were significantly greater in *Babesia* sp. BQ1 (Lintan)-infected group than in *B. divergens*-infected group during the first 3 weeks of infection. Similarly, no IgM production was detected in sera from *B. divergens*-infected sheep. Antibody responses during *Babesia* spp. and *Plasmodium* spp. infection were shown to be associated with protection against high parasitemia and disease (babesiosis or malaria) but not against parasitization *per se* (23, 24). In our study, once the humoral response had reached a plateau (2 or 3 wpi), no parasite could be detected in blood smears from the ear vein whereas parasitemia in blood from the jugular vein remained unchanged. Thus antibodies seemed to be able to decrease the number of *Babesia* sp. BQ1 (Lintan) in capillary blood. They were also able to limit the multiplication of *Babesia* sp. BQ1 (Lintan), because parasitemia no longer increased once antibody production had begun, but were unable to eliminate the infection.

In contrast the 2 groups of infected sheep showed marked differences in the cellular responses. No specific proliferation of PBMC from *Babesia* sp. BQ1 (Lintan)-infected sheep

was detected whereas a significant increase in proliferation of PBMC from *B. divergens*-infected sheep was observed from 1 wpi. The importance of cellular responses in the protective immune response of cattle against *B. bovis* infection is well established and involves mononuclear phagocyte-mediated type-1 mechanisms (23). IFNγ is involved in the production of NO molecules by monocyte/macrophages, which show high babesicidal activity, and in directing the production of opsonic IgG2 antibody (23). Goff et al (2001) (16) showed that induction of the IL10 message in the spleen was followed by reduced levels of IL12, IFNγ and TNFα expression and that IL10 production was associated with the higher susceptibility to *B. bovis* of adult cattle compared to calves. The immune responses of cattle, during infection by *B. bigemina*, predominantly involved a Th1 response associated with the detection of IFNγ, TNFα and iNOS and no or weak detection of IL4 and IL10 (25). In our study, IFNγ production was not detected in PBMC supernatant from *Babesia* sp. BQ1 (Lintan)-infected sheep but was detected in *B. divergens*-infected sheep and inversely for IL10. This suggests that IFNγ and IL10 have important roles in the course of infection by *Babesia* sp. BQ1 (Lintan) and to *B. divergens* in sheep. No IL10 was produced during *B. divergens* infection but IFNγ could be expressed and exert protective activity, which might explain the weak infectivity of *B. divergens* in sheep. In contrast, IL10 was produced early during *Babesia* sp. BQ1 (Lintan) infection, and this could be responsible for the absence of IFNγ production and the higher pathogenicity of these species of *Babesia* in sheep. Unfortunately, no NO production was detected in either the PBMC supernatant or plasma from the two groups of infected-sheep (data not shown). This absence of NO production could be explained by the lower in vitro expression of iNOS in sheep and goat macrophages than in bovine macrophages (26, 27). Furthermore Goff et al (2002) (28) demonstrated that iNOS expression in bovine monocytes, in the presence of *B. bovis* merozoites, is dependent on both IFNγ and TNFα. It would therefore be interesting to measure TNFα production in cells from *Babesia* sp. infected sheep to see whether the absence of NO production is linked with the absence of TNFα production.

Although cellular responses could be involved in the course of infection by *Babesia* sp. in sheep and in limiting parasite multiplication, they were unable to eliminate the infection. Indeed, sheep were persistently infected by *B. divergens* despite the specific-PBMC proliferation induced by *B. divergens* antigens and IFNγ production. However the early production of IFNγ by PBMC from *B. divergens*-infected sheep (between 2 and 4 wpi) could explain the absence of detection of *B. divergens* in the blood between 8 and 14 dpi for sheep Bd2 and between 8 dpi and 4 wpi for sheep Bd3. No IFNγ production was detected after 6 or 7 wpi, and parasitemia then increased to reach a plateau. Goff et al (2001) (16) have also shown the importance of the kinetics of cytokine production during *B. bovis* infection in calves and adults: if IL12 and IFNγ are produced before IL10, the innate immune response

will be protective. In contrast, if IL10 is influential first, the type 1 response will either be delayed or inhibited until it is too late to arrest the disease process. However, IL10 production by PBMC from *B. divergens*-infected sheep was not detected at time points studied and so could not explain the decrease of IFNγ production.

In conclusion, cellular responses and the production of IFNγ and IL10 have key roles in the course of infection by *B. divergens* and *Babesia sp.* BQ1 (Lintan) : IFNγ is associated with the low infectivity of *B. divergens* and IL10 with the higher ability of *Babesia sp*. BQ1 (Lintan) to infect sheep. Further investigations are needed to explore the mechanisms regulating the production of these cytokines during infection and to identify the molecules of *B. divergens* and *Babesia* sp. BQ1 (Lintan) involved in immunomodulation of the host immune responses.

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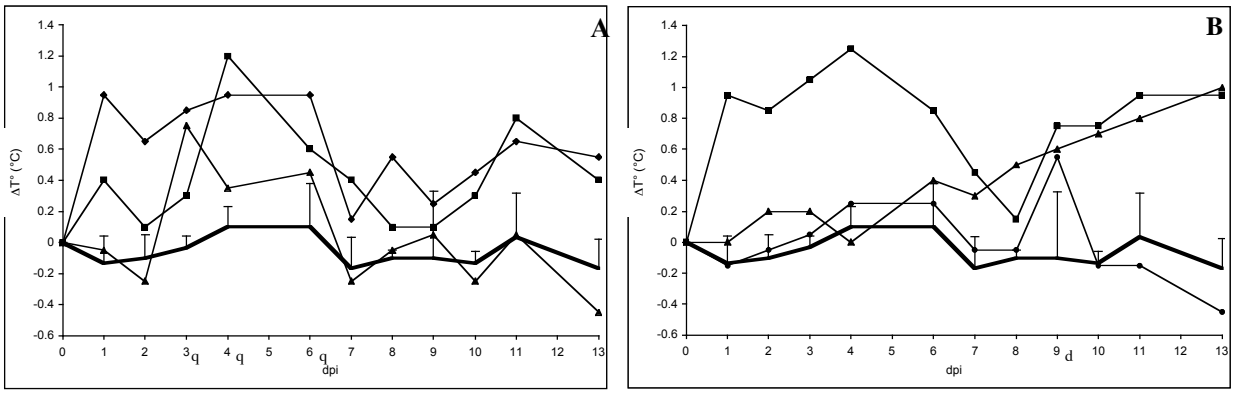
LIST OF FIGURES

Figure 1



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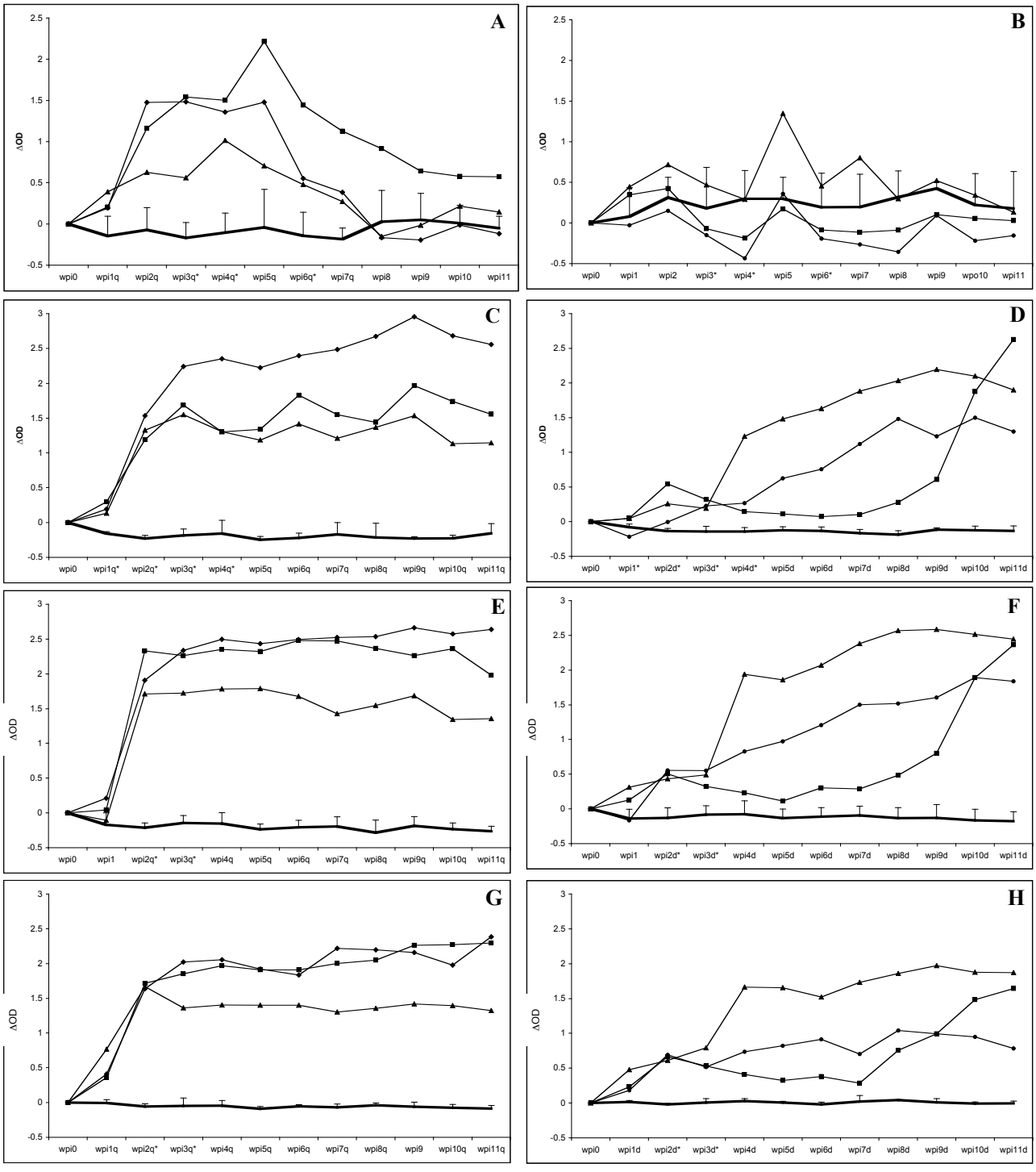
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C
ΔOD
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D
ΔOD
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ΔOD
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F
ΔOD
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G
ΔOD
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H
ΔOD
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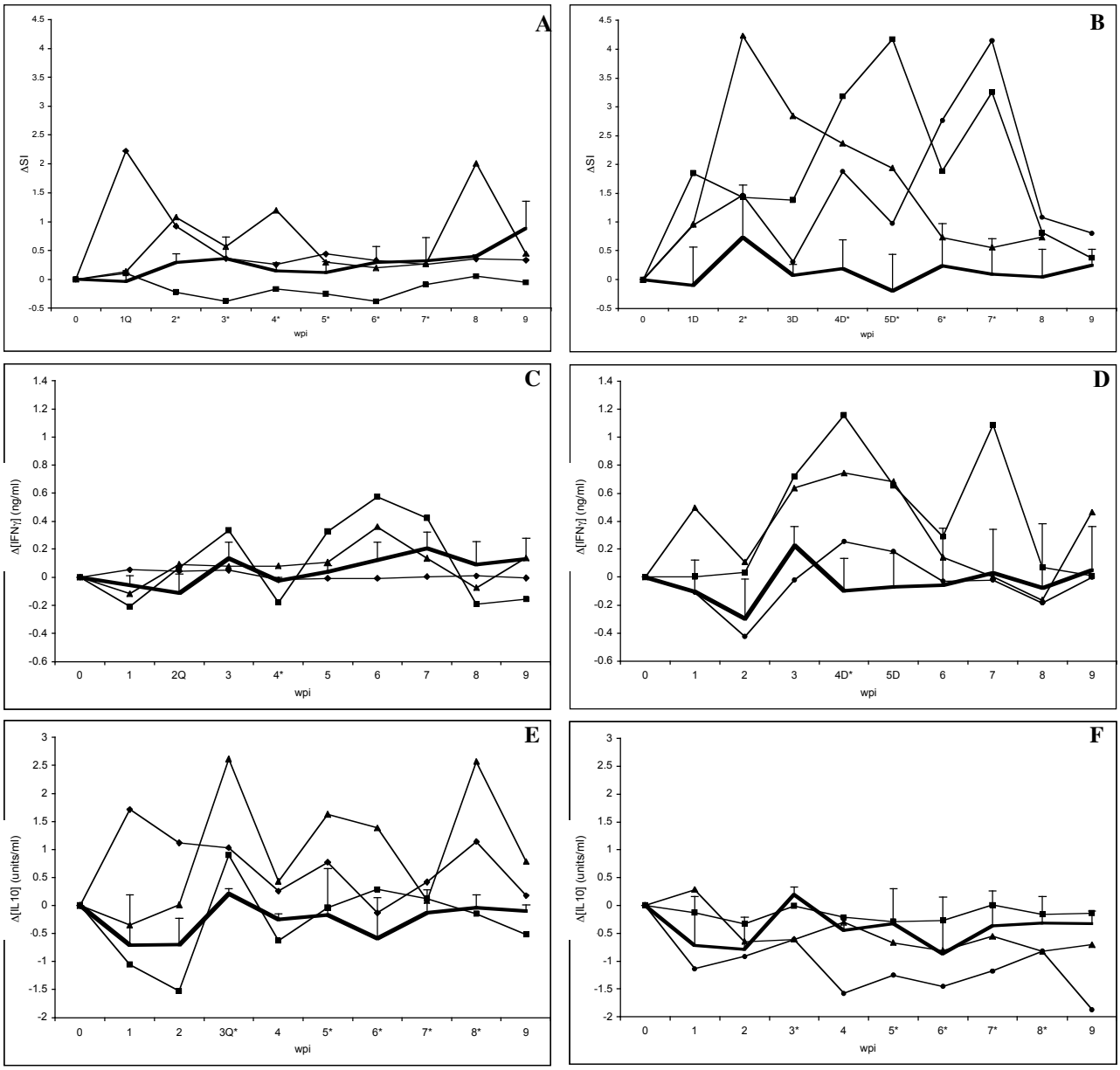
Figure 3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ΔSI
wpi
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Δ[IFNγ] (ng/ml)
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E
Δ[IL10] (units/ml)
wpi
F
Δ[IL10] (units/ml)
wpi

Table 1: in vitro susceptibility of erythrocytes to *B. divergens* and *Babesia* sp. BQ1 (Lintan) from each infected sheep (expressed as percentage of in vitro susceptibility of erythrocytes from control sheep) and quantity of iRBC inoculated in each sheep.

Sheep	Inoculation dose (iRBC)	Susceptibility to *Babesia* sp. BQ1 (Lintan) (%)	Susceptibility to *B. divergens* (%)
Bd1	12×10^8		102
Bd2	13×10^8		60
Bd3	11×10^8		90
BQ1-1	10×10^8	32	
BQ1-2	8.5×10^8	67	
BQ1-3	8×10^8	83	

Table 2: Course of parasitemia of 3 *B. divergens*-infected sheep and 3 *Babesia* sp. BQ1 (Lintan)-infected sheep. The evolution of in vivo parasitemia was estimated daily until 21 days post infection (dpi) and then weekly until 11 weeks post infection (=78 dpi) by measuring the number of days needed to obtain 1% parasitemia in standardised cultures ($d_{1\%}$) subtracted from the maximum duration (32 days) allowed for the culture ($32-d_{1\%}$). Grey squares indicate that parasites were detected in Giemsa-stained blood smears from the ear vein. (*) indicates significant differences ($p<0.05$) between the 2 infected-sheep group by the Mann-Whitney non parametric test.

dpi	0	1	2	3	4	6	7	8*	9*	10*	11*	13	14*	15*	16*	17	18	20	21	29*	36*	43*	50*	57	64*	71	78
wpi	0						1						2*						3	4*	5*	6*	7*	8	9*	10	11
Bd1	0	15	22	22	24	22	22	0	0	0	0	0	0	0	22	0	22	22	23	20	19	20	15	22	0	22	21
Bd2	0	0	22	23	24	23	22	0	0	11	0	21	0	0	22	22	22	21	21	20	8	15	20	20	19	20	20
Bd3	0	23	22	22	23	15	16	0	0	0	0	0	0	0	0	0	0	0	0	0	9	11	12	12	19	18	19
BQ1-1	0	0	18	17	20	22	23	24	23	24	23	20	22	24	23	22	23	20	20	22	22	22	22	22	22	22	23
BQ1-2	0	0	16	17	20	21	21	22	23	23	23	25	23	23	24	24	24	22	23	23	22	22	22	22	22	20	23
BQ1-3	0	17	22	23	23	22	23	22	23	23	24	23	23	24	24	22	22	21	22	24	24	23	23	23	22	24	24

Article N°5 : The development and evaluation of a loop-mediated isothermal amplification (LAMP) method for detection of *Babesia* spp. infective to sheep and goats in China. (Developement et évaluation de la méthode LAMP pour détecter *Babesia* spp. infectant les moutons et les chèvres en Chine.)

Article publié dans Experimental Parasitology, 2008, 120, 39-44.

Ce travail a été réalisé au laboratoire de parasitologie vétérinaire de la province du Gansu, à Lanzhou.

La méthode LAMP permet d'amplifier l'ADN dans des conditions isothermiques. Elle est caractérisée par l'utilisation de 4 amorces différentes désignées pour reconnaître 6 régions distinctes du gène cible. L'amplification se fait en 1 seule étape, à température constante (65°C en général).

Dans cette étude nous avons mis au point la technique LAMP pour détecter *Babesia sp*. BQ1 (Lintan) et *Babesia sp*. Xinjiang chez les petits Ruminants en Chine. Afin de déterminer les amorces spécifiques de ces 2 parasites, les séquences du gène de l'ARNr 18S de *B. ovis* (AY260178), de *Babesia sp*. BQ1 (Lintan) (AY260181), *Babesia sp.* Xinjiang (DQ159073), *Theileria sp*. China1 (AY262115) et *Theileria sp*. China2 (AY262120) ont été alignées afin d'identifier les régions variables. Les 4 amorces LAMP sont : pour *Babesia sp.* BQ1 (Lintan) : F3 : CGTTTCGGATATTGTCCGT ; B3 : GCGCATACTAGGCATTCCTC ; FIP : AGCCCAGGACATCTAAGGGCATTTTGACTCCTGCGCTTGAAGC et BIP : CCATCGAGTTTGTCCTGTCCCGTTTTAAAATCAATCCCCGACACGA et pour *Babesia sp.* Xinjiang : F3 : TTGTTCTCGTGTTCCCTT ; B3 : AAATACGAATGCCCCCAA ; FIP : AAGCCTGCTTGAAACACTCTAATTTTCGGGTTTCGTCTACTTCG et BIP : GAGCATGGAATAATAGAGTAGGACTTTTCCGTTCCTATTAACCATTACCA. La technique LAMP mise au point a été comparée à la technique nested PCR pour les 2 mêmes parasites.

Afin d'évaluer la spécificité de la LAMP et de la PCR, des échantillons d'ADN de *Theileria sp*. China1, de *Theileria sp*. China2, de *Theileria sp*. isolé au Japon, de *B. bovis*, de *Babesia sp.* BQ1 (Lintan) et de *Babesia sp*. Xinjiang ont été testés. Des amplicons ne sont observés qu'avec les échantillons d'ADN autologues. Le séquençage de ces amplicons correspond à la région cible choisie du gène de l'ARNr 18S des 2 *Babesia* étudiées. La sensibilité de la LAMP est excellente et meilleure que celle de la PCR nichée : elle permet de détecter 0,02 pg et 0,2 pg d'ADN de *Babesia sp.* BQ1 (Lintan) et de *Babesia sp*. Xinjiang respectivement, ce qui correspond à une parasitémie de 2.10^{-8} et 2.10^{-7} (0,000002 % et 0,00002 %). L'utilisation de la LAMP pour détecter les 2 espèces de *Babesia* a été évaluée chez des moutons infectés expérimentalement et naturellement. Chez les moutons infectés

expérimentalement, la LAMP permet de détecter *Babesia* sp. BQ1 (Lintan) entre le 3ème et le 63ème jour d'infection *et Babesia* sp. Xinjiang entre le 14ème et le 21ème jour d'infection. Des échantillons sanguins d'animaux de la région du Gansu (365) et de la région du Xinjiang (145) ont été testés : *Babesia* sp. BQ1 (Lintan) et *Babesia sp.* Xinjiang ont été détectés chez 14,3 % (52/365) et 3,5 % (5/145) des animaux respectivement. La technique LAMP mise au point dans cette étude peut donc être une méthode alternative à l'examen microscopique d'étalements sanguins afin de détecter *Babesia* sp. BQ1 (Lintan) et *Babesia sp.* Xinjiang. C'est une méthode sensible, spécifique, rapide (<1h), simple et ne nécessitant pas de matériels spécialisés. Elle permet de détecter les protozoaires précocément au cours de l'infection. Elle a permis de montrer que l'infection par *Babesia sp.* BQ1 (Lintan) et à *Babesia sp.* Xinjiang est une maladie relativement fréquente dans certaines régions de Chine.

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The development and evaluation of a loop-mediated isothermal amplification (LAMP) method for detection of *Babesia* spp. infective to sheep and goats in China

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ABSTRACT

The loop-mediated isothermal amplification (LAMP) reaction is a method that amplifies with high sensitivity, efficiency, and rapidity, deoxyribonucleic acid (DNA) under isothermal condition in simple incubators. Two primer sets for the LAMP method were designed using the nucleotide sequences of 18S rRNA gene of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 isolated in China. The primers were used to detect parasite DNA extracted from infected blood and purified parasites by LAMP. The specific ladder bands were amplified from the autologous genomic DNA of two *Babesia* species, respectively, and did not cross-react with the genomic DNA of *Theileria* sp. China 1, *Theileria* sp. China 2, *B. bovis*, *Theileria* sp. (Japan) and sheep. The LAMP was sensitive enough to detect 0.02 pg and 0.2 pg genomic DNA of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005, respectively, from 10-fold serially diluted samples corresponding to the amount of DNA present in 50 μl of 0.000002% and 0.00002% parasitemic erythrocytes. Furthermore, DNA extracted from blood of intact (non-splenectomized) sheep experimentally infected with *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 was amplified by the LAMP from week 1 to 9 and week 2 and 3 post-infection, respectively, demonstrating the high sensitivity of these primers. Of 365 samples collected from Gansu province, 14.3% (52/365) were positively detected by the LAMP. Of 145 samples collected on filter papers (Whatman) from the grazing sheep in Xinjiang province, 3.5% (5/145) were positive. These results show that the LAMP could be an alternative diagnostic tool for the detection of babesial infection in sheep and goats.

1. Introduction

The piroplasms of small ruminants are common parasites in the north of China, and they cause serious problems for the livestock industry. The rate of infection is 15–80% and the mortality of lambs is more than 50% in some regions (Luo and Yin, 1997; Yin et al., 2002). In the past two decades, the pathogen was considered to be an unidentified *Theileria* transmitted by *Haemaphysalis qinghaiensis*. However, recently, several strains of *Babesia* spp. infective to sheep and goats were also isolated from different places in China, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. (Tianzhu), *Babesia* sp. (Hebei), *Babesia* sp. (Madang), *Babesia* sp. (Liaoning) and *Babesia* sp. Xinjiang-2005. The symptoms of babesiosis; fever, hemolytic anaemia, icterus, and sometimes death could be observed in sheep parasitized by *Babesia* in the field (Bai et al., 2002; Guan et al., 2001, 2002). These strains can be divided into two groups based on the sequences of 18S rRNA gene, one including, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. (Ningxian), *Babesia* sp. (Tianzhu), *Babesia* sp. (Hebei), *Babesia* sp. (Madang), and *Babesia* sp. (Liaoning) and the other just containing *Babesia* sp. Xinjiang-2005 (Liu et al., 2007a; Schnittger et al., 2003, 2004). This suggests that ovine babesiosis, together with ovine theileriosis, may be wide-spread in the north of China causing enormous economic loss in the small ruminant industry. To control the disease, it is important that an accurate, highly efficient, simple method should be developed for diagnosis and epidemiological surveillance of the disease in China.

Recently, Notomi et al. (2000) reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP) that has the advantages of high specificity, sensitivity and efficiency, simple operation and equipment but also amplifies under isothermal conditions. This makes it possible to diagnose disease quickly and accurately. The original method has been improved on and used to diagnose viral, bacterial and

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protozoan diseases of humans, animals and even plants (Alhassan et al., 2007; Fukuta et al., 2003; Kuboki et al., 2003; Thekisoe et al., 2005; Toriniwa and Komiya, 2006;), and has potential for development as a the routine diagnostic method.

In this study, we developed a LAMP method for diagnosing ovine babesiosis in China based on the sequences of 18S rRNA gene of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 and evaluated its specificity and sensitivity in the blood samples collected from experimentally infected and naturally infected sheep.

2. Materials and methods

2.1. Animals

Eight sheep, 5–7 months of age, were purchased from a *Babesia*- and *Theileria*-free area. Four sheep were splenectomized 2 months before the study. Blood films taken from the ears of the sheep were fixed with methanol, stained with Giemsa and examined every week for the presence of haemoprotozoan parasites. Only negative sheep were used in the experiment.

2.2. Infection of animals

Four piroplasm strains were used in the study, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. Xinjiang-2005, *Theileria* sp. China 1 and *Theileria* sp. China 2. Four splenectomized sheep were infected with cryopreserved parasites. The rectal temperature was measured and blood smears were examined every day post-infection for presence of parasites. When the parasitemia was 5–20%, blood was collected from the jugular vein for purifying merozoites and extracting genomic parasite DNA of parasites using Alsever's solution as anticoagulant. Four intact (non-splenectomized) sheep were divided into two groups and infected with the *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005, respectively. Disease monitoring was performed by taking rectal temperatures and microscopic examination of Giemsa-stained blood smears for the presence of piroplasms. Jugular vein blood was also collected in anticoagulant for extracting parasite DNA every week for a period of 12 weeks.

2.3. Purification of Babesia spp. merozoites

Babesia sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 merozoites were prepared from the blood collected at peak parasitemia from experimentally infected splenectomized sheep. Infected blood was centrifuged at 1000*g* for 10 min at 4 °C and the packed cells were washed three times with 10 mM Tris–HCl (pH 7.4), 150 mM NaCl (Tris–saline) by centrifugation as before and the buffy coat removed. A cellulose powder column (CF-11, Whatman) was used to remove the remaining leukocytes. The packed infected erythrocytes were suspended with 1 volume Tris–saline and incubated in 37 °C water bath for 5 min. AH-1 hemolysin (final concentration: 300 HU/ml; provided by Professor Chihiro Sugimoto) was added for lysing erythrocytes in 37 °C water bath for 10 min. Lysis was stopped by addition of 0.01 volumes of 500 mM EDTA and followed by centrifugation at 1000*g* for 10 min at 4 °C to remove cellular debris and intact erythrocytes. The supernatant was recovered and further centrifuged at 4000*g* for 15 min at 4 °C to pellet the merozoites. The pellet of merozoites was washed with Tris–saline by further centrifugation until it was almost free of hemoglobin and then stored at −70 °C.

2.4. Genomic DNA preparation

Total DNA of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 were prepared from purified merozoites with the Puregene DNA Purification Kit (www.gentra.com) according to the manufacturers instructions. So that DNA samples could be considered free of sheep genomic DNA, *Theileria* sp. China 1, *Theileria* sp. China 2 and experimentally infected intact sheep DNA were directly extracted from the anticoagulated blood of infected sheep. The DNA samples of *Theileria* sp. (Japan) isolated from serow, and *B. bovis* were kindly provided by the Research Unit of Advanced Preventive Medicine, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan. Three hundred and sixty-five blood samples were collected from the sheep on pastureland in Zhuoni, Biandu, Lintan and Xiahe counties, Gansu province from April to June 2004. One hundred and forty-five blood samples were collected on filter paper (FTA card; Whatman, WB120205) from the grazing sheep in Bayituohai, Kashi, Yingtamu, Quluhai counties, Xinjiang province in April 2005. The DNA samples were extracted from the blood. Aqueous DNA preparations were frozen at −70 °C until use. The DNA samples on FTA cards were prepared according to manufacture's instructions before use.

2.5. Specific primers of LAMP and PCR

The sequences of 18S rRNA gene of *B. ovis* (AY260178), *B. motasi* (AY260180), *Babesia* sp. BQ1 (Lintan) (AY260181), *Babesia* sp. Xinjiang- (DQ159073), *Theileria* sp. China 1 (AY262115), *Theileria* sp. China 2 (AY262120) were used for reference in this study. To obtain the specific primer for the LAMP and PCR, multiple sequence alignments were performed using the program algorithm of Clustal V in the MegAlign component of the DNASTAR programme (Version 4.01 DNASTAR, Madision, Wis.) (Figs. 1 and 2). The specific primers were designed using the PrimerExplorer V2 and Primer5.0 software for LAMP and nested PCR, respectively, in variable region of 18S rRNA gene sequence. The oligonucleotide sequences of primers are shown in Table 1.

2.6. LAMP procedures

The reaction was performed in 25.0 μl mixture containing 12.5 μl LAMP buffer (40 mM Tris–HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M betaine and 2.8 mM concentration of each deoxynucleoside triphosphate (dNTP)), 0.9 μl primers mix (FIP and BIP at 40 pmol each and F3 and B3 at 5 pmol each), 2.0 μl extracted DNA or a piece of FTA card, 1.0 μl (8 U) *Bst* DNA polymerase (New England Biolabs, M0275L) and 8.6 μl distilled water. The reaction mixture was incubated in a heat block, at 63 °C for 30 min (for *Babesia* sp. BQ1 (Lintan)) or 60 °C for 45 min (for *Babesia* sp. Xinjiang-2005) and then inactivation was carried out at 80 °C for 2 min. The LAMP products were loaded on 1.5% agarose gel in a Tris–acetic acid–EDTA (TAE) buffer for electrophoresis and visualized and photographed under UV light after staining with ethidium bromide.

2.7. Nested PCR amplification

The PCR was performed in an automatic thermocycler in a total reaction volume of 50 μl containing 5.0 μl 10× PCR buffer (Mg^{2+} Plus), 4.0 μl dNTPs of 2.5 mM each of the four deoxynucleotide triphosphates, 1.25 U Taq polymerase, 2.0 μl template DNA or a piece of FTA, 1.0 μl each primers (20 pmol) and 36.75 μl distilled water. For the primary PCR cycle parameters used an initial were followed by initial step at 94 °C 3 min, 40 cycles at 94 °C for 30 s, 55 °C for 40 s and 72 °C for 1 min with a final extension step at 72 °C for 10 min. Subsequently, the nested PCR was performed using 1.0 μl of the primary product as template and the primary PCR mixture. The condition of reaction was as follows: an initial step at 94 °C for 3 min, 40 cycles at 94 °C for 30 s, 55 °C (*Babesia* sp. Xinjiang-

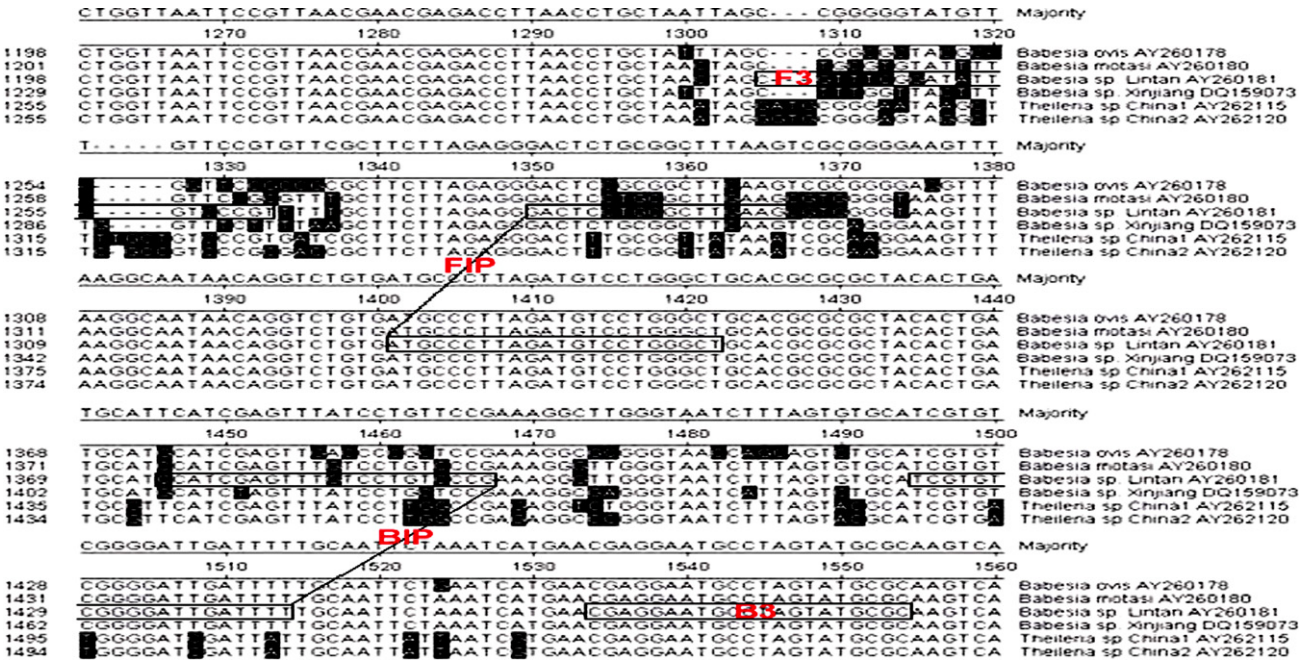

Fig. 1. The multiple sequence alignments of target site of the *Babesia* sp. BQ1 (Lintan) LAMP primers.

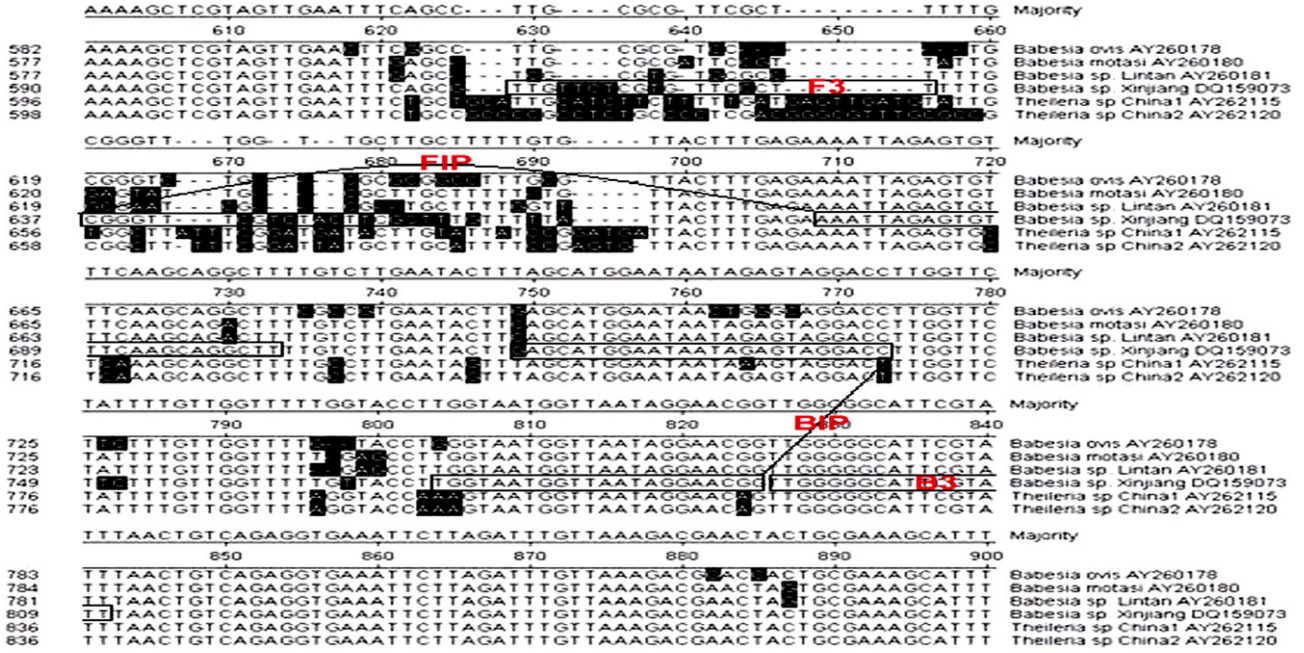

Fig. 2. The multiple sequence alignments of target site of the *Babesia* sp. Xinjiang-2005 LAMP primers.

2005) or 52 °C (*Babesia* sp. BQ1 (Lintan))for 40 s and 72 °C for 1 min with a final extension step of 72 °C for 10 min. Finally, the nested PCR products were visualized by UV transillumination in a 1.5% agarose gel following electrophoresis and staining with ethidium bromide.

2.8. Sequencing of LAMP products

LAMP products were diluted appropriately with distilled water and amplified by PCR with primers that bind to the F2 region (5′-GACTCCTGCGCTTGAAGC-3′ for *Babesia* sp. BQ1 (Lintan), 5′-CGGGTTTCGTCTACTTCG-3′ for *Babesia* sp. Xinjiang-2005) and to the complementary strand of the B1c region (5′-AAAATCAATCCCC GACACGA-3′ for *Babesia* sp. BQ1 (Lintan), and 5′-CCGTTCCTATTAAC CATTACCA-3′ for *Babesia* sp. Xinjiang-2005). The PCR products were cloned into pGEM-T Easy vector (Promega, USA), and transformed into *Escherichia coli* JM109 competent cells for sequencing by the BigDye Terminator Mix of TakaRa Company (China).

2.9. Evaluation of the LAMP using the samples from experimentally infected sheep

Two intact sheep (non-splenectomized) were infected with *Babesia* sp. BQ1 (Lintan) and a further two another infected with

Table 1
The LAMP and nested PCR primers

Parasite	Primer set	Sequence (5′–3′)
B. sp. BQ1 (Lintan)	BLTLAMP	F3: CGTTTCGGATATTGTCCCGT B3: GCGCATACTAGGCATTCCTC FIP: AGCCCAGGACATCTAAGGGCATTTTGACTCCTGCGCTTGAAGC BIP: CCATCGAGTTTGTCCTGTCCCGTTTTAAAATCAATCCCCGACACGA
B. sp. Xinjiang-2005	BXJLAMP	F3: TTGTTCTCGTGTTCCCTT B3: AAATACGAATGCCCCCAA FIP: AAGCCTGCTTGAAACACTCTAATTTTCGGGTTTCGTCTACTTCG BIP: GAGCATGGAATAATAGAGTAGGACCTTTTCCGTTCCTATTAACCATTACCA
B. sp. BQ1 (Lintan)	INNER PRIMERS BLT	BLT911-AS: AGGACTACGACGGTATCTGA BLT623-S: ATCGCGTGCTTTTGGT
B. sp. Xinjiang-2005	INNER PRIMERS BXJ	BXJ632-AS: AAGGGAACACGAGAACAA BXJ368-S: CTGAGAAACGGCTACCA
B. spp. and *Theileria* spp.	OUTER PRIMERS TBall	TBall 136-S: CATGGATAACCGTGCTAATT TBall 936-AS: ATCGTCTTCGATCCCCTAACT

Babesia sp. Xinjiang-2005. Blood smears were made using ear vein blood for microscopic examination daily for the first two weeks and then weekly. Jugular vein blood was also collected for examination with LAMP.

3. Results

3.1. The specificity of LAMP and nested PCR

In present study, we used the DNA samples from the *Theileria* sp. China 1, *Theileria* sp. China 2, *Theileria* sp. isolated from serow in Japan, *B. bovis*, *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 to evaluate the specificity of the LAMP and PCR. At the same time, DNA samples extracted from *Babesia*-free sheep and distilled water were used as the negative control and blank control, respectively. When the primer sets BLTLAMP and BXJLAMP were applied to amplify all these samples, only the autologous DNA samples of two *Babesia* spp. and the positive LAMP products showed typical ladder bands (Figs. 3A and 4C). Similar results were obtained in nested PCR and the segments amplified were about 288 bp (*Babesia* sp. BQ1 (Lintan)) and 264 bp (*Babesia* sp. Xinjiang-2005), respectively (Figs. 3B and 4D). In addition, sequencing showed that they were, respectively, amplified from the target location of 18S rRNA gene of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005.

3.2. Comparison of sensitivity of LAMP and nested PCR

The DNA samples of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 were initially diluted to 100 ng/μl and then diluted serially 10-fold. These were then used to evaluate the sensitivity

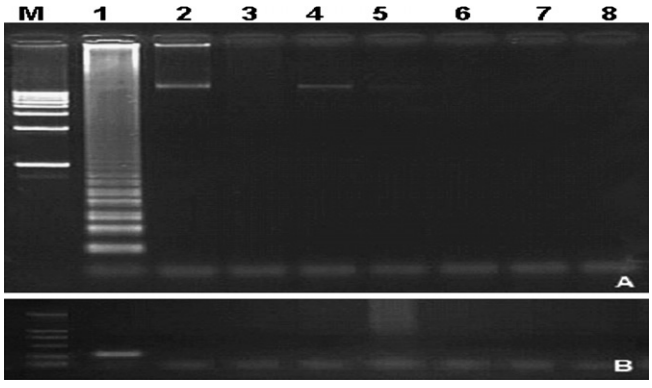

Fig. 3. The specificity of LAMP (A) and nested PCR (B) for *Babesia* sp. BQ1 (Lintan). Lanes 1–9: *Babesia* sp. BQ1 (Lintan), *Babesia* sp. Xinjiang-2005, *B. bovis*, *Theileria* sp. China 1, *Theileria* sp. China 2, *Theileria* sp. (Japan), Sheep DNA, distilled water.

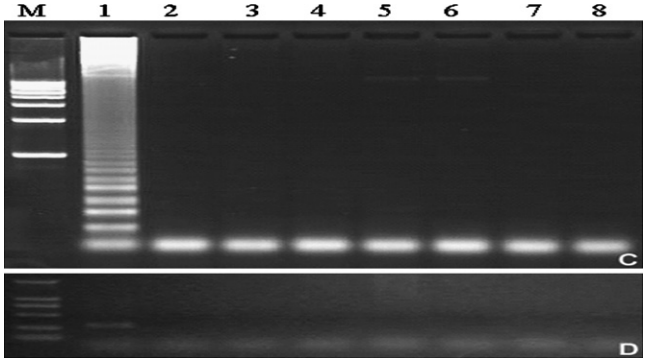

Fig. 4. The specificity of LAMP (C) and nested PCR (D) for *Babesia* sp. Xinjiang-2005. Lanes 1–8: *Babesia* sp. Xinjiang-2005, *Babesia* sp. BQ1 (Lintan), *B. bovis*, *Theileria* sp. China 1, *Theileria* sp. China 2, *Theileria* sp. (Japan), sheep DNA, distilled water.

of LAMP and PCR. The results showed that the LAMP of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 could detect 0.02 pg and 0.2 pg DNA in the samples, respectively (Figs. 5E and 6G). This corresponds approximately to the amount of DNA in 50 μl of 0.000002% and 0.00002% parasitemic erythrocytes (Bone et al., 1983; Mclaughlin et al., 1986).The two nested PCR could just detect the 2.0 pg *Babesia* DNA samples (Figs. 5F and 6H).

3.3. Evaluation of the LAMP using the samples from experimentally infected sheep

The earliest time that parasites could be detected with BLT-LAMP primer sets in the blood was on the 3rd and 5th day, respec-

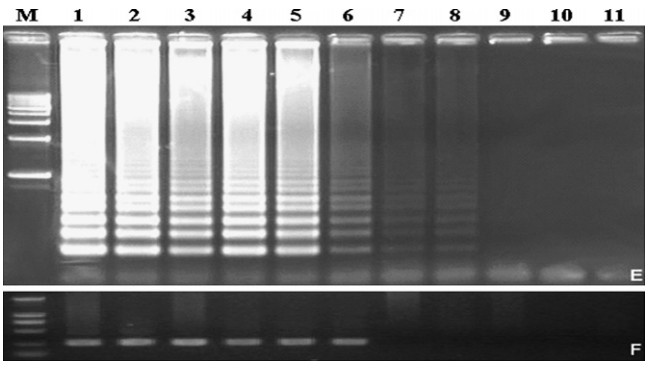

Fig. 5. The sensitivity of LAMP (E) and nested PCR (F) for *Babesia* sp. BQ1 (Lintan). Lanes 1–9: 100 ng/μl, 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 0.1 pg/μl, 0.01 - pg/μl, 0.001 pg/μl. 10–11: sheep DNA, distilled water.

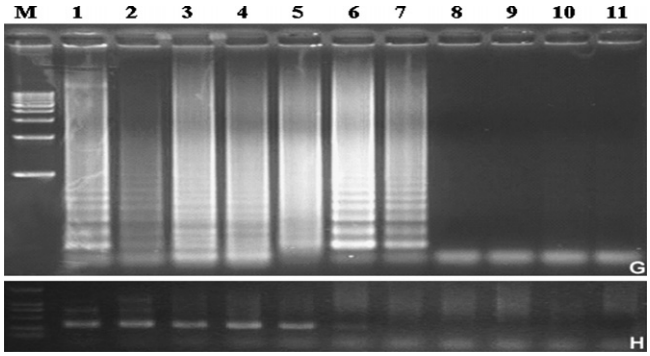

Fig. 6. The sensitivity of LAMP (G) and nested PCR (H) for *Babesia* sp. Xingjiang-2005. Lanes 1–9: 100 ng/μl, 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 0.1 pg/μl, 0.01 pg/μl, 0.001 pg/μl. 10–11: sheep DNA and distilled water.

Table 2
Examination of the field samples using the LAMP and nested PCR

Original of samples	Numbers of sample	Primer sets	Positive numbers
Gansu	365	BLTLAMP	52
		INNER PRIMERS BLT	8
Xinjiang	145	BXJLAMP	5
		INNER PRIMERS BXJ	0

tively, and the latest time was 63rd and 56th day post-infection. In contrast *Babesia* sp. BQ1 (Lintan) first appeared in blood smears on the 8th day and lasted 35 and 14 days, but we did not find parasites on 21st day post-infection. For *Babesia* sp. Xinjiang-2005, the earliest time detected by LAMP was 14th day post-infection. However, we could not detect parasites by microscopic examination during the 12 weeks post-infection.

3.4. Evaluation of the LAMP using field samples

Three hundred and sixty-five samples collected from Gansu province and 145 samples collected from Xinjiang province were evaluated with the LAMP and nested PCR. The results showed that 14.3% (52/365) and 3.5% (5/145) of the samples were positive for *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005, respectively. Eight positive samples for *Babesia* sp. BQ1 (Lintan) (2.2%) by LAMP were detected using nested PCR in 365 samples. Furthermore, nested PCR could not detect positive samples from 145 filter papers (Table 2).

4. Discussion

The diagnosis of babesial infections in vertebrate hosts has been mainly conducted by microscopic examination of blood smears because of its ease and rapid application (Bishop and Adams, 1973). However, the main shortcoming of this method is that expertise is required for examining the smears because the parasites are very similar in morphology. It is also difficult to differentiate these parasites when co-infections occur. The development of serological tests for babesiosis has resolved some of problems for distinguishing *Babesia* species, especially when a recombinant protein has been used as the diagnostic antigen, and this improves the specificity of these serological tests (Boonchit et al., 2006; Bose et al., 1990; Huang et al., 2006; Kappmeyer et al., 1999). Unfortunately the cross-reaction between closely related *Babesia* species occurs (Allred, 2003; Tenter and Friedhoff, 1986). However, a clear differentiation of these parasites is crucial for an understanding their epidemiology. The application of DNA amplification in the diagnosis of babesiosis, especially in epidemiological investigations can resolve these problems. It is more sensitive and specific than microscopic examination and serological methods (Aktas et al., 2005; d'Oliveira et al., 1995). Amplification of parasite DNA using a specific PCR has been applied to various *Babesia* species (Alhassan et al., 2005; Birkenheuer et al., 2003; Gubbels et al., 1999; Krause et al., 1996; Liu et al., 2007b; Song et al., 2004). In addition, the reverse line blot (RLB) is a useful method for detecting co-infection with piroplasms in the vertebrate and invertebrate hosts and it can differentiate the different geographical strains (Garcia-Sanmartin et al., 2006; Gubbels et al., 1999; Schnittger et al., 2004). However, the need for complex procedures and sophisticated facilities such as a thermal cycler restricts their development as routine diagnostic methods. Fortunately, the loop-mediated isothermal amplification (LAMP) developed by Notomi et al. (2000) can overcome this problem.

We established a LAMP method for detecting *Babesia* spp. infective to sheep and goats in China, using two primer sets that target in the 18S rRNA gene of these parasites. The specificity of the primers was evaluated by *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 DNA, *Theileria* sp. China 1, *Theileria* sp. China 2, *B. bovis*, *Theileria* sp. (Japan), sheep DNA and distilled water. The results showed that the primer sets of BLTLAMP and BXJLAMP only amplified the autologous DNA samples of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 in typical ladder bands. In contrast, no ladder bands were obtained from any other control. This indicates that the primer sets are specific for *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 and can be used to examine for ovine babesiosis as well as distinguish between them. The sensitivity of the test was also evaluated and showed that LAMP methods were 10- to 100-fold more sensitive than nested PCR. The comparisons of LAMP and microscopic examination were made with samples for *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 from sheep. For *Babesia* sp. BQ1 (Lintan), from 3rd to 63rd day post-infection, the parasites could be detected by the LAMP but the parasites could only be found on blood films between the 8th and 35th day post-infection. For *Babesia* sp. Xinjiang-2005 group, the parasites could be detected using the LAMP between the 14th and 21st day post-infection. However, we could not detect parasites by microscopic examination of blood smears from sheep infected with *Babesia* sp. Xinjiang-2005 for 12 weeks post-infection. The LAMP method was also evaluated in 365 field samples collected from Lintan, Biandu, Xiahe and Zhuoni counties, Gansu province. Eight (2.2%) of these samples were identified as infected with *Babesia* sp. BQ1 (Lintan) using nested PCR, whereas there were 52 (14.3%) positive samples when examined with the LAMP method. For *Babesia* sp. Xinjiang-2005, there were 5 (3.5%) positive in 145 blood samples on filter paper collected from four counties, Kashi, Bayituohai, Yingtamu, Quluhai, in Xinjiang province using the LAMP method, but no positive samples were detected by nested PCR. This demonstrates that the LAMP method has higher sensitivity than microscopic examination, or even nested PCR.

We used a novel DNA amplification method, LAMP, to identify *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 in sheep blood. Compared to PCR, it does not require complicated thermal cycling steps and has the advantage of reaction simplicity and detection sensitivity. An isothermal reaction of 1 h is enough to amplify 10^9 of the target DNA, which can be easily evaluated by visual inspection of the turbidity or fluorescence of the reaction mixture (Notomi et al., 2000; Mori et al., 2001). Because PCR or other molecular biological techniques are best conducted only in well-equipped laboratories, these methods are often impracticable for field diagnosis. Our results indicate that the two *Babesia* spp. are prevalent in China but thus far epidemiological surveillance of ovine babesiosis has never been undertaken in China. Our studies

provide a tool for monitoring prevalence of the disease in China. Finally, however, care must be taken to avoid contamination during sample preparation and appropriate controls must be used to avoid cross-contamination when the LAMP method is used.

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Article N° 6 : Merozoite proteins from *Babesia sp.* BQ1 (Lintan) as potential antigens for serodiagnosis by ELISA. (Protéines de mérozoïtes de *Babesia sp*. BQ1 (Lintan) utilisables comme antigènes potentiels de diagnostic sérologique par ELISA)

Article accepté dans Parasitology

Ce travail a été réalisé au Laboratoire de parasitologie vétérinaire de la province du Gansu, à Lanzhou et au Laboratoire de l'équipe ITPH à Nantes.

Dans cette étude, une technique ELISA, utilisant un extrait soluble de mérozoïtes de *Babesia sp.* BQ1 (Lintan) (BQMA) a été mise au point. Les mérozoïtes d'une lignée monoclonale de *Babesia sp*. BQ1 (Lintan) cultivée *in vitro* dans des hématies de moutons ont été isolés puis lysés par sonication afin de récupérer les protéines solubles. Après détermination des concentrations optimales des différents réactifs (antigènes, sérum, conjugué), les caractéristiques de la technique ELISA développée ont été déterminées. L'analyse de 198 sérums de moutons non infectés par *Babesia sp*. a permis de fixer le seuil de positivité à 30 %, ce qui correspond à une spécificité de 95.5 % ± 1 %. Chez les animaux infectés expérimentalement, les anticorps détectés par ELISA apparaissent dès la 1ère semaine après infestation et des niveaux élevés d'anticorps se maintiennent pendant au moins 8 mois. Chez les animaux infestés naturellement, le niveau d'anticorps évolue en plateau pendant au moins 1 an. Aucune réaction croisée n'a été détectée entre BQMA et les sérums de moutons infectés par différents parasites des hématies de moutons (*Theileria lunwenshuni*, *T. uilenbergi*, *Anaplasma ovis*) ou par différentes souches de *Babesia* décrites en Chine (*Babesia sp*. Hebei, *Babesia sp*. Xinjiang). En revanche, une forte réaction croisée a été démontrée avec le sérum d'animaux infectés par *Babesia sp.* Tianzhu, ce qui peut être expliqué par la proximité phylogénétique entre ces 2 parasites. Afin d'augmenter la spécificité de l'ELISA, 4 antigènes de mérozoïtes ont été identifiés par spectrométrie de masse. Ces antigènes sont spécifiques de *Babesia* sp. BQ1 (Lintan) et *Babesia* sp. Tianzhu, ils sont reconnus précocément, de façon intense et par tous les animaux infectés par *Babesia* sp. BQ1 (Lintan). Enfin, l'ELISA développée avec BQMA a permis d'estimer la séroprévalence de l'infection à *Babesia* sp. BQ1 (Lintan) dans certaines régions de Chine (de 47 à 82 %). En conclusion, l'ELISA mise au point est un bon outil pour détecter les infections précoces et chroniques par *Babesia* sp. BQ1 (Lintan).

Merozoite proteins from *Babesia* sp. BQ1 (Lintan) as potential antigens for serodiagnosis by ELISA

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Running title: ELISA to detect *Babesia sp*. BQ1 (Lintan) infection

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SUMMARY

Babesia sp. BQ1 (Lintan) is a *Babesia* isolated from sheep infested with *Haemaphysalis qinghaiensis* in China, and is closely related to *B. motasi* based on the 18S rRNA gene sequence. In the present study, an ELISA was developed with merozoite antigens of *Babesia* sp. BQ1 (Lintan) (BQMA) purified from in vitro culture. When the positive threshold was chosen as 30% of the antibodies rate, evaluated with 198 negative sera, the specificity was 95.5%. Except for *Babesia* sp. Tianzhu, there was no cross-reaction between BQMA and positive sera from *Babesia* sp. BQ1 (Ningxian)-, *Babesia* sp. Hebei-, *Babesia* sp. Xinjiang-, *Theileria luwenshuni*-, *T. uilenbergi*-, or *Anaplasma ovis*-infected sheep, which are the dominant haemoparasites of small ruminants in China. Specific antibodies against *Babesia* sp. BQ1 (Lintan) were produced 1 or 2 weeks post infection and a high level of antibodies persisted for more than 8 months in experimentally-infected sheep. This ELISA was tested on 974 sera collected from field-grazing sheep in 3 counties of Gansu province, northwestern China to evaluate the seroprevalence of *Babesia* sp. BQ1 (Lintan) infection and the average positive rate was 66.84%. The feasibility of increasing the specificity of this BQMA-based ELISA, by using some BQMA antigens for serodiagnosis is discussed.

Key words: *Babesia* sp. BQ1 (Lintan), antigen, ELISA, merozoite, cross-reaction, antibody kinetics, prevalence

INTRODUCTION

Babesiosis is a tick-transmitted haemoprotoazoan disease of domestic animals causing fever, anemia, hemoglobinuria and even death. To date, three *Babesia* species infective for small ruminants, *B. ovis*, *B. motasi* and *B. crassa*, have been recognized as valid species. *B. ovis* and *B. motasi* are two old-line parasites and the vector ticks are *Rhipicephalus bursa* and *Haemaphysalis punctata*, respectively (Alani and Herbert, 1980; Uilengberg *et al*. 1980; Levine, 1985; Friedhoff, 1988, 1997). Nevertheless, Uilenberg (2006) recently considered that *B. motasi* may well consist of at least two species or subspecies, namely a low pathogenicity group and a high pathogenicity group. *B. crassa* is a large *Babesia* species isolated from Iran. It is generally recognized as a valid *Babesia* species infective for small ruminants, based on morphology, pathogenicity, serology and phylogeny data (Hashemi-Fesharki and Uilenberg, 1981; Schnittger *et al*. 2003) although its vector tick is still unknown.

In China, Chen (1982) and Zhao *et al*. (1986) published the first reports of ovine babesiosis in Sichuan and Heilongjiang province, respectively, and identified the pathogens as *B. ovis*. After that, several strains were isolated in China. These strains can be divided into two groups based on the 18S rRNA gene sequences. One group includes *Babesia* sp. Liaoning, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Madang and *Babesia* sp. Hebei, and the other just contains *Babesia* sp. Xinjiang (Liu *et al*. 2007). Recent analysis based on the ribosomal DNA internal transcribed spacer (ITS) sequences suggested that, except for *Babesia* sp. Xinjiang, all these strains should be considered as *B. motasi* (= "*B. motasi* group in China") (Niu *et al*. 2009).

To date, the main detection method for *Babesia* sp. BQ1 (Lintan) is still the microscopic examination of blood smears, because of its ease and rapid application. However, in subclinical or latent infections, parasites may not be observed because of the low parasitemia. Guan *et al*. (2008) developed a loop-mediated isothermal amplification (LAMP) method for the diagnosis of *Babesia* sp. BQ1 (Lintan) infection. This simple and rapid technique can detect parasites only up to 63 days post-infection (dpi). An enzyme-linked immunosorbent assay (ELISA) has proved useful for the detection of sub-clinical cases and large-scale field surveys (Birkenheuer *et al*. 2003) because it is sensitive, can be automated for high throughput and is easily standardized. Our aim in this study was to develop an indirect ELISA specific for infection of *Babesia* sp. BQ1 (Lintan), using merozoite antigen (BQMA) derived from in vitro culture, which would be suitable for sero-epidemiological survey of the disease in China. This ELISA was used to assess both the humoral immune response in experimentally-infected sheep and the prevalent situation of *Babesia* sp. BQ1 (Lintan) on a small-scale. The serological relationships between the Chinese *Babesia* strains were also explored. Finally, the main proteins from BQMA, potentially suitable for use in serodiagnosis, were also identified by Western blot and mass spectrometry.

MATERIALS AND METHODS

Parasites

A monoclonal line (G7) of *Babesia* sp. BQ1 (Lintan) derived from in vitro culture by limiting dilution, as previously described (Malandrin *et al*. 2004) was maintained and cryopreserved in liquid nitrogen at the Veterinary school of Nantes (Ecole Nationale Vétérinaire de Nantes (ENVN), France) and at the Lanzhou Veterinary Research Institute (LVRI), China (Unpublished data). *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Hebei, *Babesia* sp. Xinjiang and the original strain of *Babesia* sp. BQ1 (Lintan) from infected sheep were cryopreserved in liquid nitrogen and provided by LVRI (Guan *et al*. 2001, 2002, 2009; Bai *et al*. 2002; Liu *et al*. 2007).

Sheep and immune sera

Six 1-1.5 year-old spleen-intact Chinese sheep (Tan mutton) purchased from a *Babesia*-free region in Jingtai county, Gansu province of China and three 1.5 year-old French spleen-intact sheep ("Vendéen" breed), born and kept in a sheep-fold without contact with ticks, were used in the *Babesia* infection experiment.

Spleen-intact sheep No. 3201 was infected with cryopreserved stabilate of *Babesia* sp. Xinjiang and parasites in blood stream were monitored with loop-mediated isothermal amplification (LAMP) (Guan *et al*. 2008). Spleen-intact sheep No. 08040, 08026 and 08020 were infected subcutaneously with 15 ml cryopreserved stabilate (10% parasitemia) of *Babesia* sp. Hebei, *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Tianzhu, respectively and presence of the parasites were detected with microscopic examination of Giemsa-stained smears made with ear venous blood. Parasites were found in all infected sheep during the first 15 days after inoculation. Sera collected from these infected sheep on 30th day p.i. were used as immune positive sera of *Babesia* sp. Hebei, *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Tianzhu in this study. Two Tan mutton sheep (No. 3216 & 2007) (Guan et al. 2008) were infected with the original strain. Three French sheep No. 3390, No. 3533 and No. 3446 were inoculated with 10^7, 10^8 and 10^9 red blood cells (iRBC) infected with the monoclonal line (G7) of *Babesia* sp. BQ1 (Lintan) by in vitro culture. Sera collected from these 5 sheep during 8 months p.i. were used to evaluate antibody kinetics. Sera collected from the 2 Chinese sheep on 30^{th} day were considered as positive control sera. All animals were kept in a sheep-fold with tick-free food and water available *ad libitum* in ENVN and LVRI. Sera positive for *Theileria luwenshuni*, *T. uilenbergi* and *Anaplasma ovis* were provided by the Vector and Vector-borne Disease (VVBD) laboratory of LVIR (Liu *et al*. 2008; Li *et al*. 2009).

Twelve 1.5-2 year-old sheep that were positive for *Babesia* sp. BQ1 (Lintan) by identification with LAMP (Guan *et al*. 2008), grazed on pasture in Lintan county, were bled every month for one year and the sera were used to evaluate annual kinetics of anti-BQMA antibodies under natural infection.

Sera collected from 198 sheep, 4-6 month-old, purchased from a *Babesia*-free region in

Jingtai county, Gansu province of China from 2005 to 2007 before experiment were involved to evaluate specificity of the ELISA and the mixture of these sera was used as the negative control sera.

Sera were randomly collected from 974 field-grazing sheep in Lintan, Zhuoni and Xiahe county of Gannan Tibet Autonomous Region, Gansu province where *Haemaphysalis* spp. (the vector tick of *Babesia* sp. BQ1 (Lintan) (Guan *et al*. 2002; Sun *et al*. 2008 and unpublished data)) are distributed, during May to June 2004.

Preparation of the soluble antigens from* Babesia *sp. BQ1 (Lintan) merozoites

An in vitro culture of *Babesia* sp. BQ1 (Lintan) in sheep erythrocytes was maintained in RPMI 1640 medium supplemented with 20% FBS in 75 cm^2 flasks. When parasitemia reached 8-10%, the cultures were harvested into 50 ml tubes and centrifuged at 2000 rpm for 10 min. The pellets of erythrocytes were transferred into flasks and supplemented with fresh erythrocytes up to 1 ml to continue cultivation. The supernatants were collected into 50 ml tubes and the free merozoites were harvested by centrifugation at 4000 rpm for 15 min. The pellets of merozoites were washed 3 times with pH 7.2 PBS. The collected merozoites were re-suspended with pH 7.2 PBS and subjected to 5 freeze-thawing cycles, sonicated (3 x 30 sec with 30 sec interval), centrifuged (13000 rpm, 15 min) and the supernatant, containing soluble protein, was used as antigen (BQMA). The protein concentration was measured with the kit of BC Assay (UP40840A, Interchim).

Western-blot procedure

BQMA were separated using SDS-PAGE. Western blot analysis was performed using the procedure previously described by Chauvin *et al*. (1995a) with some modifications. Briefly, the protein bands were transferred to nitrocellulose (NC) membranes of 0.45 µm pore size (RPN303E, Amersham). The NC sheet was then cut into strips 0.5 cm or 0.25 cm in width and blocked with 10% skimmed milk powder in 0.1 M Tris-buffered saline (pH 7.6) with 0.1% Tween (TBST), overnight at +4 ^{o}C. The NC strips were incubated for 1 hour with each test serum diluted at 1/100 in TBST. Following 4 washes with TBST, the strips were incubated with monoclonal anti-goat/sheep IgG-Alkaline phosphatase conjugate, antibody produced in mouse (A8062-5 ml, Sigma) diluted at 1/2000 in TBST for 1 hour. After 5 washes with TBST, positive signals were revealed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (B1911, Sigma). The approximate molecular weights were calculated by comparing their migrations with the standard molecular weight markers (TIANGEN, Beijing, China or ProSieve Color protein markers, Lonza, Levallois-Perret, France) using polynomial regression.

ELISA procedure

An ELISA procedure previously described by Chauvin *et al*. (1995b) was employed in this study with some modifications. The optimum concentration of BQMA, serum and conjugate were determined using different concentrations for each reagent. The dilutions with

the highest difference between positive and negative sera were then selected for use in the assay. Briefly, microplates (Nunc) were coated with 5 µg/ml of BQMA in 0.1M pH 9.6 carbonate buffer at 37 °C for 1 hour and then at 4 °C overnight. After washing 3 times with PBS containing 0.1% Tween 20 (PBST), the plates were blocked with 150 µl 2% gelatin in carbonate buffer at 37 °C for 30 min. After drying the plate, the samples, blank (PBST), standard positive and negative control (dilution of 1:200) were distributed in duplicate and the plates incubated at 37°C for 1 hour. After washing as above, a peroxidase conjugate of monoclonal anti-goat/sheep IgG clone GT-34 (A-9452, Sigma) diluted at 1:1000 was added to each well and the plates were again incubated at 37°C for 1 hour. After washing, 50 µl TMB (UP664781, Interchim, France) were added to each well and incubated at room temperature for 15 min. The reaction was stopped by adding 50 µl of 0.1M H_2SO_4 and the plates were read at 450 nm with an ELISA automat (MRX microplate reader, USA). The results are expressed as percentage of the specific antibody mean rate (AbR%) determined with the following formula:

$$\text{AbR\%} = \frac{\text{(Sample mean OD- Negative control mean OD)}}{\text{(Positive control mean OD- Negative control mean OD)}} \times 100$$

Mass spectrometric analysis

Mass spectrometry analyses were conducted by the platform "Biopolymers-Interaction-Structural Biology" located at the INRA Center of Angers-Nantes (http://www.angers-nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs).

The protein bands to be identified were manually excised from Coomassie stained SDS-PAGE-gel and in-gel trypsin digestions were applied to each band.

LC-MS/MS analysis

Nanoscale capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses of the digested proteins were performed using a Switchos-Ultimate II capillary LC system (LC Packings/Dionex, Amsterdam, the Netherlands) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Micromass/Waters, Manchester, UK). Chromatographic separation was conducted on a reverse-phase capillary column (Pepmap C18, 75 µm i.d., 15 cm length, LC Packings) at a flow rate of 200 nL/min. The gradient consisted of a linear increase from 2% to 40% of acetonitrile in 50 min, followed by a rapid increase to 50% of acetonitrile within 10 min. Mass data acquisitions were piloted by the Mass Lynx software (Micromass/Waters) using the so-called "data dependent acquisition" mode: MS data were recorded for 1 sec on the mass-to-charge (*m/z*) range 400-1500, after which the three most intense ions (doubly, triply or quadruply charged ions) were selected and fragmented in the collision cell (MS/MS measurements).

Protein identification – Databank searching.

Raw data were processed using the Protein Lynx Global Server software (version 2.1, Micromass/Waters). Proteins were identified by comparing the collected LC-MS/MS data with the Uniprot sequence databank (http://www.uniprot.org/) (version 14.7; 7409116 sequences). Databank searches were performed using the Mascot server 2.2 program (Matrix Science). The mass tolerance was set at 250 ppm for parent ions (MS mode) and 0.3 Da for fragment ions (MS/MS mode); one missed cut per peptide was allowed, and the oxidation of Methionines was set as a variable modification.

Validation of protein identifications

For MS/MS experiments, protein identification was based on a minimum of two MS/MS spectra matching the databank sequence, with individual MASCOT ion scores above the significance threshold ($p<0.05$), i.e. individual peptide scores above 52 in the present case. Results were validated and exported in an analytical report using the OVNIp software developed by INRA Nantes (http://wwwappli.nantes.inra.fr:8180/OVNIp/). Protein identifications generated by single MS/MS spectra were validated only if the expected value was below 0.005 and/or when the MS/MS spectra displayed a wide series of intense fragments that could be assigned, in turn, to major predicted fragments (i. E. b or y ions) of the proposed peptide sequence. These single hits were carefully inspected to validate protein identification.

RESULTS

Evaluation of positive threshold value and specificity of ELISA

The positive threshold value and specificity were determined with 198 negative sera collected from 4-6 month-old lambs purchased from a *Babesia*-free region in Jingtai county, Gansu province of China, according to Chauvin's method as described previously (1995b). The AbRs of each serum were calculated and divided into 14 classes based on the number of AbRs, i.e., first class: less than -10%; second: from -10% to -5%; third: from -5% to 0%, and so on, the last class comprising more than 50%. For calculated positive threshold values of 20%, 25%, 30%, 35%, and 40% respectively, the numbers of false positive sera were 15, 11, 9, 6, and 3, and the corresponding specificities were 92.4 ± 0.8%, 94 .4 ± 0.9%, 95.5 ± 1.0%, 97.0 ± 1.1%, and 98.5 ± 1.3% (Figure 1). We chose 30% as the positive threshold value of the ELISA in this experiment.

Cross-reactivity with other haemoparasites

Cross-reactivities between BQMA and positive sera against *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Hebei, *Babesia* sp. Xinjiang, *Theileria lunwenshuni*, *T. uilenbergi*, and *Anaplasma ovis* were evaluated with ELISA and western blot techniques. The tests were repeated twice and the mean OD and standard diversity were calculated with Excel 2000. No cross-reactions were observed between BQMA and anti-*Babesia* sp. BQ1

(Ningxian), -*Babesia* sp. Hebei, -*Babesia* sp. Xinjiang, -*T. lunwenshuni*, -*T. uilenbergi*, and –*A. ovis* antibodies in the sera by ELISA. In contrast, anti-*Babesia* sp. Tianzhu antibodies cross-react with BQMA (Figure 2).

Western blot analysis confirmed a strong reaction with positive sera from *Babesia* sp. BQ1 (Lintan) or *Babesia* sp. Tianzhu infected sheep. Antibodies specifically recognized at least nine common proteins, with molecular weights of 171, 140, 92, 74, 48, 45, 35, 33 and 32 kDa. Anti-*Babesia* sp. BQ1 (Ningxian) antibodies could recognize 3 proteins (140, 48 and 33 kDa) that co-migrated with those of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu. No specifically recognized antigens, compared with that of negative sera, were obtained for the reactions with positive sera of *Babesia* sp. Hebei, *Babesia* sp. Xinjiang, *T. lunwenshuni*, *T. uilenbergi*, and *A. ovis*, (Figure 3).

Kinetics of anti-*Babesia *sp. BQ1 (Lintan) antibodies in experimentally- and naturally-infected sheep

Two Tan mutton sheep (Chinese sheep) (No. 3216 and 2007) and three Vendean sheep (French sheep) (No. 3390, 3533 and 3446) were experimentally inoculated with *Babesia* sp. BQ1 (Lintan) infected erythrocytes, and the sera collected during infection were assessed by ELISA (Figure 4). Animals experimentally infected with *Babesia* sp. BQ1 (Lintan) had seroconverted by 1 week post-inoculation and the highest antibodies level was attained after 2 or 3 weeks. The high level of antibodies usually lasted 8 months (240 dpi) although decreased after 3 months (84 dpi) in sheep 3446 (The OD values on point of 0, 84 and 240 dpi was 0.19, 2.96 and 2.13, respectively.).

Twelve 1.5-2 year-old sheep grazed on pasture in Lintan county were bled every month for one year to evaluate the kinetics of anti-BQMA antibodies production during natural infection with *Babesia* sp. BQ1 (Lintan) by the ELISA (Figure 5). No fluctuation of antibodies titer was apparent in naturally-infected sheep.

Estimation of serologic prevalence of* Babesia *sp. BQ1 (Lintan) in different counties of Gansu province, China with ELISA

The ELISA was applied to 974 sera collected from field-grazing sheep in Lintan, Zhuoni and Xiahe county of Gannan Tibet Autonomous Region, Gansu province, northwestern China during May to June 2004, to evaluate the infection situation in these areas. When the positive threshold value was taken as 30%, the results showed that the positive rates for Lintan, Zhuoni and Xiahe County were 82.38%, 66.04% and 46.67%, respectively, and the average positive rate in these regions was 66.84% (Table 1).

Identification of potential antigens for serological diagnosis

Western blot was performed with sera from 3 French sheep infected with *Babesia* sp. BQ1 (Lintan) to study the kinetics of the recognition of antigens during infection. Three antigens, out of approximately 20 molecules were selected because they were recognized

early during infection (between 6 and 9 dpi), with high intensity, by all *Babesia* sp. BQ1 (Lintan)-infected sheep. All of them seemed to cross-react only with sera from *Babesia* sp. Tianzhu-infected sheep (figure 3). The 3 corresponding proteins were identified by comparing the collected LC-MS/MS data with the Uniprot sequence databank. Table 2 indicates only the proteins identified as proteins of *Babesia* spp.. Host proteins were also identified but they were probably contaminants because they were not recognised by antibodies from sheep sera taking before infection.

DISCUSSION

Sero-diagnostic techniques of babesiosis are essential tools for epidemiological studies and for the evaluation of preventive measures, e.g. vaccination, premunition and tick control. The enzyme-linked immunosorbent assay (ELISA) provides a quantitative result, is more sensitive and non-subjective, can be automated for high throughput and is easily standardized, compared with the complement fixation test (CFT) and indirect fluorescent antibody test (IFAT) (Tenter and Friedhoff, 1986; Brüning, 1996). Thus, a number of ELISAs have been designed recently for the diagnosis of *Babesia* spp. infection (Molloy *et al*. 1998; Ikadai *et al*. 2000; Goff *et al*. 2003). Few studies, however, have focused on the development of simple, rapid and stable routine serological techniques to detect ovine babesiosis. Current detection in both domestic and wild animals is mainly based on determination of the sero-prevalence by IFAT, and a cross-reaction between *B. motasi* and *B. crassa* has been demonstrated (Lewis *et al*. 1981; Habela *et al*. 1990; Kjemtrup *et al*. 1995; Papadopoulos *et al*. 1996; Ferrer *et al*. 1998). Duzgun *et al*. (1991) developed an ELISA for *B. ovis* using a recombinant *B. bovis*-derived antigen with cross-reactivity between *B. ovis* and *B. bovis*. In the present study, an ELISA was developed to detect infection of *Babesia* sp. BQ1 (Lintan) using soluble antigens of merozoite (BQMA) derived from in vitro culture. The calculated specificity of this ELISA was 95.5±0.97% with a positive threshold value of 30%.

The ELISA described in this manuscript was then used to investigate the seroconversion and persistence of specific antibodies during experimental and natural infection of sheep with *Babesia* sp. BQ1 (Lintan). Comparison of the kinetics of antibody levels in French sheep and Chinese sheep experimentally infected by *Babesia* sp. BQ1 (Lintan) revealed that the humoral immune response of different sheep breeds to this parasite was identical. The production of specific antibodies against *Babesia* sp. BQ1 (Lintan) was similar to those of other ovine *Babesia*, *Babesia*, *B. motasi*, *B. crassa* and *B. ovis*, first produced 5-7 dpi then peaked between 14 and 28 dpi and persisted for at least 330 dpi (12-13 months) (Uilenberg *et al*. 1980; Christensson and Thunegard, 1981; Hashemi-Fesharki and Uilenberg, 1981; Habela *et al*. 1990).

The antibody levels in experimentally-infected sheep (No. 3446) decreased during

infection whereas those of 12 naturally-infected grazing sheep showed no significant fluctuations over one year in prevalent areas. This could be explained by the presence of two activity peaks per year in *H. qinghaiensis*, the vector of *Babesia sp*. BQ1 (Lintan), one in April-July and the other in September (Teng, 1991). In any one year, grazing animals may therefore be infected several times by *Babesia* sp. BQ1 (Lintan), which each time may stimulate the immune response and thus explain the maintenance of a high level of specific antibodies. The decrease in the level of antibodies in experimentally-infected sheep could be explained by the absence of periodic antigen stimulation and by the decrease of parasitemia, as observed in *Babesia canis*-infected dogs (Brandao *et al*. 2003).

Guan *et al*. (2008) recently showed that the positive rate determined by LAMP for *Babesia* sp. BQ1 (Lintan) was 14.3% (52/365) in Gannan Tibet Autonomous Region, the most important stock-raising region of Gansu Province, People's Republic of China. The sero-epidemiological analysis established with ELISA indicates that infection with *Babesia* sp. BQ1 (Lintan) poses a very serious problem, the positive rate attaining 66.84% in Gannan Tibet Autonomous Region. The difference in prevalence measurements obtained with the 2 methods is due to the different parameters detected by these methods. The LAMP technique, developed by Guan *et al*. (2008), is able to detect the parasite for 2 months after infection in experimentally-infected sheep. In contrast, high levels of antibodies persist for at least 8 months and can be detected by BQMA-based ELISA (this study). Therefore, LAMP should be used to identify sheep recently infected with *Babesia* sp. BQ1 (Lintan) whereas ELISA can detect both early and latent infection.

Merozoite antigens from *Babesia* sp. BQ1 (Lintan) do not show cross-reactivity with sera from sheep infected with *Babesia* sp. Hebei, *Babesia* sp. Xinjiang, *T. luwenshuni*, *T. uilenbergi* or *A. ovis,* which are the dominant haemoparasites of small ruminants in China. Otherwise, the positive serum of *Babesia* sp. Tianzhu gives a strong cross-reaction with BQMA in both ELISA and Western blot and a weak cross-reaction between *Babesia sp*. BQ1 (Ningxian) and *Babesia sp*. BQ1 (Lintan) was detected only by Western blot. Based on internal transcribed spacer (ITS) gene sequences, Niu *et al*. (2009) demonstrated that these parasites should be included in two *Babesia* species, *B. motasi* and *Babesia* sp. Xinjiang. In *B. motasi*, the molecular phylogeny (Niu *et al*. 2009) and serological evidence (this study) suggest that the "*B. motasi* in China" group contains two species or subspecies, one including *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu, explaining the strong cross-reactivity between these 2 strains, and the other with *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei, explaining the absence or the weak cross reactivity between these 2 strains and *Babesia sp*. BQ1 (Lintan).

We have preliminarily studied 3 potential serodiagnosis antigens in order to increase the specificity of the BQMA-based ELISA and limit cross reaction with other *Babesia* sp. in China. Some of them, such as heat shock protein 70 or enolase (antigen 2 and 3 respectively,

table 2), have been used to detect other Apicomplexa such as *B. orientalis* (He *et al*. 2009), *Theileria* sp. (China) (Miranda *et al*. 2006), *Plasmodium vivax* (Na *et al*. 2007), and *Neospora caninum* (Shin *et al*. 2004). This preliminary study now needs to be confirmed by testing the corresponding recombinant proteins and developing an ELISA able to detect only *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu-infected sheep and thereby distinguish the 2 species or subspecies of the group of "*B. motasi* in China".

In conclusion, an ELISA has been developed for the diagnosis and epidemiological study of *Babesia* sp. BQ1 (Lintan). This ELISA is a rapid, cheap and robust technique for detecting sheep infected with *Babesia* sp. BQ1 (Lintan) early after infection and during the persistent phase of the parasite. This ELISA provides an important tool for further investigating the prevalence of this parasite in pasture-grazing small ruminants in Inner Mongolia, Xinjiang, Qinhai, Gansu and Sichuan provinces of China. Further studies on antigens are needed to distinguish *Babesia* sp. BQ1 (Lintan) by ELISA from some other *Babesia* spp. in China.

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LEGENDS OF TABLE

Table 1. Examination of anti-*Babesia* sp. BQ1 (Lintan) antibodies from field samples with ELISA

Table 2. Identification of proteins by mass spectrometry

LEGENDS OF FIGURES

Figure 1. Distributions of mean antibody rates (AbR) of negative sera for evaluation of the positive threshold value. Animal number: the number of sheep corresponding sera that have AbR value in the AbR class; the number ion top of each column represents the animal number in this AbR class.

Figure 2. Cross-reaction of merozoite antigen of *Babesia* sp. BQ1 (Lintan) with positive sera of *Babesia*, *Theileria* and *Anaplasma* infective to sheep in China, as determined by ELISA (AbR: antibodies rate).

Figure 3. Western blot of merozoite antigens derived from in vitro culture of *Babesia* sp. BQ1 (Lintan). Lanes: M, the molecular weight markers (kDa); 1, a pool of negative sera, 2-9, positive sera of *A. ovis*, *T. uilenbergi*, *T. lunwenshuni*, *Babesia* sp. Xinjiang, *Babesia* sp. Hebei, *Babesia* sp. Tianzhu, *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. BQ1 (Lintan). Standard molecular weight markers are indicated on the left (kDa). Arrows and numbers on the right show the antigens recognized by sera and their molecular weights (kDa).

Figure 4. Kinetics of anti-*Babesia* sp. BQ1 antibody in 3 French sheep experimentally-infected with culture (No. 3390, 3533 and 3446) and 2 Chinese sheep infected with blood (No. 3216 and 2007). The bold curve shows the mean OD value corresponding to each time point and the error bars indicate the standard deviation.

Figure 5. Kinetics of anti-*Babesia* sp. BQ1 (Lintan) antibodies production in 12 naturally-infected Chinese sheep studied by ELISA. The bold curve shows the mean OD value corresponding to each month and the error bars indicate the standard deviation.

Figure 6. Western blot analysis of IgG response to BQMA of a representative sheep infected with *Babesia* sp. BQ1 (Lintan). Standard molecular weight markers are indicated on the left (M, kDa). Arrows and numbers on the right show the 4 antigens which could be potentially used for the serodiagnosis of *Babesia* sp. BQ1 (Lintan) infection.

Figure 1

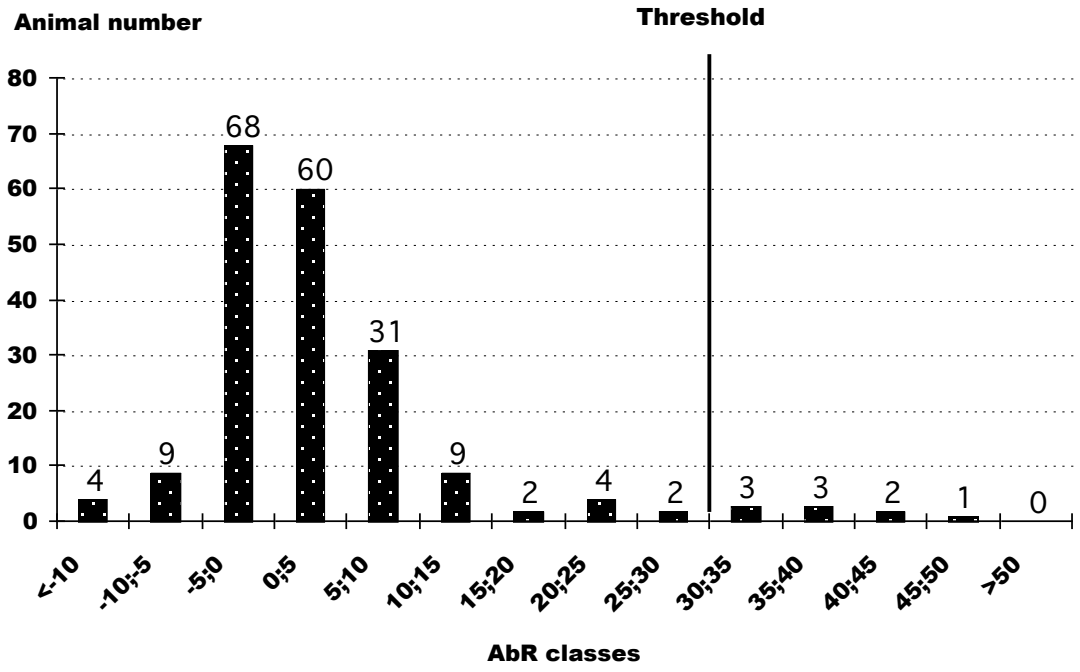
Animal number
Threshold
80
70
60
50
40
30
20
10
0
4
9
68
60
31
9
2
4
2
3
3
2
1
0
<-10
-10;-5
-5;0
0;5
5;10
10;15
15;20
20;25
25;30
30;35
35;40
40;45
45;50
>50
AbR classes

Figure 2

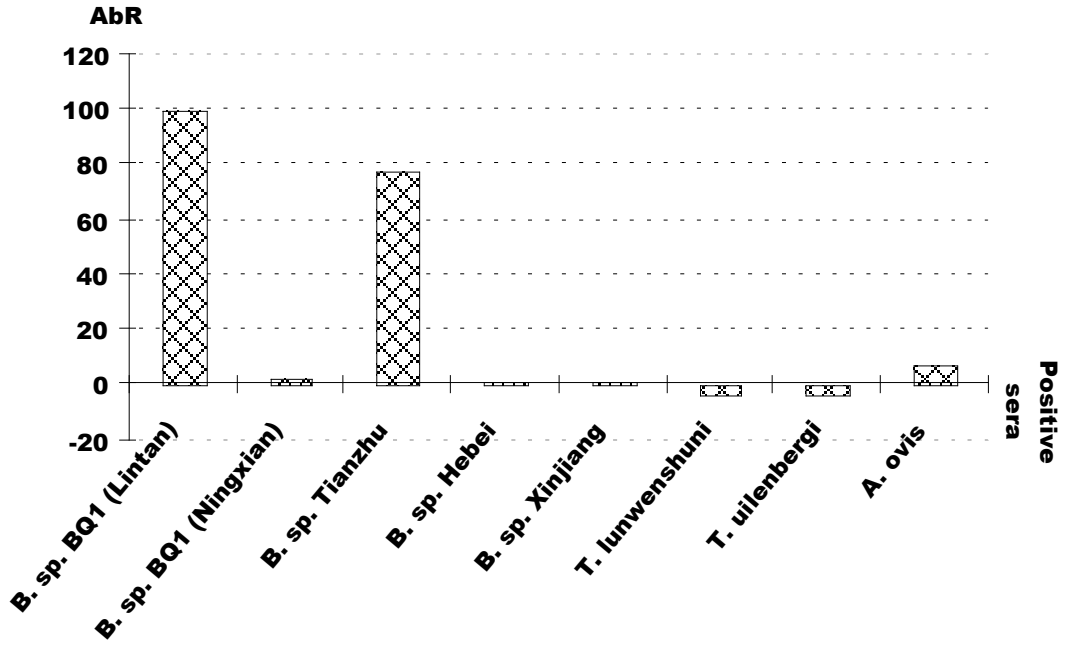
AbR
120
100
80
60
40
20
0
-20
B. sp. BQ1 (Lintan)
B. sp. BQ1 (Ningxian)
B. sp. Tianzhu
B. sp. Hebei
B. sp. Xinjiang
T. lunwenshuni
T. uilenbergi
A. ovis
Positive sera

Figure 3

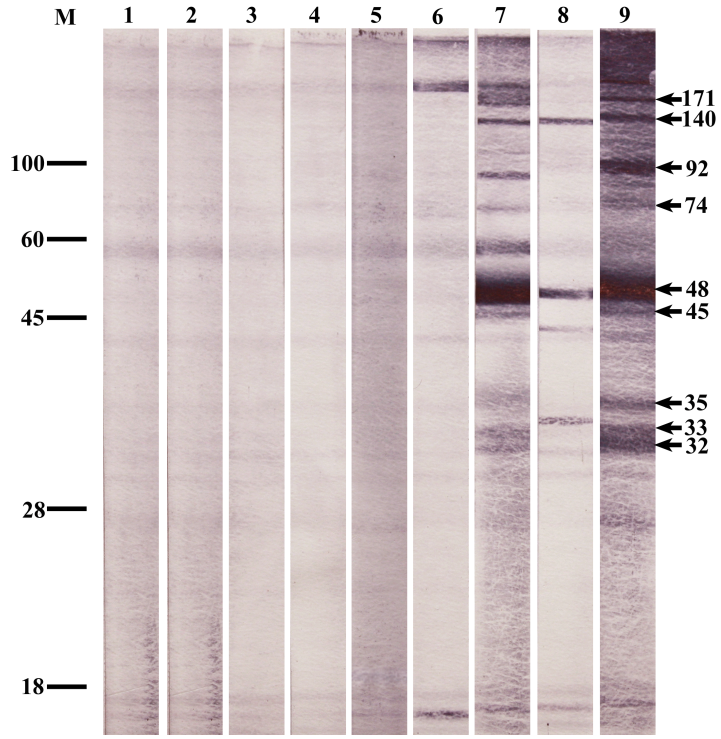

Figure 4

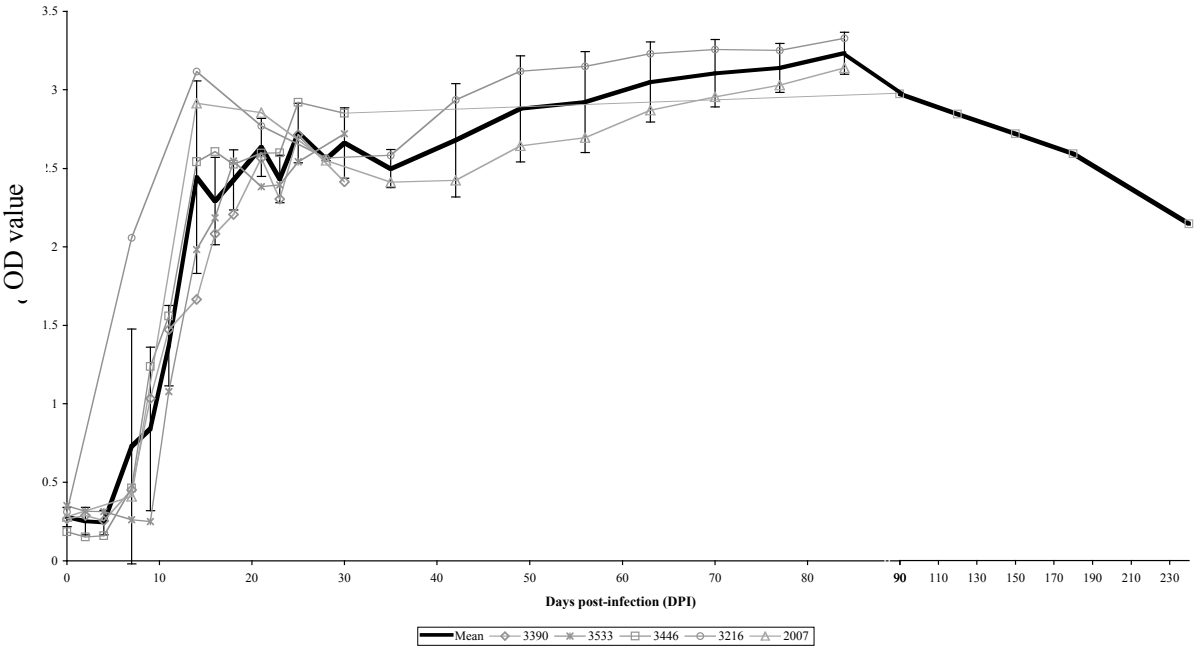
OD value
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
90
110
130
150
170
190
210
230
Days post-infection (DPI)
Mean
3390
3533
3446
3216
2007

Figure 5

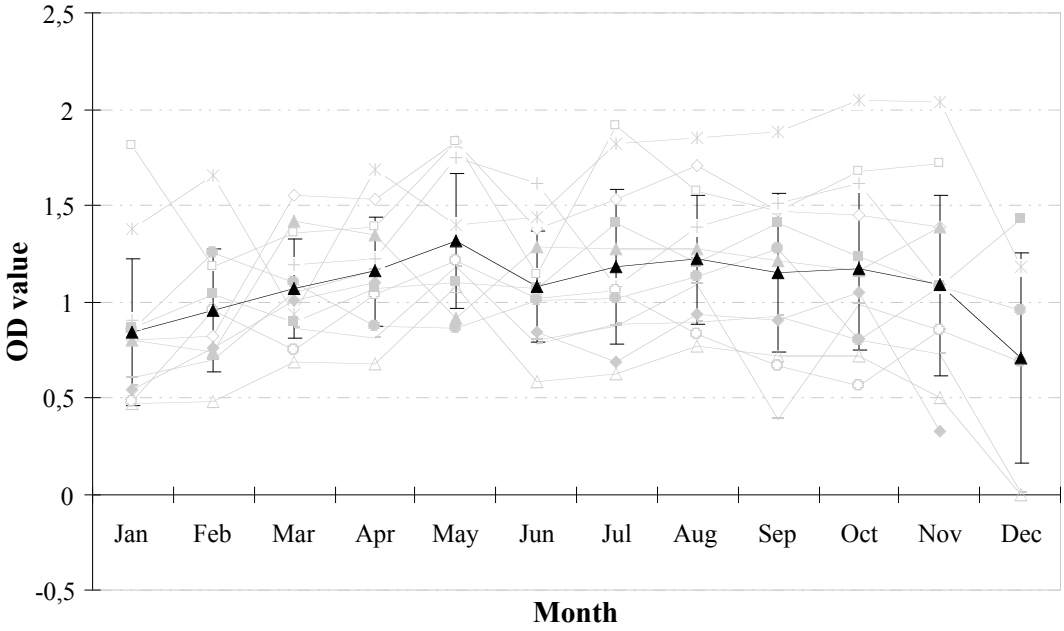
2,5
2
1,5
1
0,5
0
-0,5
OD value
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month

Figure 6

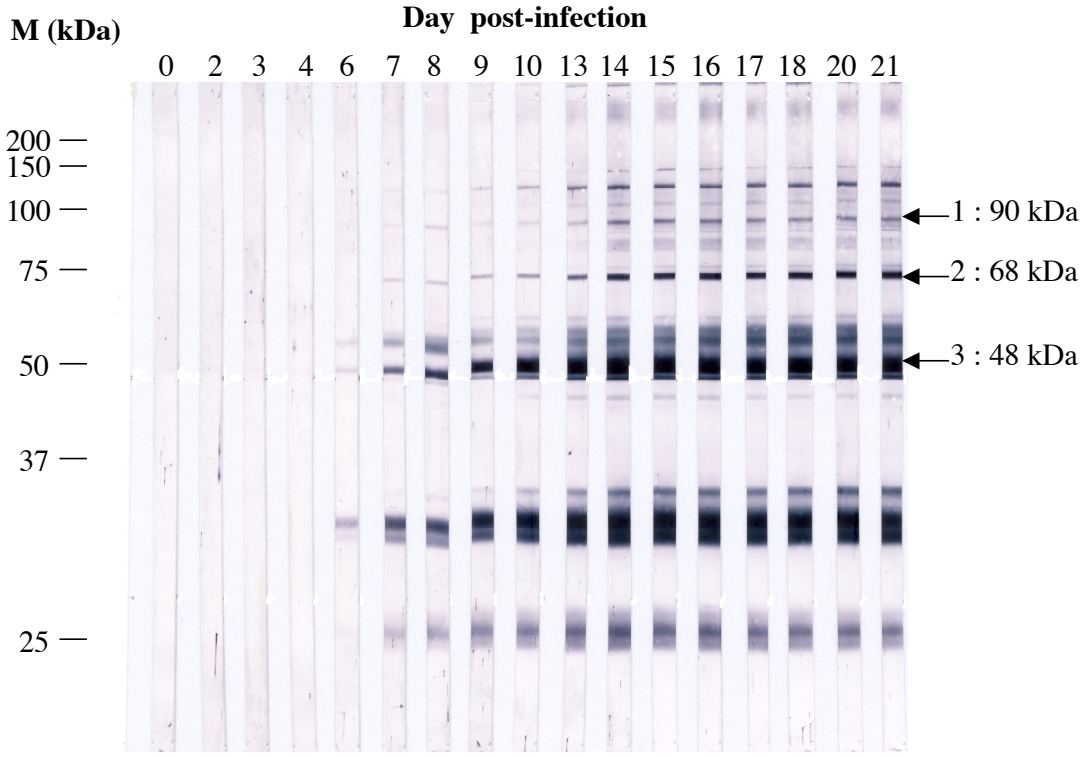
M (kDa)
Day post-infection
0 2 3 4 6 7 8 9 10 13 14 15 16 17 18 20 21
200
150
100
75
50
37
25
1 : 90 kDa
2 : 68 kDa
3 : 48 kDa

Table 1

County	Total number	Positive number	Positive rate
Lintan	261	215	82.38%
Zhuoni	533	352	66.04%
Xiahe	180	84	46.67%
Total	974	651	66.84%

Antigen	Protein entry	Description	Species	MW exp	MW theoret	Nb of peptides (Nb of unique peptides)	PEPTIDE SEQUENCE	Modifier	Peptide scores
1	A7ARM1	Cell division control protein 48, putative	*B. bovis*	90	88.9	3 (3)	1-NAPAIIFIDEVDSIAPK		87
							2-LDQLIYIPLPDLPAR		65
							3-AVASECSANFISIK	Carb (C)	56
	A7ATV4	ClpB, putative	*B. bovis*		105.1	3 (3)	1-ILDSALVEAAQLSDR		100
							2-FQPVYVDQPSVEETISILR		54
							3-NNPILLGDPGVGK		53
	A7ARM5	Heat shock protein 90, putative	*B. bovis*		91.1	1 (1)	1-HELINNLGTIAK		94
	A7AWG3	Elongation factor 2	*B. bovis*		94.0	1 (1)	1-AYLPVAESFGFTTALR		102
	A7AVE1	Cell division cycle protein ATPase, putative	*B. bovis*		103.1	1 (1)	1-VINQILTEIDGVNVK		54
2	Q2V2L6	Heat Shock Protein 70	*B. caballi*	68	72.2	15 (10)	1-SDATINFTNTIFDVKR		131
							2-AIITVPAYFNDAQR		91
							3-IINEPTAAAIAYGIDR		87
							4-VEIITNEMGNR		83
							5-FNDKEVQNDMK	Ox (M)	82
							6-SDATINFTNTIFDVK		82
							7-VEIITNEMGNR	Ox (M)	77
							8-DYAPEEISAMVLK		69
							9-DYAPEEISAMVLKK	Ox (M)	68
							10-VEGPIIGIDLGTTYSCVGIYK	Carb (C)	66
							11-DYAPEEISAMVLK	Ox (M)	66
							12-AIITVPAYFNDAQR		62
							13-NIVITPDKGR		61
							14-EVEALCNPIISK	Carb (C)	57
							15-QALEVYLDSMKR	Ox (M)	53
	Q0WY83	Heat Shock Protein 70	*B. ovis*		70.5	7 (7)	1-IINEPTAAAIAYGLDK		80
							2-DAVITVPAYFNDSQR		80
							3-MKEIAELYLGK	Ox (M)	79
							4-FHLTGIAPAPR		72
							5-IHEVVLVGGSTR		66
							6-TTPSYVAFTDTER		64
							7-ALESSGLDKR		54
	A7AP56	Heat Shock Protein 70	*B. bovis*		71.6	3 (3)	1-AKFESLNEDLFVK		111
							2-FESLNEDLFVK		65
							3-ITPSYVSFTDDGHK		58

3	Q2LEY6	Elongation factor 1-alpha	*B. bovis*	48	49.7	14 (8)	*1-NMITGTSQADVAMLVVPAE AGGFEAAFSK*	Ox (M)	126
							2-THINLVVIGHVDSGK		118
							3-EHALLAFTLGVK		108
							4-VPFVAISGFMGDNMVER	Ox (M)	103
							5-NMITGTSQADVAMLVVPAE AGGFEAAFSK	Ox (M)	102
							6-EHALLAFTLGVK		98
							7-EHALLAFTLGVK		79
							8-VPFVAISGFMGDNMVER	Ox (M)	75
							9-YYYTVIDAPGHR		69
							10-IGGIGTVPVGR		66
							11-STTTGHLIYK		64
							12-THINLVVIGHVDSGK		61
							13-NMITGTSQADVAMLVVPAE AGGFEAAFSK		54
							14-QTVAVGVIK		53
	A7AVP6	EIF-4A-like DEAD family RNA helicase, putative	*B. bovis*		53.0	5 (4)	1-GSYVAIHASGFR		99
							2-VNIVINYDMPDSTDSYLHR	Ox (M)	96
							3-VNIVINYDMPDSTDSYLHR	Ox (M)	78
							4-VACLGISHTR	Carb (C)	71
							5-DFFLKPEILR		57
	A7AP71	Enolase	*B. bovis*		48.4	2 (2)	1-SGETEDTFIADLVVALGTGQIK		84
							2-SAEEALDLLVESIKK		57
	A7ARA6	Dihydrolipoyl dehydrogenase	*B. bovis*		52.0	1	1-NIIIATGSEVTTFPGDALK		70

Note: ox (M): oxidation of methionine ; Carb (C): carbamidomethylation of cystein, Nb: number, MW Exp: experimental molecular weight, MW Theorit: theoretical MW, unique peptide: in terms of amino acid sequence

Article N° 7 : A member of HSP90 family from *Babesia* sp. BQ1 (Lintan) infective for small ruminants in China: Molecular characterization, phylogenetic analysis and antigenicity. (Un membre de la famille des HSP90 chez *Babesia sp.* BQ1 (Lintan) infectant les petits Ruminants en Chine : caractérisation moléculaire, analyse phylogénétique et antigénicité)

Article soumis à Journal of Clinical Microbiology

Ce travail a été réalisé au laboratoire de parasitologie vétérinaire de la province du Gansu, à Lanzhou.

Dans ce travail, une protéine Hsp90 de mérozoïte de *Babesia sp*. BQ1 (Lintan) (BQHsp90) est décrite pour la 1ère fois. Grâce à une banque d'ADNc de *Babesia sp.* BQ1 (Lintan), un clone de 699 paires de bases (pb) contenant une queue polyA de 94pb, ayant une identité de 81% avec l'extrémité 3' du gene HsP90 de *Babesia bovis* (XM_001611504) et du gène Hsp90 (AF136649). La séquence complète de l'ADNc de BQHsp90 a été obtenue par RACE PCR : c'est un ADNc de 2399pb contenant un ORF de 2154pb et. La séquence a été déposée sur GenBank (GQ397856). L'ADN génomique est de 2573pb avec 3 exons et 2 introns de 404pb et 142 pb localisés au niveau des sites 1423/1424 et 1924/1925 respectivement.
La protéine codée par cet ADNc a été analysée par des outils de bioinformatique : elle est de 717 acides aminés avec un pI théorique de 5,0 et un poids moléculaire de 83 kDa. Un domaine conservé de liaison à l'ATP est localisé entre les résidus 29 et 183, une signature de la famille HSP90 en position 27-36, un site TRP binding au niveau de la terminaison –COOH ainsi qu'un « nuclear localization signal ». BQHsp90 semble être une protéine cytoplasmique. L'analyse 3D montre qu'elle contient 2 domaines distincts : un domaine NH2-terminal et un domaine COOH-terminal reliés par une région hautement chargée de 67 acides aminés.

En utilisant le gène et la protéine Hsp90, une analyse phylogénétique de *Babesia sp.* BQ1 (Lintan) et des autres *Babesia* ovines a été réalisée. Elle confirme que les genres *Babesia, Theileria, Cryptosporidium, Eimeria, Toxoplasma* et *Plasmodium* sont classés dans des branches différentes. Au sein du genre *Babesia*, les parasites peuvent être classés en 3 groupes : le groupe « *B. bovis* », le groupe « *Babesia sp*. Xinjiang » et un autre groupe regroupant les 4 souches chinoises de *Babesia* ovines étudiées (groupe « *B. motasi*-like). Au sein de ce 3ème groupe, 2 sous-groupes peuvent être distingués, l'un contenant *Babesia sp*. BQ1 (Lintan) et *Babesia sp.* Tianzhu et l'autre *Babesia sp*. BQ1 (Ningxian) et *Babesia sp*. Hebei. L'analyse de l'antigénicité de la protéine recombinante rBQHsp90 montre qu'elle est bien reconnue au cours d'une infection de moutons à *Babesia sp*. BQ1 (Lintan). Afin d'évaluer l'utilisation potentielle de la BQHsp90 en tant qu'antigène de diagnostic, la spécificité de cette protéine doit être analysée, notamment en utilisant des sérums d'animaux

infectés par d'autres hémoparasites des moutons et par les autres souches de *Babesia* ovines présents en Chine.

A member of the HSP90 family from *Babesia* sp. BQ1 (Lintan) infective for small ruminants in China: Molecular characterization, phylogenetic analysis and antigenicity

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Abstract

Heat shock protein 90 (Hsp90) is a key component of the molecular chaperone complex essential for activating many signalling proteins involved in the development and progression of pathogenic cellular transformation. A Hsp90 gene (BQHsp90) was cloned and characterized from *Babesia* sp. BQ1 (Lintan), a Chinese ovine *Babesia* isolate, by screening a cDNA expression library and performing rapid amplification of cDNA ends (RACE). The full-length cDNA of BQHsp90 is 2399bp with an open reading frame (ORF) of 2154bp encoding a predicted 83 kDa polypeptide with 717 amino acid residues. It shows significant homology and similar structural characteristics to Hsp90 of other apicomplex organisms. Phylogenetic analysis, based on the Hsp90 amino acid sequences, showed that the *Babesia* genus is clearly separate from other apicomplexa genera. Five Chinese ovine *Babesia* isolates were divided into 2 phylogenetic clusters, namely *Babesia* sp. Xinjiang (previously designated a new species) and a '*Babesia motasi*' cluster containing the other 4 isolates. These 4 '*Babesia motasi*' isolates could be further divided into 2 subclusters, one containing *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu, and the other *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei. Finally, the antigenicity of BQHsp90 with prokaryotic expressed rBQHsp90 protein is also discussed.

Key words: *Babesia* sp. BQ1 (Lintan), Hsp90, *Babesia motasi*, Phylogenetic analysis

Introduction

Heat shock proteins (HSPs) are some of the phylogenetically conserved and ubiquitously expressed protein families in bacteria, mammals and plants. These proteins play essential roles in stress tolerance and the folding, activation and assemblage of many proteins. According to their homology, function and size, they can be divided into different families, e.g., HSP110, HSP90, HSP70, HSP60, HSP40, and small HSP (5, 16). The molecular chaperone heat shock protein 90 (Hsp90, "90" being the average molecular mass) is important in the folding and functioning of many proteins involved in cell survival, especially those participating in cell cycle regulation and signal transduction (29). Due to its broad functions, it is highly abundant in both stressed and non-stressed cells, and constitutes 2.8% of the total cellular protein (4). Recently, it has also been implicated in studies of evolution and some new phylogenetic relationships have been revealed (10, 35). Furthermore its contributions to the immune response have led to encouraging studies of its use as an antigen or adjuvant in vaccine, especially for cancer (16, 18, 37).

In protozoa, Hsp90 is involved in the invasion and growth of the parasites in host cells. Inhibition of Hsp90 by geldanamycin decreases the invasion of host cells by *Toxoplasma gondii* (2, 9) and *Eimeria tenella* (30). Hsp90 has also been reported to participate in the morphological differentiation of *T. gondii* and *Leishmania donovani* in order to adjust to changes in ambient temperature, pH and other stress-inducers (9, 40). In vector-borne parasites, Hsp90 plays a fundamental role in circulation between invertebrate and vertebrate hosts which is responsible for parasite stress because of the totally different host body temperatures (1, 40). Since then, Hsp90 has been considered as a target in the design of anti-protozoan drugs (1, 21).

However, little is known about the Hsp90 gene of piroplasms. Gerhardsa et al. (1994) showed that a 87 kDa Hsp90 protein was expressed by *Theileria parva* during both the sporozoite and schizont stages but not in the piroplasm stage, although the corresponding transcript was detected (13). *T. parva* and *T. annulata*-induced IκB kinase (IKK) activity does not require functional Hsp90 in the schizont stage, unlike that of other microbial pathogens (19). Ruef et al. (2000) based their demonstration of the paraphyly of piroplasms on phylogenetic analysis of the *Babesia bovis* Hsp90 gene (32). In our study, a full-length Hsp90 cDNA was cloned and characterized from a Chinese ovine *Babesia* isolate, *Babesia* sp. BQ1 (Lintan), and named BQHsp90. Five Chinese ovine *Babesia* isolates together with *T. annulata*, *T. parva*, *B. bovis* and other apicomplexa parasite species were subjected to phylogenetic analysis based on the Hsp90 amino acid sequences. Finally, the antigenicity of a recombinant BQHsp90 (rBQHsp90) expressed in the prokaryotic system was evaluated by western blot and ELISA.

MATERIALS AND METHODS

Parasites

A monoclonal line (G7) of *Babesia* sp. BQ1 (Lintan) merozoites was grown in vitro in sheep erythrocytes as described by Guan et al. (submitted to Veterinary Parasitology) and infected sheep blood of *Babesia* sp. BQ1 (Lintan) was cryopreserved in liquid nitrogen at the Vector and Vector-borne disease (VVBD) laboratory, LVRI, China. *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesia* sp. Tianzhu and *Babesia* sp. Xinjiang were isolated from infected sheep and cryopreserved in the VVBD laboratory.

Anti-*Babesia* sp. BQ1 (Lintan) immune sera

A spleen-intact sheep was injected subcutaneously with blood (30 ml) from *Babesia* sp. BQ1 (Lintan)-infected sheep, cryopreserved in liquid nitrogen when 10% parasitemia had been reached. Immune sera were collected from the jugular vein one month post-infection. Sera collected before infection were used as negative sera. All sera were incubated with a volume of BL21 (DE3) lysates at 37^{o}C for 1 hour to block any anti-*Escherichia coli* antibodies present in the sheep sera and then centrifuged at 13000 x g for 5 min at 4 ^{o}C (5417R, Eppendorf, German). The supernatants were dispensed into 1.5 ml tubes and frozen at -20 ^{o}C until use.

Purification of *Babesia* sp. BQ1 (Lintan) merozoites from culture

Babesia sp. BQ1 (Lintan) merozoites were purified from the in vitro culture as follows. Briefly, the culture was harvested into 50 ml tubes and centrifuged at 800 _ g for 10 min (DL-5M, Xiangyi, China) when parasitemia had reached 8-10%. The pellets of erythrocytes were washed twice with PBS and suspended in 2 volumes of 7% glycerol. The suspension was left to settle for 20 min at room temperature (RT), then centrifuged at 800 x g for 10 min at RT, after which the pellets of erythrocytes were re-suspended with 10 volumes of PBS at 4 ^{o}C until 95% of the erythrocytes were lysed. The lysates were first centrifuged at 800 x g for 10 min at 4 ^{o}C. The supernatants were collected and centrifuged at 3650 x g for 15 min at 4 ^{o}C. The pellets of merozoites were then washed twice with PBS and frozen at -70 ^{o}C until use.

Construction and immunoscreening of a *Babesia* sp. BQ1 (Lintan) cDNA expression library

Total RNA was extracted from the above-mentioned crude purified merozoites of *Babesia* sp. BQ1 (Lintan) with TRIZOL® Reagent (15596-026, Invitrogen, USA), and the polyadenylated mRNA was purified with a mRNA Purification Kit (27-9258-02, Amersham, UK) according to the manufacturer's instructions. Double strand cDNA with a directional *EcoR I* or *Hind III* Linkers at both ends was synthesized from mRNA by following the instructions of a cDNA synthesis kit (69991-3, Novagen, USA). After digestion with *Hind III* and *EcoR I*, excess linkers and small cDNA products (<300 bp) were removed through a gel filtration column. The cDNA was then ligated to the *EcoR I* and *Hind III* sites of the λ screen

vector (69984-3, Novagen, USA). The recombinant phage DNA was packaged by using PhageMaker packaging extracts (69985-3, Novagen, USA). The phage titer of the primary library was determined following the manufacturer's instructions. A primary cDNA library with a size of 1 x 10^6 PFU was constructed. The phage lambda library was amplified and frozen at -70 ^oC. The amplified library (3.5 x 10^9 PFU) of the *Babesia* sp. BQ1 (Lintan) merozoite cDNA expression library was immunoscreened using immune serum collected from sheep infected with *Babesia* sp. BQ1 (Lintan) (12). Ninety three positive plaques were revealed by primary screening of the library on plates. Phage plugs were removed from the plates according to the sites of positive signal on the membrane and subjected to re-screening until all the signals on the membranes were positive. Pure phage stock was converted to plasmid by using the in vivo auto-subcloning capabilities of the loxP-cre system of λ screen vector in host strain BM25.8 and the plasmids were used for sequencing.

DNA sequencing

Recombinant plasmid isolated from BM25.8 was transformed into the host strain JM109. The purified recombinant plasmid DNA was produced using the TakaRa MiniBEST Plasmid Purification Kit (TakaRa, China) and an insert cDNA was sequenced with the λ screen vector-specific primers (SP6 promoter and T7 terminator) using the BigDye terminator cycle sequencing system (Applied Biosystems) and automated sequencer (Applied Biosystems) from the TakaRa company (China). Sequence analysis was done using the Lasergene software package for Windows (DNASTAR, Madison, WI) and the NCBI database. The nucleotide sequence of an EST fragment showed high homology with the Hsp90 gene of *B. bovis* based on the BLASTn in GenBank and was designated BQHsp90.

Amplification of the full length cDNA of BQHsp90

A SMART^{TM} RACE cDNA amplification kit (Clontech Laboratories, USA) was used to amplify the 5' end of BQHsp90 from cDNA of *Babesia* sp. BQ1 (Lintan) with Gene specific primer (GSP1) (5'- ACGCTCAGTCCACCTCCTCCATCTT -3') designed from 3' end of BQHsp90 EST fragment according to the manufacturer's instructions. Amplified PCR fragments were routinely cloned into pGEM-T easy vector (Promega, USA) and nucleotide sequences determined by the TakaRa Company (China). The full length cDNA sequence of BQHsp90 was assembled with ligation of the 5' and 3' ends using the Lasergene software package for Windows (DNASTAR, Madison, WI) and the open reading frame (ORF) was determined using ORF Finder (www.ncbi.nlm.nih.gov/gorf).

Characterization of BQHsp90

Multiple sequence alignment (MSA) was performed on the deduced BQHsp90 amino acid sequence and those of *B. bovis* (AAF61428), *T. annulata* (XP_952473), *T. parva* (AAA30132), and *Plasmodium falciparum* (CAA82765) using MEGA 4. The putative signal peptide was predicted using SignalP (www.cbi.dtu.dk/services/SignalP). The molecular mass (Mw) and theoretical isolectric point (pI) was calculated on line

(www.expasy.org/tools.pi_tool.html). Motif scan and transmembrane topology prediction were done using MyHits (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The potential protein subcellular location was predicted from the server (http://psort.ims.u-tokyo.ac.jp/form2.html). The three-dimensional domain structure was predicted and assessed by SWISS-MODEL (http://www.expasy.org/swissmod/SWISS-MODEL.html).

The structure of the BQHsp90 genomic sequence was confirmed by performing PCR analysis with BQHsp90-G primers (F203-TGCCTTGGAAAAGATCCGTTATGAG, R2231-CGCTCAGTCCACCTCCTCCATCTTA) designed from the sequence data of the BQHsp90 cDNA clone with initial denaturation at 94 °C for 1 min, then 94 °C for 30 s; 60 °C for 30 s; and 72 °C for 2 min, for 35 cycles, then at 72 °C for 10 min. Sequencing was performed as described above. Locations and exon-intron structures of the BQHsp90 genomic sequences were predicted using the GENSCAN programme (http://genes.mit.edu/GENSCAN.html) and ClustalW.

Phylogenic analysis

Hsp90 genomic DNA (gDNA) was amplified with primers UHSP90 (F443: CGGTGTCGGTTTCTACTCGGCTTAC, R2231: CGCTCAGTCCACCTCCTCCATCTTA) designed on the conserved region of Hsp90 from Chinese *Babesia* isolates infective for small ruminants, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesia* sp. Tianzhu and *Babesia* sp. Xinjiang (at 94 °C for 1 min as initial denaturation, then 94 °C for 30 s; 63°C for 30 s; and 72 °C for 2 min, for 35 cycles, then at 72 °C for 10 min). The PCR products were processed as described above for sequencing by the TakaRa company. The prediction of introns and splicing sequences, and the deduction of amino acid sequences was performed with GENSCAN (http://genes.mit.edu/GENSCAN.html) and the clustal W programme in MEGA 4 software. Several Hsp90 protein sequences of Apicomplexa species that are important for humans and domestic animals were identified i.e. *B. bovis*, *T. annulata*, *T. parva*, *Toxoplasma gondii*, *Eimeria acervulina*, *E. tenella*, *Crytosporidium parvum*, *C. muris*, *P. vivax* and *P. falciparum* and downloaded from the Blast server (www.ncbi.nlm.nih.gov/Blast) (Table 1). MSA and evaluation of the phylogenetic relationships were performed using the ClustalW programme in MEGA 4 and DNAStar.

Prokaryotic expression of the BQHsp90 gene

A pair of specific clone primer BQHsp90-cDNA within the ORF was designed with software Primer 5.0 (Full-F17: 5' -AGTATCTACCTAGCGACATCTTTCT -3'; Full-R2207: 5' -TCAGTCCACCTCCTCCATCTTA -3'). The BQHsp90 containing the entire ORF was amplified from cDNA of *Babesia* sp. BQ1 (Lintan) (initial denaturation at 94 °C for 1 min, then 94 °C for 30 s; 55 °C for 30 s; and 72 °C for 2 min, for 35 cycles, then at 72 °C for 10 min) and the PCR product was ligated into pGEM-T-easy (pGEM-BQHsp90) for sequencing. The Champion™ pET Directional TOPO® Expression Kit (Invitrogen, USA) was used for the expression of BQHsp90. The ORF of BQHsp90 was amplified from recombinant plasmid

pGEM-BQHsp90 with a pair of expression primers (BQHsp90-F75-TOPO: *CACC*ATGGCGACGGAGAGTCAGGAG, BQHsp90-R2228-TOPO: TCAGTCCACCTCCTCCATCTTAGGG) as follows: initial denaturation at 94 °C for 1 min, then 94 °C for 30 s; 65°C for 30 s; and 72 °C for 2 min, for 30 cycles, then at 72 °C for 10 min. The PCR product was gel-purified with Agarose Gel DNA Extraction Kit (TakaRa, China). The pET200/D-TOPO® cloning reaction was set up and recombinant plasmid was constructed into One Shot® TOP10 Chemically Competent *E. coli* for characterization following the user manual. After sequencing, the recombinant plasmid extracted from One Shot® TOP10 was transformed into BL21 Star™ (DE3) One Shot® Chemically Competent *E. coli* for expression. To induce recombinant BQHsp90 (rBQHsp90) expression, IPTG was added to a final concentration of 1 mM, and expression was induced for 4 h at 37°C. The rBQHsp90 was purified from supernatants of the lysates by Ni affinity chromatography according to the manufacturer's protocol (Invitrogen, USA)

Immunogenicity analysis of rBQHsp90 by Western blot and ELISA

For Western blot, the purified recombinant BQHsp90 was electrophoresed by SDS-PAGE under reducing conditions and then transferred to nitrocellulose (NC) membranes of 0.45 µm pore size (RPN303E, Amersham). The NC sheets were blocked with 10% skimmed milk powder in 0.1 M Tris-buffered saline (pH 7.6) and 0.1% Tween (TBST) overnight at 4 ^{o}C, and then incubated for 1 hour with test sera diluted at 1/100 in TBST. Following 4 washes with TBST, the sheets were incubated with monoclonal anti-goat/sheep IgG-Alkaline phosphatase conjugate (A8062-5ml, Sigma) diluted at 1/2000 in TBST for 1 hour. After 5 washes with TBST, positive signals were revealed using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (B1911-100ml, Sigma).

Anti-Hsp90 antibodies levels were evaluated by ELISA in sera from 4 sheep experimentally infected by *Babesia* sp. BQ1 (Lintan) using rBQHsp90 as antigen. Briefly, microplates (Nunc) were coated with 2 µg/ml of rBQHsp90 in 0.1M pH 9.6 carbonate buffer at 37°C for 1 hour and then at 4 °C overnight. After washing 3 times with PBS containing 0.1% Tween 20 (PBST), the plates were blocked with 150 µl of 2% gelatin in carbonate buffer at 37 °C for 30 min. After drying the plate, blank (PBST) and sera (dilution of 1:200) were distributed in duplicate and the plates were incubated at 37°C for 1 hour. After washing as above, a peroxidase conjugate of monoclonal anti-goat/sheep IgG clone GT-34 (A-9452, Sigma) diluted at 1:1000 was added to each well and the plates were again incubated at 37°C for 1 hour. After 3 washes in PBST and 3 in PBS, 50 µl TMB (Sigma) were added to each well and incubated at room temperature for 15 min. The reaction was stopped by adding 50 µl of 0.1M H_2SO_4 and the plates were then read at 450 nm with an ELISA automat (Bio-RAD, USA). Differences between the rBQHsp90 specific antibodies levels of sera recovered before infection and 1 month after infection were detected by student's t-test.

RESULTS

Construction and immunoscreening of cDNA expression library of *Babesia* sp. BQ1 (Lintan) merozoites

Analysis of the cDNA expression library of *Babesia* sp. BQ1 (Lintan) merozoites revealed sequences more than 200bp in length involved in 10 EST with high homology to *B. bovis* genes. Except for 2 hypothetical proteins, these included gliding-associated protein 45 (GAP45), p200, Rab1b, Histone H2A protein, cyclophilin, RNA recognition motif containing protein, membrane protein and Hsp90 by sequence alignment. In clone 45, which belongs to the HSP90 family, a 699bp length containing a 94bp poly(A) tail but no entire ORF was found. It showed 81% identity with the 3' end of the Hsp90 gene of *B. bovis* and the entire 3' end of the Hsp90 gene (Accession number: XM_001611504 and AF136649) when aligned by BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and was designated BQHsp90.

Amplification and Characteristics of BQHsp90 full-length

A 5' RACE reaction was carried out to obtain a complete cDNA sequence of BQHsp90. BQHsp90 is a 2399bp cDNA containing a 2154bp ORF which encodes a predicted 717 amino acid residues protein with a theoretical pI of 5.0 and mass of 83 kDa. The sequence was deposited in GenBank under the accession number **GQ397856**. The multiple sequence alignment (MSA) of the deduced BQHsp90 amino acid sequence with the Hsp90 of *B. bovis*, *T. annulata*, *T. parva* and *P. falciparum* revealed identities of 92.0%, 81.9%, 81.7% and 70.6%, respectively (Figure 1). No signal sequence was identified in the transcript using the SignalP software. SMART and MyHits analysis revealed a conserved ATP-binding domain between positions 29 and 183. This superfamily of homodimeric ATPases is found in several ATP-binding proteins, such as histidine kinase, DNA gyrase B and phytochrome-like ATPases. MyHits analysis revealed that BQHsp90 contains a Hsp90 family signature at position 27-36 and a TRP binding site at the COOH- terminus. In addition, a nuclear localization signal (NLS) was detected (Figure 1). The PSORT II prediction indicated that BQHsp90 is probably a cytoplasmic protein (reliability: 76.7).

Genomic DNA (gDNA) and cDNA of BQHsp90 were amplified with primer BQHsp90-G and sequenced. The BQHsp90 gDNA and cDNA were 2573bp and 2029bp in length respectively. Exon-intron structure analysis with GENSCAN revealed that the BQHsp90 gDNA consisted of two introns and three exons, and the introns were 403bp and 142bp in length and located at sites 1423/1424 and 1924/1925, respectively (Figure 2). The predicted splice sites of the introns conformed to the GT/AG rule.

Simple 3D analysis of the BQHsp90 domains revealed two clearly distinguishable components, notably the NH2-terminal domain and COOH-terminal domain attached by a highly charged linker region of 67 amino acids (Figure 1). The 3D structure of the NH2-terminal domain (from 1 to 217 amino acids) was constructed by using the structure of

Hordeum vulgare (Barley) Hsp90 (PDB No.: 2jkiB) as template (70.5%) similarity (41). It consists of nine-stranded β-sheets and nine helices (five α-helices and four 3,10 helices) that are ligated by 17 coils (Figures 3a, b, c). The 3D structure of the COOH-terminal domain was inferred from that of *Leishmania major* Hsp90 (PDB No.: 3hjcA) (61.7% similarity), which was positioned between 285 and 689. It has 12-stranded β-sheets and 21 helices (14 α-helices and seven 3,10 helices) (Figures 3 d, e, f). The two 3D structural assessments of overall quality showed that the Z-score was -7.14 and -8.43 for the NH2-terminal domain and COOH-terminal domain, respectively.

Phylogenetic analysis

Hsp90 gene fragments were amplified from all genomic DNA of *Babesia* sp BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesia* sp. Tianzhu and *Babesia* sp. Xinjiang. The sequence sizes were 2333bp, 2339bp, 2340bp, 2332bp and 2166bp, corresponding to the accession numbers GQ443608, GQ443604, GQ443605, GQ443606 and GQ443607, respectively. Exon-intron analyses of these genomic Hsp90 with GENSCAN revealed that the variety of sequence lengths resulted in different sized introns (in GenBank database). Multiple sequence alignments based on the gDNA and deduced amino acid sequences of Hsp90 in 5 Chinese *Babesia* isolates revealed that the major varieties are present in the charged linker region (Figure 4). The identities of the 5 Chinese *Babesia* isolates were determined on the basis of the nucleotide and amino acid sequences of Hsp90 (Table 2). The minimum identities of nucleotide and amino acid sequences in 5 Chinese *Babesia* isolates were present in *Babesia* sp. Xinjiang (less than 80% and 90%). The identities of the amino acid and nucleotide sequences were more than 97.8% and 93.2%, respectively, for *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, and *Babesia* sp. Tianzhu. In addition, the maximum identities were found for *Babesia* sp BQ1 (Lintan) and *Babesia* sp. Tianzhu (99.3% and 99.4% for the amino acid and nucleotide sequence) and for *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei (99.5% and 98.8% for the amino acid and nucleotide sequence). The Bootstrap test of phylogeny for the apicomplexa Hsp90 aminoacid dataset generated identical tree topologies for Neighbor-Joining (NJ) analyses, using the clustalW programme in software MEGA 4 (Figure 5). The relationships were in agreement with the traditional taxonomic classification as all *Babesia*, *Theileria*, *Cryptosporidium*, *Eimeria*, *Toxoplasma* and *Plasmodium* were classified into separate branches. The *Babesia* group could be divided into 3 clades: *Babesia* sp. Xinjiang, *B. bovis* and the four other Chinese ovine *Babesia* isolates. These 4 Chinese ovine *Babesia* isolates could be further divided into 2 subclades, one including *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu, and the other *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei.

Expression and Antigenic analysis of rBQHsp90

An entire BQHsp90 ORF was successfully inserted into the pET200/D-TOPO® vector and expressed in BL21 Star™ (DE3). The recombinant BQHsp90 protein (rBQHsp90) was

expressed in two forms i.e. as soluble protein and inclusion bodies. The soluble rBQHsp90 protein was purified from supernatant of recombinant BL21 (Figure 6A). Western blot analysis showed that *Babesia* sp. BQ1 (Lintan) infected sheep serum could specifically recognize the rBQHsp90 protein. No-reactions from rBQHsp90 were detected with negative sera, or from the pET200/D-TOPO® vector control with either positive or negative sera (Figure 6B). The anti-Hsp90 IgG levels evaluated by ELISA had significantly increased in sera obtained from sheep after infection with *Babesia* sp. BQ1 (Lintan) compared with the levels in non-infected sheep ($P<0.01$) (Figure 7).

DISCUSSION

A Hsp90 gene from ovine *Babesia* (*Babesia* sp. BQ1 (Lintan)) has been cloned and characterized for the first time. The protein encoded by the BQHsp90 gene is a 83kDa member of the HSP90 family, sharing high homology with *B. bovis*, *T. annulata*, and *T. parva* Hsp90, and high sequence identities around the N-terminal and C-terminal domains. *In silico*, several Hsp90 conserved signatures, such as the ATP-binding domain, Hsp90 signature, TRP binding site, GXXGXG motif and nuclear localization signal, were identified on BQHsp90 protein. BQHsp90 is a cytoplasmic Hsp90 protein according to PSORT II analysis. Comparison of the genomic DNA and cDNA sequences of BQHsp90 showed that the BQHsp90 gene contains two introns. This is in agreement with reports that all Hsp90s contain introns but that the number of introns differs from one to another (8, 14). The structural analysis of BQHsp90 also showed high similarity with the 3-dimensional structure of other Hsp90 proteins i.e. presence of a N-terminal domain and COOH-terminal domain linked by a charged linker region. As the BQHsp90 protein exhibits several structural characteristics common to the HSP90 family, it probably shares some important functions such as ATPase activity. For example, Kumar et al. (2007) and Zhang et al. (2008) used 3-dimensional structure analysis to show that conserved domains of Hsp90 were involved in nucleotide binding, ATPase activity, co-chaperone binding and intersubunit interactions (22, 41). It might therefore be possible to use inhibitors of BQHsp90 functions to develop therapeutic treatments against *Babesia* sp. BQ1 (Lintan), as described for toxoplasmosis or malaria (9, 21, 22).

Although small-subunit (SSU) rRNA genes are commonly used to determine the molecular phylogenies of eukaryotes and prokaryotes and some new species were discovered based on SSU rRNA gene sequences (20, 27), several authors (6, 31, 35) have demonstrated that, due to the extremely variable rate and mode of rRNA evolution, unsound methods or artefactual grouping may also produce phylogenies if we just used small-subunit (SSU) rRNA genes in phylogenetic analysis. Thus, together with conserved protein-coding genes, it makes phylogenetic analysis more sound and close to virtual evolutional relationship of organisms.

For instance, Fukuda and Endoh (2008) used both the Hsp90 and β-tubilin genes to determine the phylogeny of the dinoflagellate *Noctiluca scintillans* and proposed a possible evolutionary position between the diploid dinoflagellates and haploid core dinoflagellates (11). In this study, we used the Hsp90 gene to carry out a phylogenetic analysis within the apicomplexa species and to clarify the phylogeny of Chinese *Babesia* strains.

MSA of the deduced BQHsp90 amino acid sequence showed that BQHsp90 shared more than 70% identity with the Hsp90 of other apicomplexa. The *Babesia*, *Theileria*, *Eimeria*, *Cryptosporidium*, *Plasmodium*, and *Toxoplasma* genera were apparent as clearly separate clusters as in traditional taxonomy. In China, several geographical strains of ovine large *Babesia* have been isolated from field-collected blood or ticks in the past two decades. Liu et al. (2007) subjected these Chinese ovine *Babesia* isolates to a phylogenetic analysis based on the 18S rRNA gene sequences, and showed that 7 Chinese isolates could be separated into two clusters, a *Babesia* sp. Xinjiang cluster and a '*Babesia motasi*' cluster containing *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu and *Babesia* sp. Hebei together with an European *B. motasi* (23). Recent phylogenetic analyses, based on ribosomal DNA internal transcribed spacer (ITS) sequences, suggest that all these strains, except *Babesia* sp. Xinjiang, should be considered as *B. motasi* (26). In our phylogeny analysis, based on the amino acid sequences of Hsp90, the results were similar to those of analyses based on ribosomal genes, 18S rRNA and internal transcribed spacer (ITS) genes in that *Babesia* sp. Xinjiang, *B. bovis* and 4 other Chinese ovine *Babesia* isolates separated into 3 distinct clusters on the phylogenetic tree. This provides further evidence that *Babesia* sp. Xinjiang appears to be a new *Babesia* species infective for small ruminants. Interestingly, the 4 Chinese '*Babesia motasi*' could be further divided into 2 subclades, one containing *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu and the other *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei. MSA indicated that *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Tianzhu were more closely related, as were *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Hebei. Thus, the results of phylogenetic analyses based on ITS or BQHsp90 sequences should provide sound molecular evidence for the taxonomic position of the four Chinese ovine '*B. motasi*'. Uilenberg provided a detailed description of the taxonomic state of *B. motasi* in 2006, declaring that it could be separated into at least two species or subspecies based on the differences in pathogenicity (low virulence in northern Europe and high virulence in southern Europe and the Mediterranean basin), infectivity to sheep and goats, serology and even morphology (38). The taxonomy of the Chinese '*B. motasi*', with one group of low virulence (*Babesia* sp. BQ1 (Lintan) subclade) (15) and another of high virulence (*Babesia* sp. BQ1 (Ningxian) subclade) (3), seems to align with this viewpoint. Nevertheless, before these Chinese ovine '*B motasi*' isolates can be classified as two species or subspecies, further evidence from biological studies of the tick vector, virulence or antigenicity will be required.

Hsp90-specific antibodies have been used as a diagnostic marker of disease progression

in some tumors and psychiatric disorders (25, 34). They are also found in patients with various autoimmune disorders such as rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus and inflammatory bowel diseases (17, 36). Hsp90 has rarely been considered for use in the diagnosis of diseases caused by pathogen invasion, due to the presence of anti-Hsp90 antibodies in the normal IgG repertoire and cross-reaction between antibodies and Hsp90 from different organisms (28, 39). Nevertheless, the level of anti-Hsp90 specific antibodies significantly increases when hosts are infected by pathogens (33, 42). De Andrade et al. (1992) showed that recombinant Hsp90 from *Leishmania donovani donovani* did not cross-react with sera from *Trypanosoma cruzi* and *Toxoplasma gondii* patients in either western blot or ELISA (7). Hsp90 from *Trichinella spiralis* was also specific and showed no reaction with irrelevant immune rat sera by western blot (24). In the present study, no reaction was detected between recombinant BQHsp90 and sera recovered from sheep before infection with *Babesia* sp. BQ1 (Lintan) and the levels of BQHsp90 antibodies significantly increased in sera from *Babesia* sp. BQ1 (Lintan) infected sheep, compared with those obtained from autologous sheep before infection. BQHsp90 could thus be a potential sero-diagnostic antigen for detecting infection with *Babesia* sp. BQ1 (Lintan). However, further studies are required to define possible cross-reactions between rBQHsp90 and antibodies against the Hsp90s of other pathogens, especially the parasites infecting small ruminants in China.

Conflict of interest

None declared

Acknowledgements

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Table 1. Information about the apicomplexa species included in this study

Species	Strain	Accession number of nucleotide	Accession number of protein
Babesia sp.	BQ1 (Lintan)	GQ397856 [1] , GQ443608 [2]	ACV04849 [1], ACV71146 [2]
Babesia sp.	BQ1 (Ningxian)	GQ443604	ACV71142
Babesia sp.	Hebei	GQ443605	ACV71143
Babesia sp.	Tianzhu	GQ443606	ACV71144
Babesia sp.	Xinjiang	GQ443607	ACV71145
Babesia bovis	MEX	AF136649	AAF61428
Theileria annulata	Ankara	XM_947380	XP_952473
Theileria parva		M57386	AAA30132
Toxoplasma gondii		AY344115	AAQ24837
Eimeria acervulina		AY459430	AAS18319
Eimeria tenella	PAPt38	AF042329	AAB97088
Cryptosporidium parvum	Iowa II	XM_626924	XP_626924
Cryptosporidium muris	RN66	XM_002142364	XP_002142400
Plasmodium vivax	SaI-1	XM_001613401	XP_001613451
Plasmodium falciparum	7	Z29667	CAA82765

Note: [1] = derived from cDNA, [2] = derived from genomic DNA

Table 2. Percent identities of hsp90 nucleotide and amino acid sequences in 5 Chinese ovine *Babesia* isolates by ClustalW

Parasite	Percent identity of amino acid sequences					Percent identity of nucleotide sequences				
	1	2	3	4	5	1	2	3	4	5
Babesia sp. BQ1 (Lintan)	1	99.3	97.8	98.0	87.8		99.4	93.2	93.2	78.5
Babesia sp. Tianzhu	2		98.2	98.3	88.1			93.5	93.5	78.5
Babesia sp. BQ1 (Ningxian)	3			99.5	87.6				98.8	78.9
Babesia sp. Hebei	4				87.8					78.8
Babesia sp. Xinjiang	5									

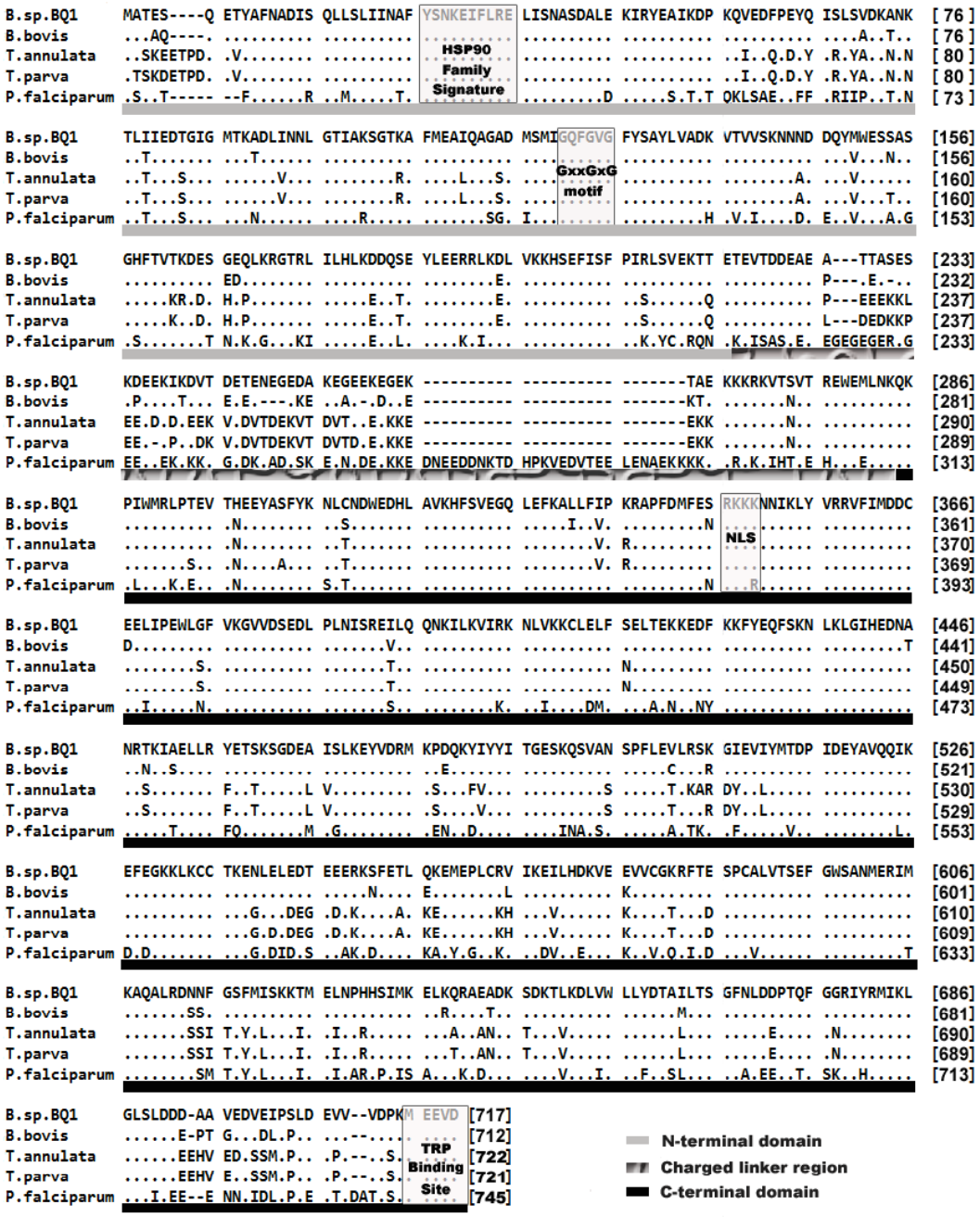

Figure 1. Multiple sequence alignment of Hsp90 proteins from *Babesia* sp. BQ1 (Lintan), *B. bovis*, *T. annulata*, *T. parva* and *P. falciparum*. The dots and short lines in the sequence represent identical residues and non-existent residues respectively.

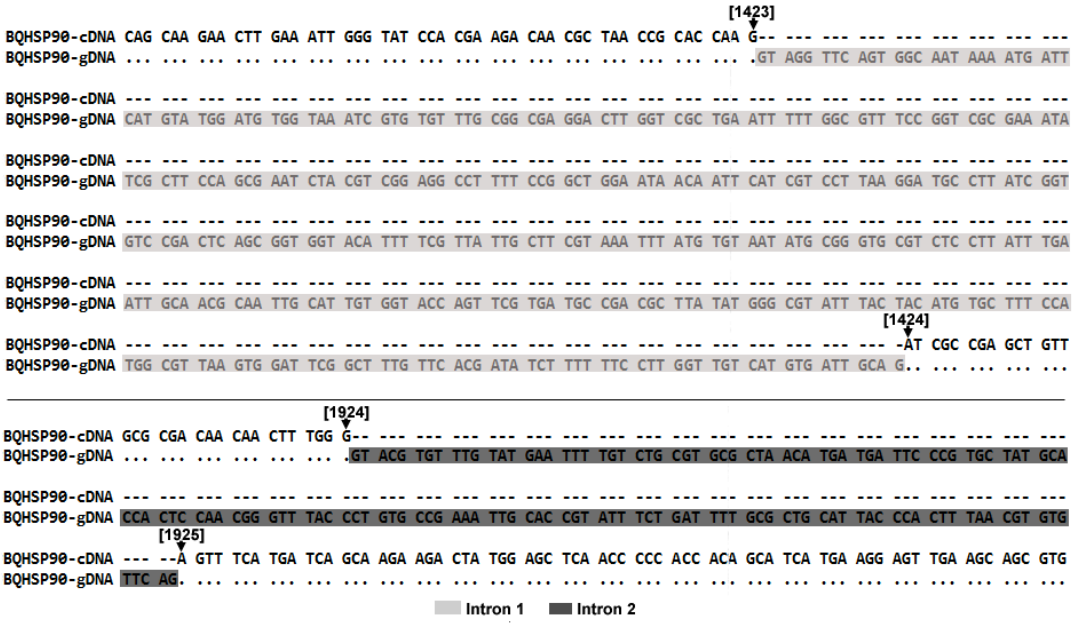

Figure 2. Sequence alignment of BQHsp90 cDNA and gDNA. The degree of darkness of background indicates the two different introns. The numbers on the cDNA sequence show the nucleotide numbers of the complete BQHsp90 sequence.

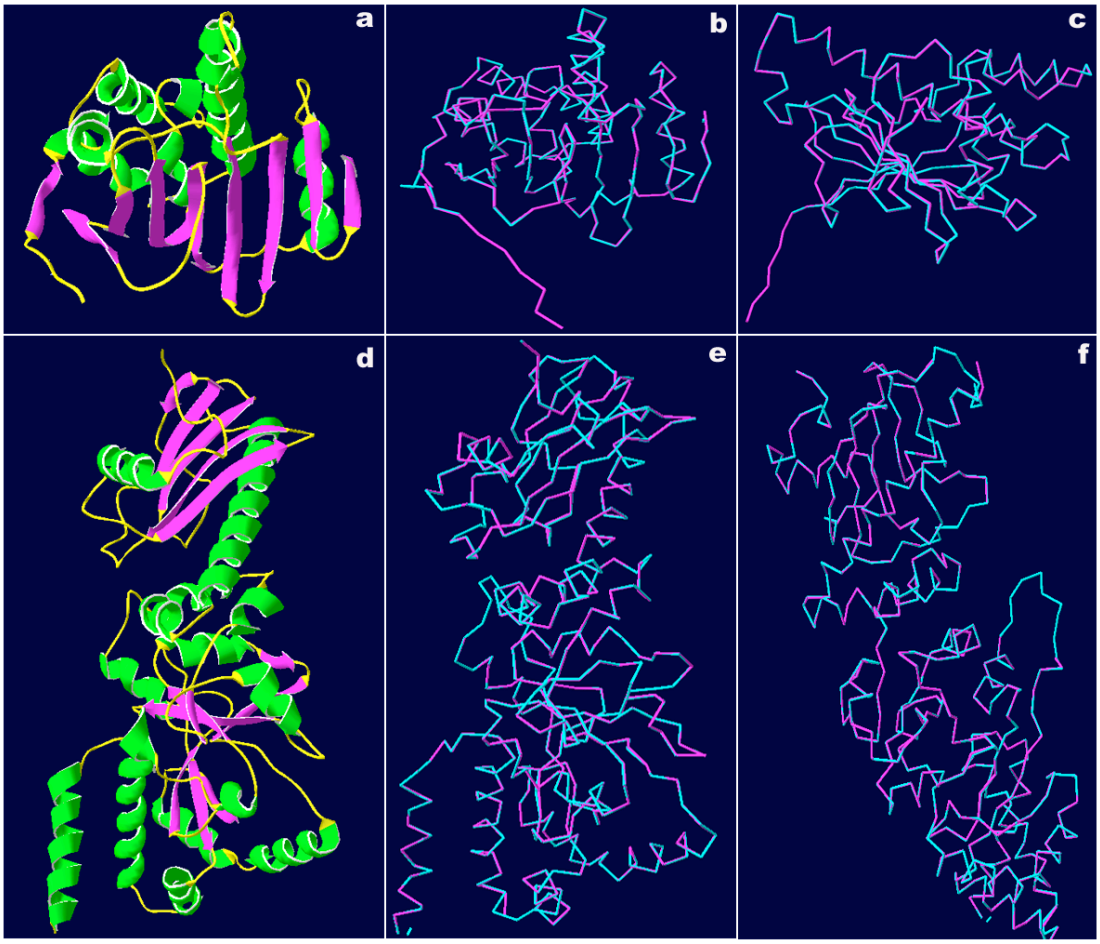

Figure 3. Model of the three-dimensional structure of BQHsp90 N- terminal domain and middle domain. a: and d: Three-dimensional structure of the N-terminal domain and middle domain of BQHsp90, α Helices are shown in bright green, ß sheets in pink and loops in yellow; b, c and e, f: Superimposition between the model (baby blue) and the template protein (pink), b and e: front, c and f: right. The colour interpretation refers to the web version of this paper.

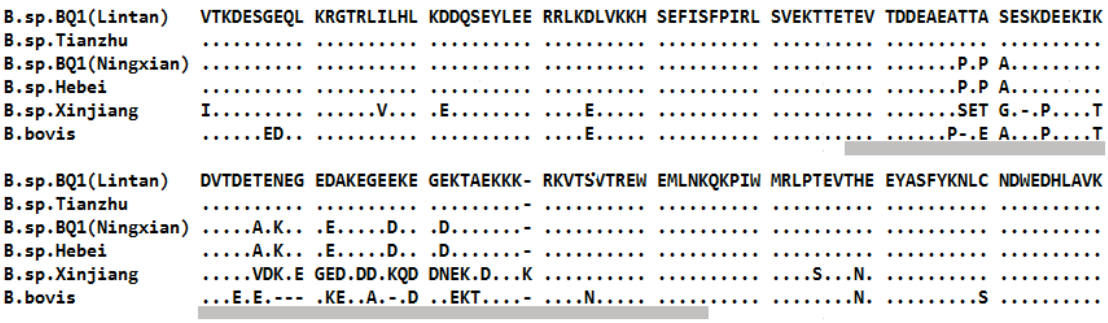

Figure 4. Alignment of *Babesia* HspP90 deduced amino acid sequences. The region of grey background indicates the charged linker region.

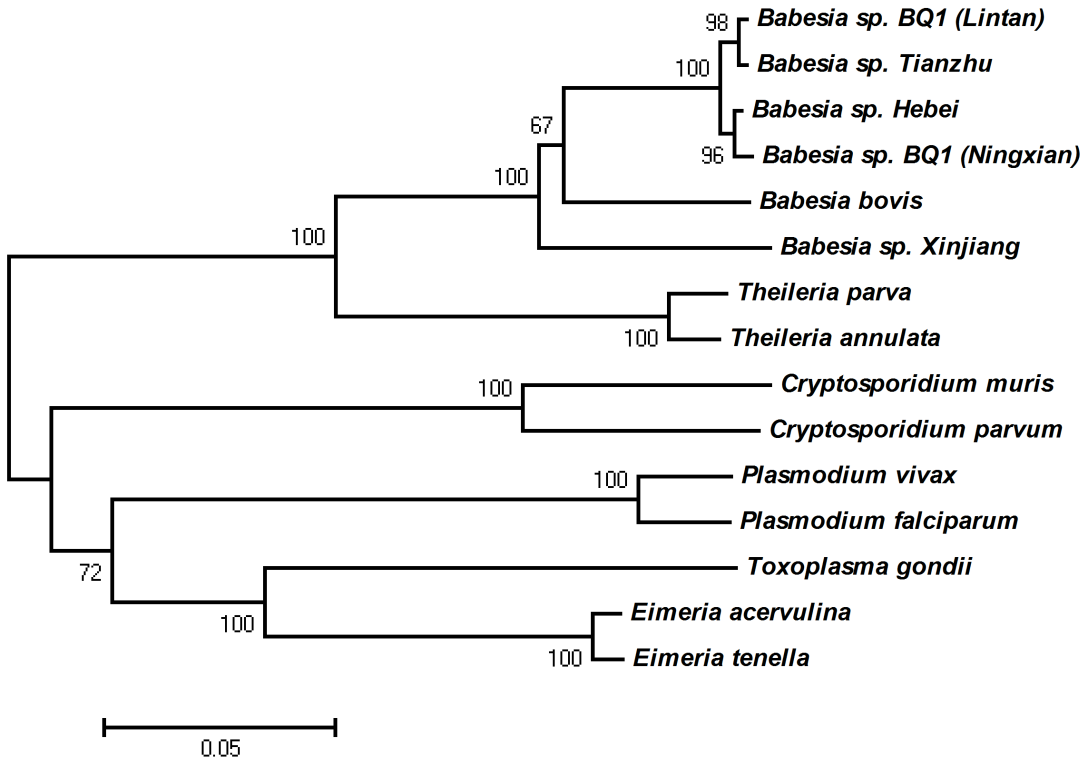

Figure 5. Phylogenetic relationships between 5 Chinese *Babesia* isolates and 10 other apicomplexa species based on the amino acid dataset of Hsp90 genes. The evolutionary history was inferred using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 564 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

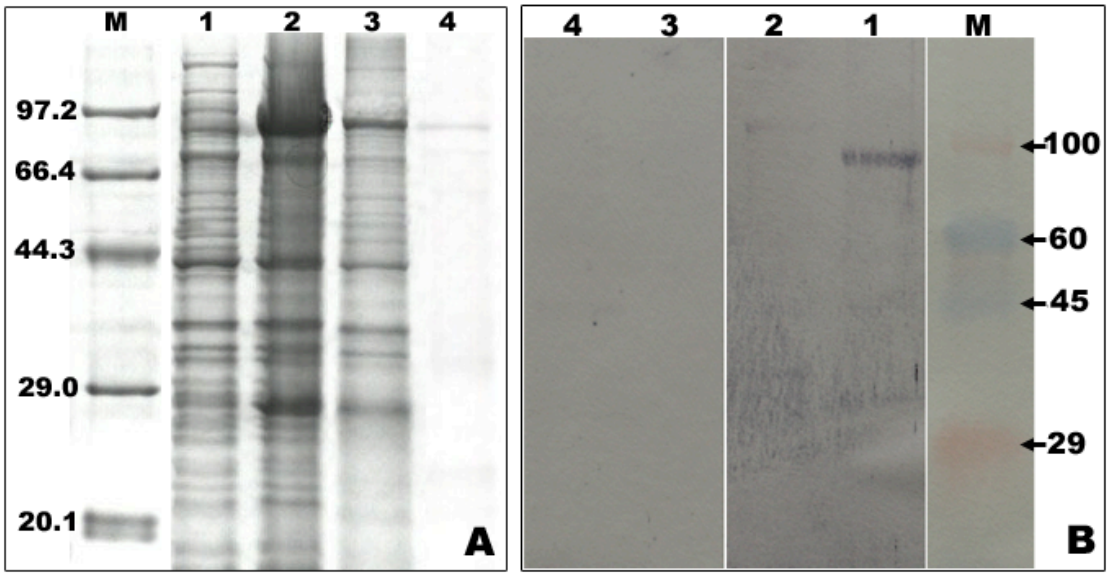

Figure 6. SDS-PAGE (A) and western blot (B) of rBQHsp90 protein. A: M is the marker of mass (kDa); 1-4 represent lysates of recombinant BL21 (DE3) before induction, lysates of recombinant BL21 (DE3) after induction (LRAI), soluble proteins in LRAI and purified rBQHsp90, respectively. B: M is the protein marker (kDa). 1 and 3 are rBQHsp90 protein reacted each with positive serum from sheep infected by *Babesia* sp. BQ1 (Lintan) and negative serum; 2 and 4 are pET200/D/*lacZ* control reacted with positive serum from sheep infected by *Babesia* sp. BQ1 (Lintan) and negative serum, respectively. The right and left numbers represent the mass of each band in the protein marker.

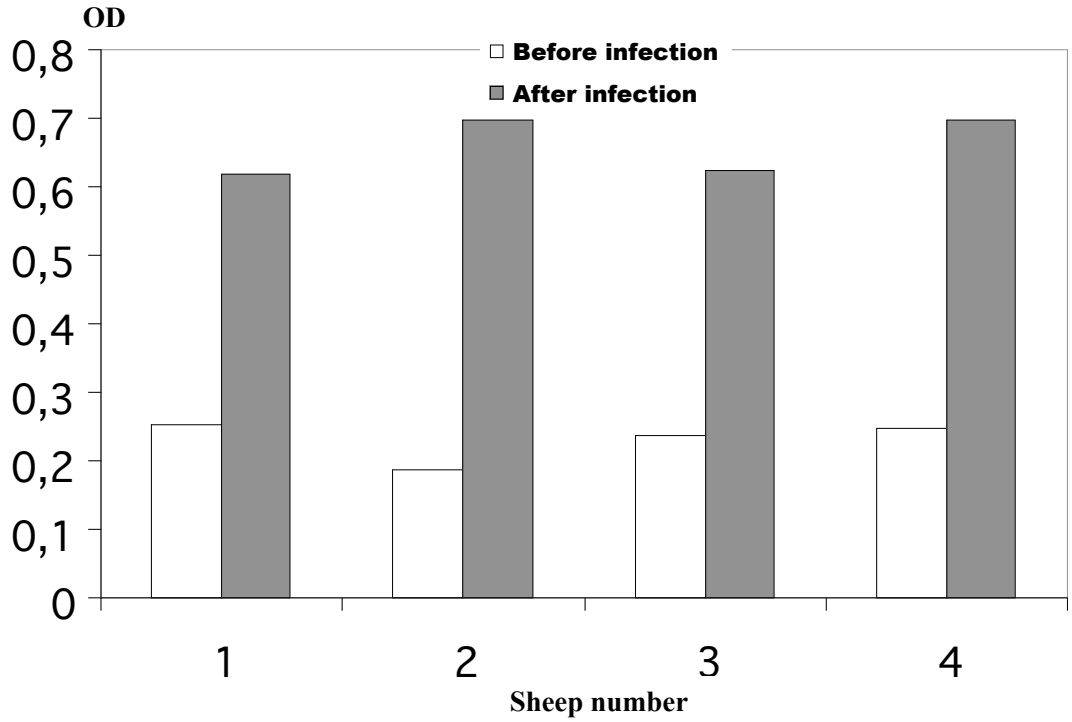

Figure 7. Level of rBQHsp90 protein specific antibody in four sheep sera before and after infection with *Babesia* sp. BQ1 (Lintan). Antibody level of specific rBQHsp90 significantly increased after infection ($P<0.01$).

Article N° 8 : A novel member of intrinsically unstructured proteins BQP35, potentially diagnostic antigen for infection of *Babesia* sp. BQ1 (Lintan) (Un nouveau membre des protéines intrinsèquement non structurées BQP35, antigène potentiel de diagnostic pour l'infection à *Babesia sp*. BQ1 (Lintan))

Ebauche d'article qui sera soumis après obtention de résultats complémentaires. L'anglais n'a pas été corrigé.

Ce travail a été réalisé au laboratoire de parasitologie vétérinaire de la province du Gansu, à Lanzhou

Un nouveau gène de *Babesia sp*. BQ1 (Lintan) (BQP35) a été cloné en criblant une banque d'ADNc de mérozoïtes de *Babesia sp*. BQ1 (Lintan) avec un sérum de mouton infecté. La séquence de l'ADNc contient 1140 paires de base (pb) avec une ORF de 936pb et code pour une protéine de 311 acides aminés avec un poids moléculaire de 35 kDa et un point isoélectrique de 7,05. L'ADN génomique correspondant a une longueur de 936pb et est sans intron. La protéine BQP35 est essentiellement une protéine nucléaire. Cependant, l'analyse par SDS-PAGE de la protéine recombinante rBQP35 a une migration anormale à 50 kDa. Ceci pourrait être du au fait que cette protéine appartient à la famille protéique IUP (intrinsically unstructured protein), ce qui a été confirmé par les analyses bioinformatiques effectuées. Nous avons montré également que la protéine BQP35 pourrait être un antigène de diagnostic sérologique. En effet, les analyses bioinformatiques et par Western blot montrent qu'elle est antigénique (une forte réaction avec les sérums de moutons infectés par *Babesia sp*. BQ1 (Lintan) est observée) et spécifique (les sérums de moutons infectés par des hémoparasites importants en Chine (*Theileria luwenshuni, T. uilenbergi, Anaplasma ovis, Babesia sp*. BQ1 (Ningxian), *Babesia sp*. Xinjiang, *Babesia sp*. Hebei, *Babesia sp*. Tianzhu) ne réagissent pas avec cette protéine). Avant d'être publiés, ces résultats doivent être complétés par d'une part la confirmation que la protéine migrant à 50 kDa est bien la protéine BQP35, ce qui pourra être fait par analyse de la bande par spectrométrie de masse, et par d'autre part l'analyse par ELISA de la cinétique de réponse anti-BQP35 de moutons infectés expérimentalement. Enfin, l'analyse de sérums de moutons non infectés ou infectés naturellement par *Babesia sp*. BQ1 (Lintan) permettra de montrer l'intérêt diagnostic de cette protéine BQP35.

A novel member of intrinsically unstructured proteins BQP35, potentially diagnostic antigen for infection of *Babesia* sp. BQ1 (Lintan)

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Abstract

A novel gene of *Babesia* sp. BQ1 (Lintan) (BQP35) was cloned by screening a merozoite cDNA expression library with infected sheep serum and using rapid amplification of cDNA ends (RACE). The nucleotide sequence of the cDNA was 1140 bp with an open reading frame (ORF) of 936 bp encoding a 35-kDa predicted polypeptide having 311 amino acid residues. Comparison of BQP35 cDNA and genomic DNA sequences shows that BQP35 has not intron. *In-silico* prediction of antigenicity indicates that it has the potentiality as a diagnostic antigen. The recombinant BQP35 (rBQP35) expressed in prokaryotic expression system and purified with Nickel-Chelating Resin showed an abnormally slow migration on SDS-PAGE. Disorder region prediction indicates it has characteristics of intrinsically unstructured proteins (IUPs). The rBQP35 could be specifically recognised by positive sera from sheep infected by *Babesia* sp. BQ1 (Lintan) in western blot. It almost had no cross-reaction with sera from sheep infected with other dominant ovine piroplasms in China, four ovine *Babesia* isolates, *Theileria luwenshuni*, *T. uilenbergi* and *Anaplasma ovis* both in ELISA and western blot. These data suggested that BQP35 has a potentiality of diagnostic antigen using in serologic detection of *Babesia* sp. BQ1 (Lintan) infection.

Key words: *Babesia* sp. BQ1 (Lintan), BQP35, recombinant protein, diagnostic antigen, IUPs

Introduction

Babesiosis is one of most ubiquitous and common tick-borne haemoprotozoan disease of domestic animals, caused by the development of protozoa of the genus *Babesia* parasites in erythrocytes. These haemoparasites are naturally transmitted by *Ixodid* ticks. Therefore, geographic distribution of *Babesia* species always correlates with the tick vector in given area in which babesiosis is an endemic disease. Acute infection in sheep can result in seriously clinical signs, such as fever, regenerative anemia, hemoglobinuria, splenomegaly, and sometimes death (Kuttler, 1988). In addition, animals remain chronically infected. Some economic losses as ill-thrift, abortions, loss of milk or meat production, draft power, impact on international trade are attributed to the disease (Bock et al., 2004). In view of these problems of *Babesia* infection, it is therefore necessary to develop some reliable diagnostic methods for this disease.

The classical diagnosis of acutely infected animals is well demonstrated by light microscopic examination of intra-erythrocytic parasites in Giemsa-stained blood smears. However, in subclinical or latent infection, parasites is hardly exposed in the smears because of low parasitemia (Böse et al. 1995). In epidemiological studies, the detection of antibodies in the sera is the optimal choise for determination of *Babesia* infection (Molloy et al. 1998). Several serological tests such as the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) have been developed for babesiosis of domestic animals and used in the detection of sub-clinical cases and field surveys (Boonchit et al. 2006; Goff et al. 2008). However, the IFAT is not suitable for analysis of a large number of serum samples due to timeconsuming and the subjective interpretation of the results (Böse et al. 1995). On the other hand, ELISA is quite sensitive and is appropriate for testing large number of samples especially in field surveys (Weiland, 1986). Previous studies of ELISAs have shown that recombinant antigens provide better options than native antigens because recombinant antigens are usually available in pure forms and offer higher specific activity (Böse et al., 1995; Tebele et al., 2000; Boonchit et al., 2006).

Ovine babesiosis is an economic important disease, caused by *B. ovis*, *B. motasi*, *B. crassa* and some unidentified *Babesia* species (Friedhoff, 1988; Uilenberg, 2006). In China, several different geographic isolates, at least dividing into two groups (*Babesia* sp. Xinjiang and other *B. motasi*-like parasites, such as *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Hebei etc…) are responsible for babesiosis of sheep and goats (Lian et al., 1997; Yin et al., 1997; Bai et al., Guan et al., 2001, 2002; 2002; Liu et al., 2007; Niu et al, 2009a). Several molecular diagnostic tools based on the DNA amplification have been developed for detecting the infection of these parasites (Guan et al., 2008; Niu et al, 2009b). Recently, we also have developed an ELISA for *Babesia* sp. BQ1 (Lintan) using merozoite antigens derived from in vitro culture for carrying out large scale sero-epidemiological survey (unpublished data). In the present study, in order to obtain

potentially serodiagnosis antigen, we screened a merozoite cDNA expression library of *Babesia* sp. BQ1 (Lintan) using sera derived from sheep experimentally infected by *Babesia* sp. BQ1 (Lintan). A previously unidentified protein (BQP35) has been obtained. Our data indicate that the recombinant BQP35 protein expressed in *E. coli* has some characters of intrinsically unstructured proteins (IUPs) as abnormally slower migration on SDS–PAGE. *In silico* analysis of amino acid sequence confirmed that BQP35 is a novel member of IUP family from *Babesia*. Serological studies showed that rBQP35 had potentiality of diagnostic antigen for detecting *Babesia* sp. BQ1 (Lintan) infection.

Materials and Methods

Parasites and immune sera

Monoclonal line (G7) of *Babesia* sp. BQ1 (Lintan) were grown in sheep erythrocytes in vitro as described by Guan et al. (unpublished) and infected sheep blood by *Babesia* sp. BQ1 (Lintan) were cryopreserved in liquid nitrogen in Vector and Vector-borne disease (VVBD) laboratory, LVRI, China.

Spleen-intact sheep was injected subcutaneously with 30 ml of infected sheep blood cryopreserved in liquid nitrogen with 10% parasitemia. Immune sera were collected from jugular vein at different timepoints post-infection. Sera collected from sheep before infection was used as negative sera. Sera from sheep experimentally infected with *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesi*a sp. Tianzhu, *Babesia* sp. Xinjiang, *Theileria luwenshuni*, *T. uilenbergi* and *Anaplasma ovis* were provided by Vector and Vector-borne disease (VVBD) laboratory, LVRI, China. All the sera were blocked with a volume lysates of *Escherichia coli* BL21 (DE3) (Novagen) at 37 ^{o}C for 1 hour and then centrifuged at 13000 rpm for 5 min at 4 ^{o}C. The supernatants were dispersed into 1.5 ml tubes and frozen at -20 ^{o}C until use.

Construction and immunoscreening of a merozoite cDNA expression library Babesia *sp. BQ1 (Lintan)*

Purification of *Babesia* sp. BQ1 (Lintan) merozoites was performed from culture. Briefly, culture was harvested when the parasitemia reached 8-10%. Lysis of erythrocytes was conducted based on the difference of erythrocyte osmotic pressure in 7% glycerol and in pH7.2 PBS. Merozoites were simply purified from lysates using differential centrifugation. Total RNA was extracted from the above crude purified merozoites of *Babesia* sp. BQ1 (Lintan) with TRIZOL® Reagent (Invitrogen), and the polyadenylated mRNA was purified with a mRNA Purification Kit (Amersham, UK) according to the manufacture's instructions. Double strand cDNA with a directional *EcoR I* or *Hind III* Linkers at both ends was synthesized from mRNA using a cDNA synthesis kit (Novagen, USA). The cDNA was then ligated to the *EcoR I* and *Hind III* sites of the λ screen vector (Novagen) after digestion. The recombinant phage DNA was packaged using PhageMaker packaging extracts (Novagen).

Phage titer of the primary library was determined following the manufactures. The phage lambda library was amplified and frozen at -70 ^{o}C. The amplified library was used for immunoscreening with anti-*Babesia* sp. BQ1 (Lintan) sheep serum according to the method previously described by Gao et al. (2007). Pure phage stock was converted to plasmid in host strain BM25.8 which was able to auto-subclone λ screen vector. Recombinant plasmids were isolated from BM25.8 and were transformed into the host strain JM109 for sequencing with the λ screen vector-specific primers (SP6 promoter and T7 terminator) by TakaRa company. Sequence analysis was done using the Lasergene software package for Windows (DNASTAR, Madison, WI) and NCBI database. Five EST fragments with high identities but without entire ORF was obtained.

Amplification of the full length cDNA using 5' end RACE

A SMART^{TM} RACE cDNA amplification kit (Clontech Laboratories, USA) was used to amplify the 5' end from cDNA of *Babesia* sp. BQ1 (Lintan) with Gene specific primer (GSP1) (5'-TCTTCGCCATCGGCTGGAAATCGTC -3' or 5'-CCGGCAGCCTCGTTGTGCTCGTCTA-3') designed from conserved region of those EST fragments. The 5'-RACE was performed according the manufacture's instruction. The touch down PCR cycling parameters were as following: 5 cycles of 90^{o}C for 30 sec and 72 ^{o}C for 3 min; 5 cycles of 94 ^{o}C for 30 sec, 70 ^{o}C for 30 sec and 72 ^{o}C for 3 min; 25 cycles of 94 ^{o}C for 30 sec, 68 ^{o}C for 30 sec and 72 ^{o}C for 3 min. Amplified PCR fragments were routinely cloned into pGEM-T easy vector (Promega, USA) and nucleotide sequences determined by TakaRa Company (China). Only congruential sequences from the 2 GSP1 were used to assemble full length cDNA sequence based the overlap using the Lasergene software package for Windows (DNASTAR, Madison, WI) and the open reading frame (ORF) was determined using ORF Finder (www.ncbi.nlm.nih.gov/gorf). Results showed that a new gene, designated as BQP35, was amplified using BLAST analysis.

Characterization of BQP35

The homological protein sequence was searched with WU-BLAST2 (http://www.ebi.ac.uk/Tools/blast2/). The putative signal peptide was predicted using SignalP (www.cbi.dtu.dk/services/SignalP). Molecular mass (Mw) and theoretical isolectric point (pI) were calculated on line (www.expasy.org/tools.pi_tool.html). Motif scan were done using MyHits (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The potential protein subcellular location was predicted on the server (http://psort.ims.u-tokyo.ac.jp/form2.html). The antigenicity was analysis with DNASTAR, Madison, WI.

To confirm genomic structure of BQP35, a pair of specific primers (5'-ATGAAGAAACGCGAGTTC-3', 5'- TCAAGCCTCTTGTGGCT-3') was designed from the sequence data of the BQP35 including entire ORF. PCR analysis was performed with genomic DNA of *Babesia* sp. BQ1 (Lintan) and *Babesia*-free sheep genomic DNA as control using the cycles, at 94 °C for 4 min as initial denaturation, then 94 °C for 30 s; 60 °C for 30 s;

and 72 °C for 1 min, for 35 cycles, then at 72 °C for 10 min. Sequencing was carried out as above described. Locations of intron were predicted with GENSCAN programme (http://genes.mit.edu/GENSCAN.html) and ClustalW.

Expression and purification of recombinant BQP35 protein in E. coli

The Champion™ pET Directional TOPO® Expression Kits (Invitrogen, USA) was employed into expression of BQP35 gene. The BQP35 gene was amplified with expression primers (forward primer: 5'-CACCATGAAGAAACGCGAGTTC-3'; backward primer: 5'-TCAAGCCTCTTGTGGCT-3') and cloned into expression vector pET200/D-TOPO®. Recombinant pET200-BQP35 was transformed into One Shot® TOP10 Chemically Competent *E. coli* for characterization following the user manual of the kit. After sequencing, the recombinant plasmid was extracted from One Shot® TOP10 and transformed into BL21 Star™ (DE3) One Shot® Chemically Competent *E. coli* for expression with the monitor of PCR and sequencing. The positive transformants were inoculated into LB medium (containing 50 μg/ml Kanamycin) and cultivated for 3.5 hours at 37°C. Recombinant BQP35 (rBQP35) expression was induced with 1 mM IPTG at 37°C for 4 h and identified on SDS-PAGE.

The rBQP35 protein were purified using ProBond™ Purification System according to the manufacture's protocol (Invitrogen, USA). Briefly, the BL21 cells were harvested from culture and then resuspended in Native Binding Buffer. Lysis was performed following five frozen and defrosted cycles and additonal sonication for 15 min of 5 sec sonication and 5 sec interval on ice. Supernatants were collected from lysates by centrifugation and 8 ml supernatant was loaded on a prepared Nickel-Chelating Resin column and bound for 1 hour at room temperature using gentle agitation. After 4 times washing with Native Wash Buffer by gravity, the rBQP35 protein bound on the Nickel-Chelating Resin was eluted with Native Elution Buffer and identified with SDS-PAGE by standard techniques (Sambrook and Russel, 2001) and western blot. For Western blot, the purified recombinant proteins was electrophoresed and transferred to nitrocellulose (NC) membranes of 0.45 μm pore size (RPN303E, Amersham). NC sheets were blocked with 10% skimmed milk powder in 0.1 M Tris-buffered saline (pH 7.6) with 0.1% Tween (TBST) for overnight at 4 ^{o}C, and then incubated for 1 hour with test sera from *Babesia* sp. BQ1 (Lintan) infected sheep and negative sera of diluted at 1/100 in TBST. The sheets were then incubated with monoclonal anti goat/sheep IgG-Alkaline phospatase conjugate (A8062-5ml, Sigma) diluted at 1/2000 in TBST for 1 hour. Positive signals were finally revealed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (B1911-100ml, Sigma).

Prediction of disorder region in BQP35

Composition of amino acid sequence of BQP35 was analysed with Composition Profiler (http://www.cprofiler.org/cgi-bin/profiler.cgi). Computer programs FoldIndex (http://bip.weizmann.ac.il/fldbin/findex), DisEMBL™ (http://dis.embl.de/) and MeDorV1.4

(http://www.vazymolo.org/MeDor/index.html) were used to analyse structure of BQP35, *B. bovis* hypothetical protein (A7ASD6) and ß-galactosidase. The amino acid sequence of BQP35 was submitted to database DisProt (http://www.disprot.org/) (Sickmeier et al., 2007) for searching homological IUPs.

Specificity of rBQP35 for Babesia sp. BQ1 (Lintan) infection

For identifying the specificity of rBQP35, sera each from *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Hebei, *Babesia* sp. Xinjiang, *Theileria luwenshuni*, *T. uilenbergi*, *Anaplasma ovis* infected sheep and negative sera were tested using rBQP35 as antigen in ELISA and western blot. Briefly, the process of ELISA was as following. Microplates (Nunc) were coated with 2 μg/ml of rBQP35 in 0.1M pH 9.6 carbonate buffer at 37°C for 1 hour and then 4°C overnight. After washing 3 times (5 min per wash) using PBS containing 0.1% Tween 20 (PBST), the plates were blocked with 150 μl of 2% gelatin in carbonate buffer at 37°C for 30 min. After drying the plate, blank (PBST) and sera (dilution of 1:200) were distributed in duplicate and the plates incubated at 37°C for 1 hour. After washing as above, a peroxidase conjugate of monoclonal anti-goat/sheep IgG clone GT-34 (A-9452, Sigma) diluted at 1:1000 was added in each well and the plates were incubated at 37°C for 1 hour again. After 3 washes in PBST and 3 in PBS, 50 μl of TMB (Sigma) were added to each well and plates were incubated at room temperature for 15 min. The reaction was stopped by addition of 50 μl of 0.1M H_2SO_4 and then the plates were read at 450 nm with an ELISA automat (Bio-RAD, USA). That of western blot was as above mentioned in "*Expression and purification of recombinant BQP35 protein in* E. coli".

Results

Cloning and characterization of the BQP35 gene

Five EST fragments (297bp-357bp) with high identities except for the bases on near poly (A) were screened from cDNA library. A full-length cDNA of BQP35 gene was amplified from *Babesia* sp. BQ1 (Lintan) cDNA using 5' end RACE and submitted to GenBank with accession number GU014487. This full-length BQP35 cDNA has 1140bp with predicted ORF of 936bp, 118-bp 5' UTR, 72-bp 3'-UTR and 14-bp poly (A). It encodes a 35-kDa polypeptide having 311 amino acids. One putative polyadenylation signal (AATAA) that is conserved in some other apicomplexa parasites (Florin-Christensen et al., 2002; Lanzer et al., 1993), is detected between 24^{th} and 28^{th} base on the downstream of stop codon TGA (Figure 1). The homologous analysis showed that BQP35 protein is likely a homologue of hypothetical protein of *B. bovis* T2Bo (accession number: A7ASD6 in EMBL and XP_001611023 in GenBank) but the identity is just 26%. The analysis of protein characteristics *in silico* indicated a pI of 7.05 and a molecular weight of 35.13kDa and the absence of a N-terminal signal peptide. One nuclear localization signals and bipartite is

located on the C-terminal (Figure 1) and no other putative domains were detected by Myhits servers. The PSORT II prediction indicated that the BQP35 is likely a nuclear protein (60.9 %: nuclear, 21.7 %: cytoplasmic,17.4 %: mitochondrial). Computer based Jameson-Wolf's plot of the predicted polypeptide revealed that most regions within the amino acid sequence had a predicted good antigenic index of 1.7. Genomic DNA (gDNA) and cDNA of BQP35 were amplified and sequenced. The results showed that BQP35 gDNA and cDNA had same length of 936-bp and no intron in the BQP35 gDNA with GENSCAN analysis.

Expression and purification of recombinant BQP35 protein

The recombinant BQP35 (rBQP35) protein was expressed in *E. coli* BL21 as two patterns, soluble protein and dissoluble inclusion body. Soluble rBQP35 was purified from supernatant of lysates with affinity chromatography. SDS–PAGE analysis revealed an unusual migration of rBQP35 protein on gel, which His6-BQP35 is approximate 50 kDa instead of the computer predicted 39.3 kDa (35.13 kDa BQP35+ 4.17 kDa fusion tag). Contrastly, positive control protein for expression, ß-galactosidase with expected molecular weight of 121 kDa migrated on the gel as usual (Fig. 2A). The western blot indicated that sera from *Babesia* sp. BQ1 (Lintan) infected sheep could specifically recognized His6-BQP35 purified using nickel-chelating resin at the about 50kDa site (Figure 2B).

Prediction of disorder region

Composition of amino acid showed that BQP35 have higher levels of charged amino acids, higher levels of certain amino acids (P, R and S), lower levels of others (C and W), and lower levels of hydrophobic amino acids, which is the favor of unstructured proteins (Uversky 2002). FoldIndex prediction showed BQP35 has three disordered regions through the amino acid sequence, with a high index of disorder prediction. The longest disordered region contains 105 amino acid residues and total number of disordered residues is 286 (91.96%). Only the regions between amino acids 78–94 and 200–207 that just possess 8.04% are probably folded (Figure 3A). Disorder analysis for *B. bovis* hypothetical protein (A7ASD6) was also carried out with FoldIdex and result indicated that it is also probably a disordered protein (Figure 3B). Considering ß-galactosidase that is the positive control during expression of BQP35, same analysis was done and as expected, the FoldIndex predicted ß-galactosidase only having 2 short segments of disorder (possess 2.36% of total residues and the longest with 10 residues) (Figure 3C). Anyway, the predictions were also submitted to DisEMBL^{TM} and MeDorV1.4 for verifying the result of prediction and similar results were obtained (not show). However, we could not obtain homological IUPs for BQP35 in the database DisProt. Thus we propose BQP35 should be a novel member of IUP family and the abnormal migration on SDS-PAGE should be rose from characteristics of IUP (Alves et al., 2004 ; García-Ortega et al., 2005; Kang et al., 2008).

Potentiality of rBQP35 protein as a diagnostic antigen

Anti-*Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu,

Babesia sp. Hebei, *Babesia* sp. Xinjiang, *Theileria luwenshuni*, *T. uilenbergi*, *Anaplasma ovis* immune sheep sera and negative sera from sheep before experiment were used to test specificity of rBQP35 with western blot. Results showed that serum positive for *Babesia* sp. BQ1 (Lintan) had strong reaction with rBQP35. Contrastly, the signals to sera of other ovine haemoparasite were very weak (Figure 4). Twelve sera taking at differents timepoints *Babesia* sp. BQ1 (Lintan) infection in 1 sheep at various timepoints, sera to other chinese ovine piroplasms and to *A. ovis* and negative sera were involved into the ELISA using rBQP35 as antigen. Results in Figure 5 showed that rBQP35 was specific for *Babesia* sp. BQ1 (Lintan) infected sheep sera although some weak cross reaction signals were revealed in the *Babesia* spp positive sera. The difference between the mean OD450 standard deviation for sera from *Babesia* sp. BQ1 (Lintan) infected sheep and those from other ovine haemoprotozoan infected sheep was significant with the Student's test of Excel 2000 ($P<0.01$). Therefore, we considered rBQP35 have good specificity for using as a diagnostic antigen in serological diagnosis.

Discussion

A novel *Babesia* sp. BQ1 (Lintan) gene encoding a predicted 35.13 kDa protein BQP35 were discribed in our study by screening a merozoite cDNA library with positive serum collected from experimentally infected sheep. This gene is 1140bp length having 936bp ORF and has not significant high homologous protein in protein dataset except for 26% identity with a *B. bovis* hypothetical protein (A7ASD6). The PSORT II prediction indicated that the BQP35 is a nuclear protein. Exon-intron structure analysis indicates no intron in genomic DNA of BQP35. Computer analysis shows that BQP35 have good antigenic index based Jameson-Wolf's plot of the predicted polypeptide. The polypeptides with good antigenic index were promising candidates of diagnostic antigens (Boonchit et al., 2006; Aboge et al., 2007). Thus, we propose BQP35 is a suitable candidate of diagnostic antigen for detecting the infection of *Babesia* sp. BQ1 (Lintan) in sheep. A prokaryotic expression system was employed to in vitro express BQP35 in *E. coli* and rBQP35 was identified with SDS-PAGE and western blot. Results showed that an unexpected "gel shifting" present on SDS-PAGE. An about 50kDa molecular mass showed on the SDS-PAGE instead of predicted 39.3 kDa but contrastly positive control protein for expression, ß-galactosidase with expected molecular weight 121 kDa migrated on the gel as usual. Similarly, a western blot signal also revealed by positive sera from *Babesia* sp. BQ1 (Lintan) infected sheep in the 50 kDa place. Therefore, it stands to reason that we resulted in an incorrect insert during construction of recombinant expression vector. However, PCR and sequencing results showed BQP35 gene segment was right inserted into the clone site of pET200/D-TOPO® (data not shown) and rBQP35 could be specially recognised by sera from *Babesia* sp. BQ1 (Lintan) infected sheep, which imply that

BQP35 should be correctedly expressed in vitro.

The anomalous migration on SDS-PAGE has been well demonstrated for lots of either natural or recombinant protein and termed as “gel shifting”. The early researchs showed that the phenomenon resulted from the post-translational modifications of proteins, e.g. phosphorylation, glycosylation (Leach, et al. 1980; Macfarlane et al. 1999; Kishi et al. 2003). However, that is not the whole things of the real facts based on some of recent evidence. These post-translational modifications are very unlikely to occur on an eukaryotic protein in these prokaryotic systems but some of recombinant proteins still showed the unusual electrophoretic behavior on SDS-PAGE (Carlotti et al., 2008; He et al., 2004; Nakajima et al., 2007). The slower migration behavior of some recombinant proteins expressed in eukaryotic cell was proved independent on the post-translational modifications (Vlves et al. (2004, 2005) and García-Ortega et al. (2005)). Their experimental data indicated that the protein structure and composition, charged number and bias of amino acide should be responsible for the abnormal behavior of proteins on SDS-PAGE. Rath et al. (2009) conclued that the anomalous SDS-PAGE migration of membrane protein is due to detergent SDS binding to protein. All the above listed evidences implied that the anomalous migration of proteins on SDS-PAGE, “gel shifting”, should be triggered by multiple factors.

Since 1989, the studies about “intrinsically unstructured proteins, IUPs” or “natively unfolded proteins” proved that these extremely flexible, essentially extended proteins having little or no ordered secondary structure under physiological condition are up to 30% of total proteins in eukaryotic cell and have activities and functions in vivo. The lack of structure is due to strong amino acid compositional bias in IUPs, e.g. rich in hydrophilic and charged residues but often lack bulky hydrophobic residues necessary for a stable 3-dimensional fold (Tompa 2002; Uversky 2002; Dunker et al. 2001). Alves et al. (2004), García-Ortega et al. (2005) and Kang et al. (2008) showed that recombinant IUPs expressed both in prokaryotic and eukaryotic cells expressed slower migration rate on SDS-PAGE due to the compositional bias of acidic and hydrophobic amino acid. Tompa (2002) and Csizmok et al (2006) considered the feature of anomalous migration on SDS-PAGE as an index for identifying IUPs. In our studies, rBQP35 also showed this feature on SDS-PAGE. For verifying the result, the structure of BQP35 was predicted *in silico* with three predictors of IUPs, FoldIndex, DisEMBL^{TM} and MeDorV1.4, and homologues was then explored for rBQP35 in IUPs database DisProt. As expected, these programs reveals that BQP35 is a novel member of IUP family. Composition of amino acid sequence of BQP35 showed that BQP35 have higher levels of certain amino acids (R S and P), lower levels of others (W and C), higher levels charged and lower levels of hydrophobic amino acids that is the favor of IUPs (Tompa 2002; Uversky 2002; Dunker et al. 2001). These compositonal bias of charged and hydrophobic amino acids just explains the anomalous migration of rBQP35 on SDS-PAGE. At the same time, the structure of ß-galactosidase that is the positive control used during expression of

BQP35 was also analysed with FoldIdex and the result showed that is total a folded protein, which is coicident with its feature on SDS-PAGE. The likely homologue of BQP35, *B. bovis* hypothetical protein (A7ASD6) were recovered through sequencing whole genome of *B. bovis* T2Bo and its structure and function were never explored. In our study, through analysis of disorder, we found the *B. bovis* hypothetical protein (A7ASD6) is also an IUP protein. We therefore considered *B. bovis* hypothetical protein (A7ASD6) and BQP35 should belong to homologous IUP protein in *Babesia* that so far we unrecognized.

As our previous purpose of this study, the potentiality of BQP35 as a candidate of diagnostic antigen was evaluated with sera from several piroplasms and *A. ovis* each infected sheep in western blot and ELISA. Results indicated that rBQP35 had strong reaction with positive sera of *Babesia* sp. BQ1 (Lintan) in western blot. Cross-reaction test showed that there were almost not reactivities between rBQP35 and sera from other four chinese ovine *Babesia* isolates, two *Theileria* species and one *Anaplasma* species infected sheep in western blot. Comparison of rBQP35 reactivities as antigen in ELISA to sera both from *Babesia* sp. BQ1 (Lintan) infected sheep and from other ovine haemoparasites infected sheep indicated that rBQP35 could specifically detect infection of *Babesia* sp. BQ1 (Lintan). Therefore, BQP35 could be considered as a good candidate of antigen for detection of *Babesia* sp. BQ1 (Lintan) infection using in sero-diagnosis. In conclusion, we firstly obtain an IUP protein from *Babesia* spp. and prelimilary understand it is a potentially diagnostic antigen for detecting *Babesia* sp. BQ1 (Lintan) infection although the application as diagnostic antigen, biochemical characters, and functions still need to reveal in future studies.

Conflict of interest

None declared

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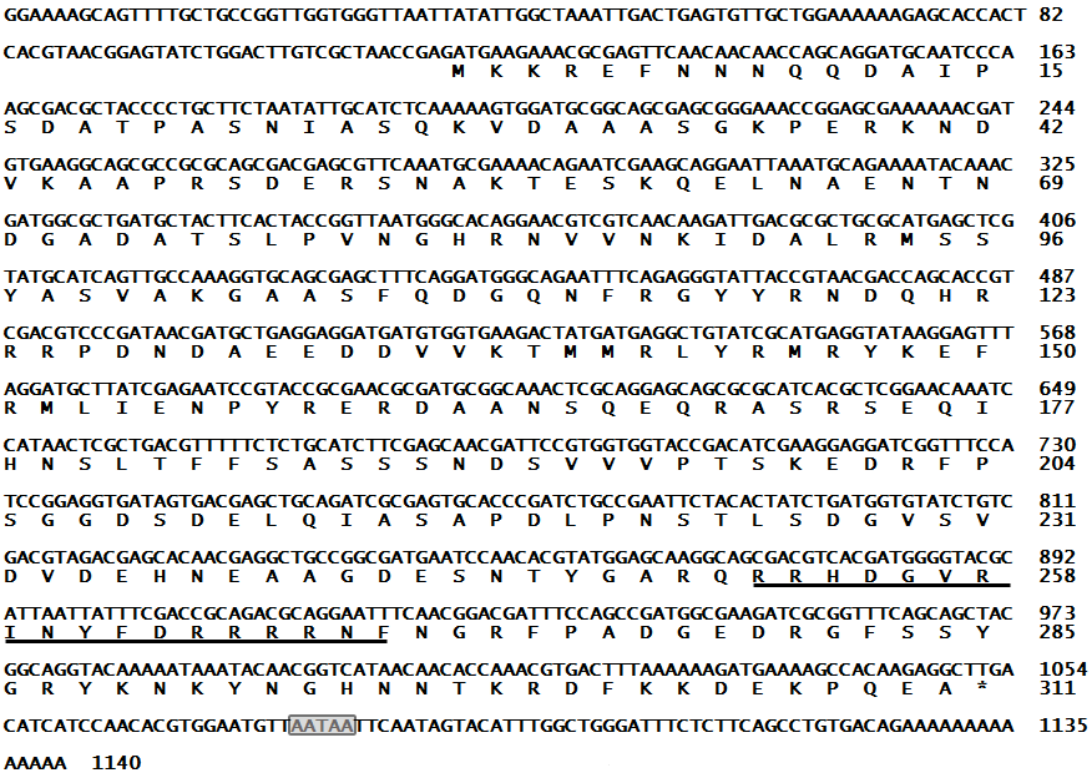

Figure 1. The full-length nucleotide sequence, including the coding region and flanking sequences of BQP35 cDNA. The predicted amino acid translated from ORF is indicated below each codon. The underlined amino acids at COOH-terminal show the nuclear localization signals and bipartite. The framed polynucleotide AATAA is the polyadenylation signal of BQP35 and the asterisk represents the stop codon

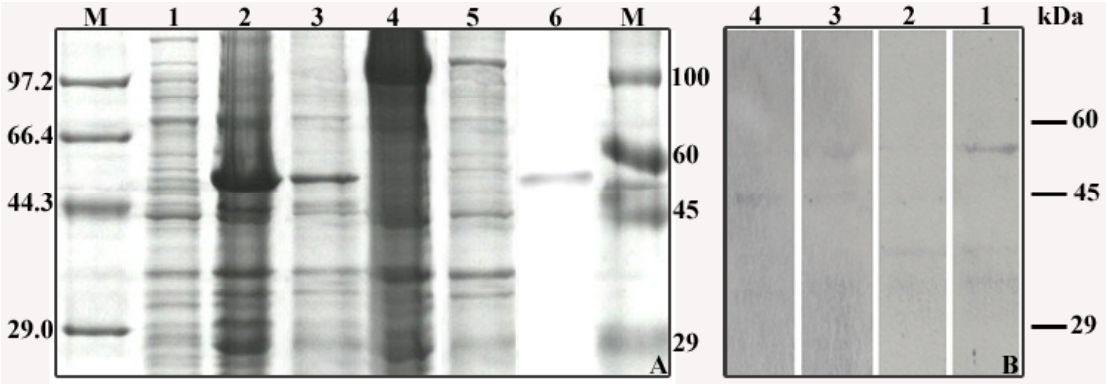

Figure 2. SDS-PAGE (A) and western blot (B) of His6-BQP35. A: The left and right M represent two different protein marker (kDa) and the molecular mass standards are indicated in sides (in kilodaltons). Lane 1, the recombinant of pET200-BQP35 before induction. Lane 2, 3 and 6, lysates of recombinant BL21, supernants of the lysates and purified His6BQP35, respectively. Lane 4 and 5, positive control pET200-_-galactosidase each in lysates and supernants of lysates; B: Molecular mass standards are indicated on the right (kDa). Lane 1 and 2, reaction of purified His6-BQP35 with each *Babesia* sp. BQ1 (Lintan) positive sera and negative sera. Lane 3 and 4, supernants of pET200-_-galactosidase with each *Babesia* sp. BQ1 (Lintan) positive sera and negative sera.

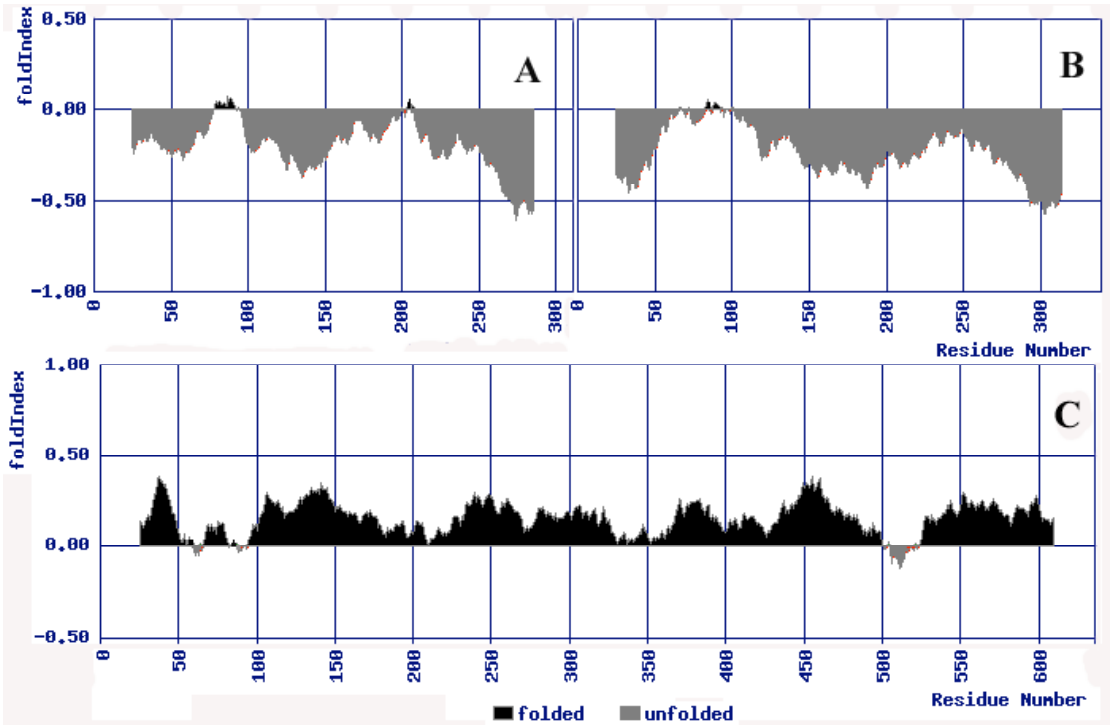

Figure 3. Disordered regions of BQP35 (A), *B. bovis* hypothetical protein (accession number: A7ASD6) (B) and _-galactosidase (accession number: NM_138342) (C) predicted by FoldIndex. The amino acid sequences were submitted to http://bip.weizmann.ac.il/fldbin/findex. The folded and unfolded segments of the proteins as predicted from this program are shown. The unfoldability of BQP35, *B. bovis* hypothetical protein and _-galactosidase is -0.165, -0.260 and 0.203, respectively.

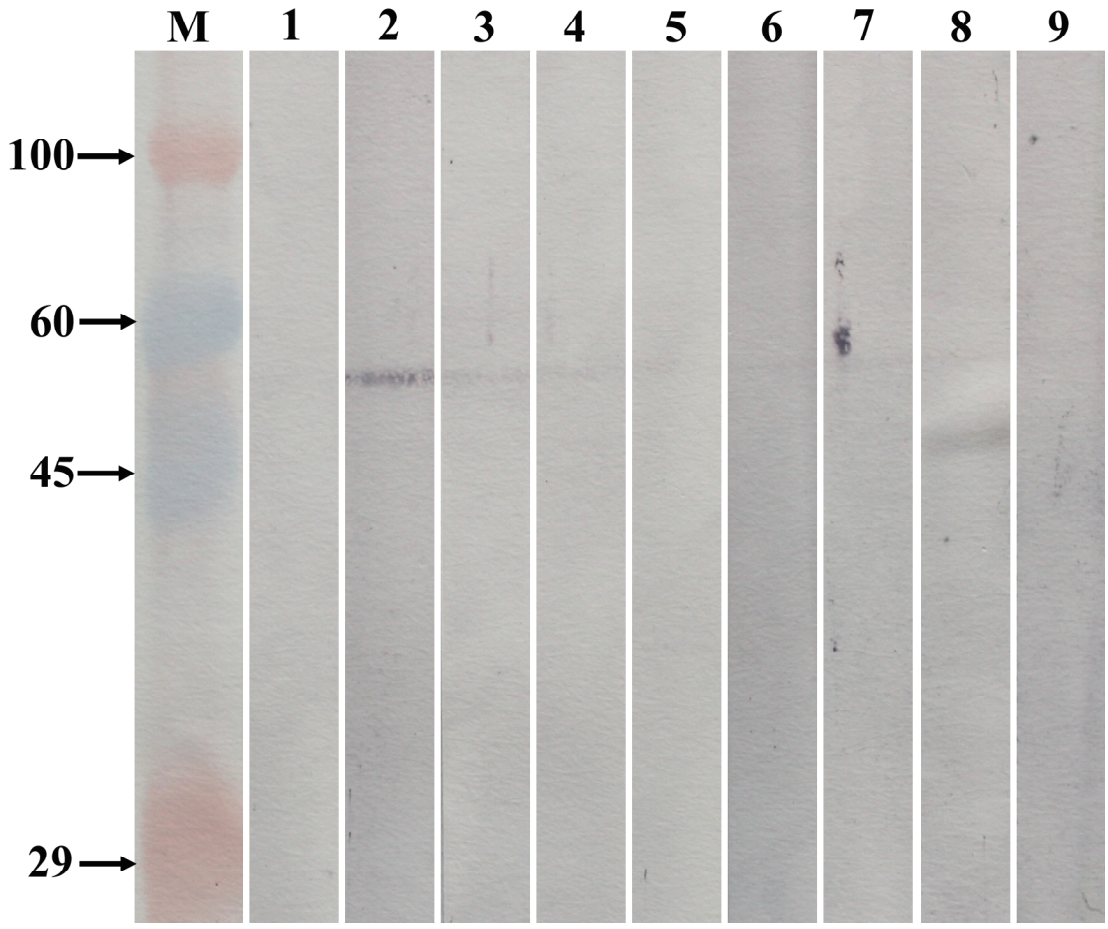

Figure 4. Test of cross-reaction between ovine haemoparasites and rBQP35 with western blot. M, standard molecular weight markers (kDa); lane 1, sheep serum before infection; lane 2-9, sera from *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Hebei, *Babesia* sp. Xinjiang, *Theileria luwenshuni*, *T. uilenbergi* and *Anaplasma ovis* each infected sheep.

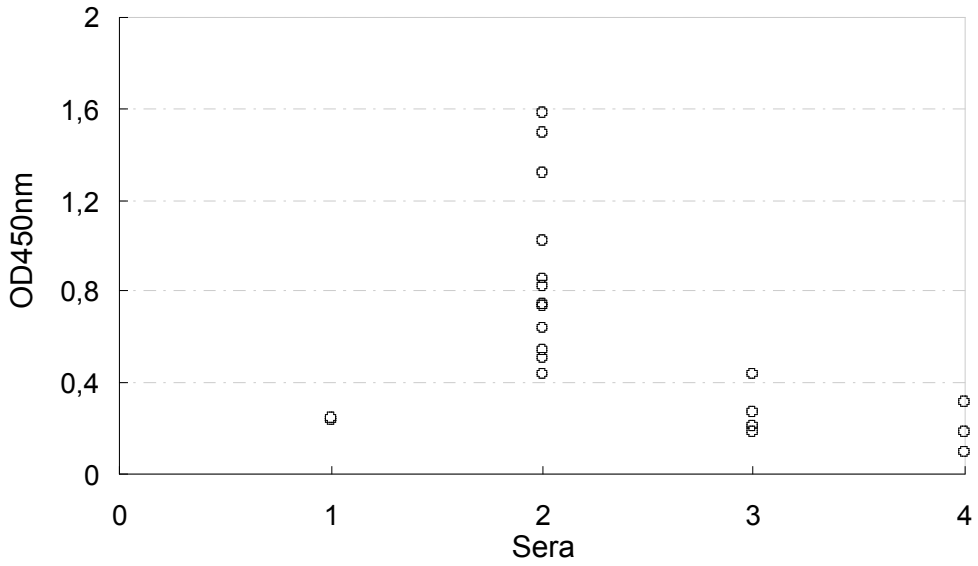

Fig. 5. The cross-reaction of rBQP35 to different sera in ELISA. Circles represent the optical densities at 450nm (OD450nm) of sheep sera before infection (column 1), *Babesia* sp. BQ1 (Lintan) infected sheep sera at different timepoints(column 2), other ovine *Babesia* isolates (*Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Hebei and *Babesia* sp. Xinjiang) infected sheep sera (column 3), *T. luwenshuni*, *T. uilenbergi* and *A. ovis* infected sheep sera (column 4). The significant difference between the mean OD450 standard deviation for sera from *Babesia* sp. BQ1 (Lintan) infected sheep and those of sera from other ovine haemprotozoan infected sheep was observed by Student's t test ($P < 0.01$).

DISCUSSION GENERALE

La piroplasmose est une maladie importante chez les moutons et les chèvres dans le Nord de la Chine. Dans les zones atteintes, le taux d'infection est de 15 à 80 % et la mortalité des agneaux peut atteindre 50 % dans certaines régions (Ren *et al*., 1982; Li *et al*., 1985; Luo *et al*., 1997). L'agent pathogène de cette maladie a d'abord été considéré comme étant une *Theileria* non identifiée puis il a été montré qu'elle était provoquée par *T. luwenshuni* et *T. uilenbergi* transmises par *Haemaphysalis qinghaiensis* (Yin *et al*., 2007). A partir de 1996, a été observée une maladie avec des symptômes de babésiose tels que fièvre, anémie hémolytique, hémoglobinurie et même mort dans des troupeaux de moutons à queue courte "Small-tailed" à l'est de la province du Gansu. Une babésie "grande forme" a été isolée. Depuis, plusieurs souches de grandes babésies ont été isolées de tiques collectées sur le terrain et de sangs de moutons dans de nombreuses aires en Chine. Les piroplasmoses ovines sont donc des maladies largement répandues et causant d'importantes pertes économiques en Chine ; elles apparaissent provoquées par diverses espèces de *Theileria* et de *Babesia* (Lian *et al*., 1997; Yin *et al*., 1997). Cependant, ces parasites ont été peu étudiés. Afin de mieux comprendre ces parasites et de développer des mesures de contrôle, nous avons ciblé notre étude sur 3 points : l'analyse phylogénétique des souches de babésies en Chine, les interactions du protozoaire avec son hôte vertébré et le développement d'outils de diagnostic. Deux souches de parasites ont été plus particulièrement étudiées : *Babesia sp*. BQ1 (Lintan) et *Babesia sp*. Xinjiang.

Cette étude a été réalisées dans 2 laboratoires complémentaires :

1. le laboratoire "Vector and Vector-borne disease" dirigé par le Pr. Hong Yin, étudie depuis de nombreuses années les theilérioses et les babésioses en Chine. De nombreux outils ont été développés afin de caractériser les parasites d'un point de vue biologique, (transmission, infection expérimentale d'animaux et de tiques) et moléculaire ;
2. l'équipe "Interactions Tique-Parasite-Hôte" dirigé par le Pr. Alain Chauvin a une bonne expérience sur la babésiose en France et notamment dans les domaines de l'épidémiologie, de l'interaction avec l'hôte vecteur et l'hôte vertébré et dans le développement d'outils de diagnostic.

1. Classification de *Babesia* spp. des petits ruminants en Chine

Traditionnellement, différents outils sont disponibles pour classer les babésies : les outils biologiques tels que la morphologie intra-érythrocytaire, les tiques vectrices et la virulence du parasite, et des outils moléculaires tels que la similarité de gènes ou de protéines.

B. ovis, B. motasi et *B. crassa* sont considérés comme des espèces valides sur la base de leurs caractéristiques biologiques et moléculaires (Hashemi-Fesharki et Uilenberg, 1981 ; Papadopoulos *et al*., 1996 ; Freidhoff, 1997 ; Schnittger *et al*., 2003 ; Uilenberg, 2006). Sept souches de babésies ovines ont été isolées en Chine : *Babesia sp*. BQ1 (Ningxian), *Babesia*

sp. BQ1 (Lintan), *Babesia sp.* Xinjiang, *Babesia sp*. Madang, *Babesia sp*. Tianzhu, *Babesia sp*. Hebei et *Babesia sp*. Liaoning (Lian *et al*., 1997 ; Yin *et al*., 1997 ; Guan *et al*., 2001, 2002 ; Liu AH *et al*., 2007). La classification des ces isolats n'est pas claire du fait du manque de données biologiques et moléculaires. Notre étude s'est focalisée sur la classification de *Babesia sp*. BQ1 (Lintan) et *Babesia sp*. Xinjiang.

Liu AH *et al*. (2007) et Niu *et al*. (2009) ont analysé la phylogénie de babésies chinoises grâce aux gènes ARNr 18S et ITS. Les résultats indiquent qu'elles peuvent être divisée en 2 groupes au moins, le groupe *Babesia sp*. Xinjiang et le groupe *B. motasi*-like. La conclusion commune de ces chercheurs est qu'il y a en Chine au moins 2 espèces de *Babesia* : *Babesia sp*. Xinjiang et *B. motasi*. Les protéines Hsp90 sont prometteuses en tant que marqueurs phylogénétiques universels pour les analyses phylogéniques du fait de leur forte conservation. De plus, la cible représentée par un gène évoluant lentement est appropriée pour étudier les relations anciennes plutôt que pour différencier les souches et les sous-espèces récentes (Barta, 1997 ; Young *et al*., 2001). Nous avons amplifié le gène Hsp90 de différents isolats de babésies chinoises, *Babesia sp*. BQ1 (Lintan), *Babesia sp*. BQ1 (Ningxian), *Babesia sp*. Tianzhu, *Babesia sp*. Hebei et *Babesia sp.* Xinjiang. L'analyse phylogénétique basée sur les séquences en nucléotides et en acides aminés coïncide avec celle basée sur la séquence des gènes ARNr 18S et ITS : il y a 2 groupes d'espèces, le groupe *Babesia sp*. Xinjiang et le groupe *B. motasi*-like. De plus, le groupe *B. motasi*-like peut être divisé en 2 sous-groupes, un incluant *Babesia sp*. BQ1 (Lintan) et *Babesia sp.* Tianzhu et l'autre *Babesia sp*. BQ1 (Ningxian) et *Babesia sp*. Hebei. Ces résultats sont partiellement confirmés par l'analyse des réactions croisées par sérologie. Par ELISA, les antigènes BQMA de *Babesia* sp. BQ1 (Lintan) ne sont pas reconnus par les sérums de moutons infectés par *Babesia sp*. BQ1 (Ningxian), *Babesia sp*. Hebei ou *Babesia sp*. Xinjiang. En revanche, une importante réaction croisée entre *Babesia sp.* BQ1 (Lintan) et *Babesia sp*. Tianzhu est mise en évidence.

De plus, nous avons caractérisé la morphologie de *Babesia sp*. BQ1 (Lintan) et *Babesia sp*. Xinjiang, ce qui révèle que cette dernière a une morphologie totalement différente des autres babésies ovines. Sa taille moyenne est de 2,42 (±0,35) µm x 1,06 (±0,22) et et sa forme typique est une forme géminée à deux mérozoïtes piriformes fins et pointus alors que pour les souches *B. motasi*-like les mérozoïtes piriformes sont épais avec une pointe émoussée. *Babesia sp*. Xinjiang est donc facile à distinguer de *B. ovis* (*Babesia* "petit forme"), de *B. motasi* (piroplasme épais) et de *B. crassa* (nombreuses formes à 4 mérozoïtes par érythrocyte) (Hashemi-Fesharki et Uilenberg, 1981 ; Lewis *et al*., 1981b ; Kuttler, 1988b). En revanche, il est difficile de différencier *Babesia sp*. BQ1 (Lintan) des autres souches de *B. motasi*-like et ses caractères morphologiques, similaires à ceux de *B. motasi* (longueur de 2,23 ± 0,2 µm ; Kuttler, 1988b) sont très variés, incluant des formes anaplasmoïdes, des formes rondes, des éléments piriformes simples, des formes de divisions, des formes géminées à deux mérozoïtes piriformes, mais aussi des formes tubulaires, des formes irrégulières ainsi que des hématies

multiparasités (figure 9). Ces aspects morphologiques sont aussi similaires aux formes observées pour *Babesia* sp. BQ1 (Ningxian) (figure 10). La taille des piroplasmes est également similaire entre *Babesia* sp. BQ1 (Lintan) (1,8-2,1 µm x 0,9-1,7 µm) et *Babesia* sp. BQ1 (Ningxian) (1,8-2,5 µm x 0,9-1,8 µm). Pour les autres souches, les caractères morphologiques ne sont pas décrits.

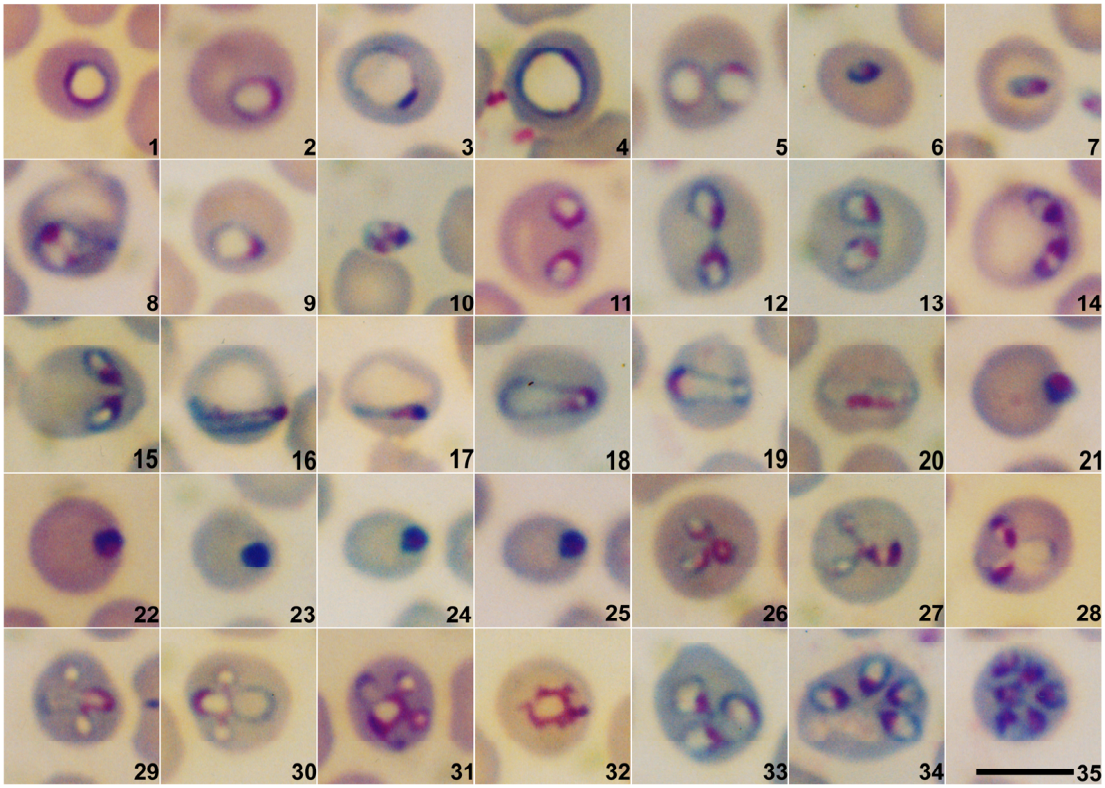

Figure 9 : formes morphologiques de *Babesia* sp. BQ1 (Lintan) dans les érythrocytes. Barre = 5 µm. 1-4 : forme ronde ; 5: double forme ronde ; 6-10: élément piriforme simple ; 11-15 : forme géminée à deux mérozoïtes piriformes ; 16-20 : forme tubulaire ; 21-25 : forme anaplasmoïde ; 26-28 : formes de division ; 29-31 : formes de division ; 32: formes irrégulières bourgeonnantes ; 33-35 : hématies multiparasitées.

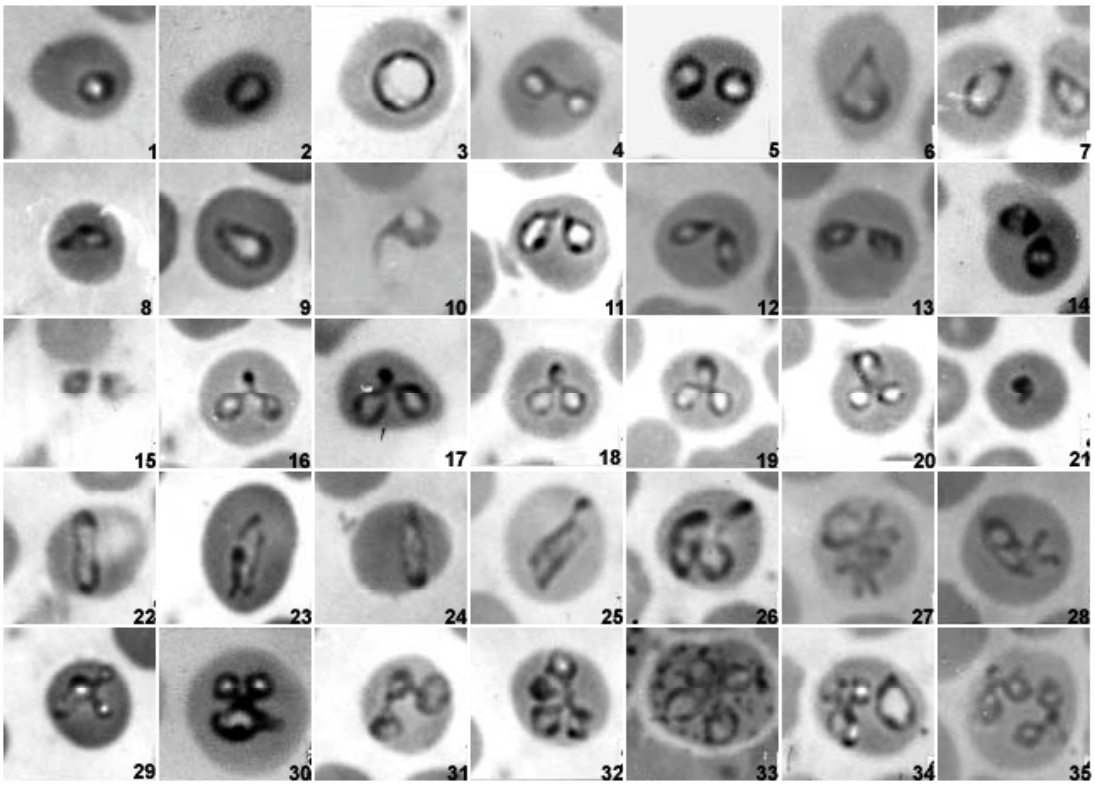

Figure 10 : formes morphologiques de *Babesia* sp. BQ1 (Ningxian) dans les érythrocytes. 1-3 : forme ronde ; 4-5: double forme ronde ; 6-10: élément piriforme simple ; 11-15 : forme géminée à deux mérozoïtes piriformes ; 16-20 : formes de division ; 21 : forme anaplasmoïde ; 22-25 : forme tubulaire ; 26-27 : formes irrégulières bourgeonnantes ; 28-32 formes de division ; 33-35 : hématies multiparasitées. (d'après Bai et al., 2002)

Parmi les espèces de babésies infectant les petits Ruminants, seul *B. ovis* transmis par *R. bursa*, a une virulence élevée pour les moutons et les chèvres. *B. crassa*, dont le vecteur est inconnu, est une espèce non pathogène. La pathogénicité de *B. motasi* est variable selon la zone géographique, les souches de l'Europe du Nord ayant une faible virulence, contrairement aux souches de l'Europe du sud (Friedhoff, 1997 ; Uilenberg, 2006). *H. punctata* est la seule tique vectrice communément reconnue, bien que des données montrent que *R. bursa* peut également transmettre *B. motasi*. En ce qui concerne les souches chinoises, la virulence et les tiques vectrices été étudiées uniquement pour *Babesia sp*. (Ningxian) (Bai *et al*., 2002). Nous les avons caractérisés pour *Babesia sp*. BQ1 (Lintan) et *Babesia sp*. Xinjiang dans notre étude (tableau 3).

Tableau 4. Caractéristiques biologiques des souches de *Babesia* spp. infectant les petits Ruminants en Chine.

Isolats	Tique vectrice	Morphologie	Virulence	Phylogénie
Babesia sp. BQ1 (Lintan)	***H. qinghaiensis*** ***H. longicornis***	Similaire à *B. motasi*	**Faible**	**Groupe *B. motasi*-like, sous-groupe de faible virulence**
Babesia sp. BQ1 (Ningxian)	*H. longicornis*	Similaire à *B. motasi*	Forte	**Groupe *B. motasi*-like, sous-groupe de forte virulence**
Babesia sp. Hebei	Non identifié	Similaire à *B. motasi*	Non connu	**Groupe *B. motasi*-like, sous-groupe de forte virulence ?**
Babesia sp. Tianzhu	Non identifié	Similaire à *B. motasi*	Non connu	**Groupe *B. motasi*-like, sous-groupe de faible virulence ?**
Babesia sp. Madang	Non identifié	Similaire à *B. motasi*	Non connu	Groupe *B. motasi*-like
Babesia sp. Liaoning	Non identifié	Similaire à *B. motasi*	Non connu	Groupe *B. motasi*-like
Babesia sp. Xinjiang	***Hy. a. anatolicum***	**Different des autres *Babesia* ovine**	**Faible**	Groupe *Babesi*a sp. Xinjiang (nouvelle espèce)

En gras apparaît les caractères mis en évidence lors de notre étude

***Babesia* sp. Xinjiang** a une faible virulence chez les moutons non splénectomisés. Aucun signe clinique ni parasite sur les étalements sanguins réalisés à partir de sang capillaire prélevé à l'oreille ne sont observés mais la détection moléculaire par la technique LAMP est positive. En revanche les moutons splenectomisés infectés expriment des symptômes de babésiose (réponse fébrile mais pas d'hémoglobinurie ni d'ictère) et une parasitémie détectable sur étalement ; ils peuvent mourir avec une parasitémie de 5 % s'ils sont immunodéprimés par injection de déxaméthasone . Nous avons également identifié le vecteur qui est *Hyalomma anatolicum anatolicum*. Une transmission transovarienne et transtadiale est observée et tous les stades larve, nymphe et adulte peuvent transmettre le parasite. ***Babesia* sp. BQ1 (Lintan)** peut être présent de façon irrégulière sur les étalement sanguins réalisés à partir de sang capillaire prélevé à l'oreille de moutons non splénectomisés infectés expérimentalement mais jamais sur ceux réalisés à partir de sang prélevé à la veine jugulaire. Une légère hyperthermie est mise en évidence chez ces animaux mais sans signe clinique ni modification de l'hématocrite. Chez les moutons ou les chèvres splénectomisés, une augmentation de la température rectale supérieure à 41°C, une diminution du taux d'hémoglobine et du nombre de globules rouges sont observés. La parasitémie peut atteindre 80 % si les animaux splénectomisés sont immunodéprimés par injection de déxaméthasone (Guan *et al*. 2002). Ces données suggèrent que *Babesia sp*. BQ1 (Lintan) a une faible virulence pour les petits Ruminants. La mise en évidence expérimentale de la tique vectrice de cet isolat n'est pas aisée. Des 1ères expériences n'avaient pas permis de révéler la présence de parasite par examen microscopique de sang de moutons infestés par *H. qinghaiensis* infectés par *Babesia* sp. BQ1 (Lintan). Les outils moléculaires (PCR nichée) nous ont permis de montrer que *H. qinghaiensis* et *H. longicornis* peuvent être les vecteurs de cet isolat mais les capacités de transmission ne semblent pas être importantes. Ces résultats sont des arguments supplémentaires pour classer *Babesia sp*. BQ1 (Lintan) et *Babesia sp*. Ningxian dans le groupe des *B. motasi*-like puisque ces 2 souches sont transmises par *Haemaphysalis* spp. (*Babesia sp*. Ningxian est transmise par *H. longicornis* (Lian *et al*, 1997 ; Yin *et al*., 1997 ; Bai *et al*., 2002)). Cependant, le seul vecteur correctement identifié de *B. motasi* est *H. punctata* (Friedhoff, 1997 ; Uilenberg, 2006). Il serait donc nécessaire de savoir si *H. punctata* est capable de transmettre ces babésies chinoises. En revanche, du point de vue de leur virulence, elles se comportent différemment puisque *Babesia sp*. BQ1 (Ningxian) a une forte virulence, même pour les animaux non splénectomisés (Lian *et al*, 1997 ; Yin *et al*., 1997 ; Bai *et al*., 2002).

En conclusion, la classification de *Babesia* spp infectant les petits Ruminants en Chine a été clarifiée par notre étude. *Babesia* sp. Xinjiang semble être une nouvelle espèce de part ses caractères biologiques et moléculaires. Dans le groupe *B. motasi*-like, il est possible que, comme pour *B. motasi* en Europe (Uilenberg, 2006), il y ait au moins 2 espèces ou sous –espèces, une de faible virulence incluant *Babesia* sp. BQ1 (Lintan) et *Babesia* sp. Tianzhu, une de forte virulence incluant *Babesia* sp. BQ1 (Ningxian) et *Babesia* sp. Hebei.

2. Interaction de *Babesia sp.* BQ1 (Lintan) avec son hôte vertébré

Babesia spp. est un parasite strict des érythrocytes de leur hôte vertébré ; ils s'y multiplient de façon asexuée. Durant cette étape de leur cycle parasitaire, les mérozoïtes extracellulaires envahissent les érythrocytes de l'hôte, se développent en trophozoïtes puis se divisent en deux mérozoïtes qui sortent alors des globules rouges par rupture de la membrane cellulaire et les mérozoïtes "fils" envahissent de nouvelles cellules. La phase intra-érythrocytaire est responsable des signes cliniques de la babésiose tels que l'hyperthermie, l'anémie, l'ictère et l'hémoglobinurie (Ristic, 1988) et bloquer ou limiter la multiplication asexuée du protozoaire est une stratégie permettant de limiter les effets pathologiques de l'infection. Pour cet objectif, les mécanismes d'interactions entre les babésies et leur hôte doivent être mieux connus, notamment la réceptivité des animaux, les différences entre race ou individu, les caractères structuraux de la surface des cellules de l'hôte et la réponse immunitaire du vertébré dirigée contre le parasite. Dans notre étude, nous avons abordé cette question des interactions *Babesia* spp. - hôte vertébre en comparant les interactions de *Babesia sp*. BQ1 (Lintan) et *B. divergens* avec le mouton. En effet, le mouton est un hôte naturel *Babesia sp.* BQ1 (Lintan). En revanche l'infection naturelle de mouton par *B. divergens* n'a jamais été décrite mais il peut en être l'hôte expérimental (Chauvin et al., 2002).

L'étude de la résistance génétique de l'hôte contre les infections à *Plasmodium* spp. a montré que certains loci tels que *char*2 et *char*4 jouent un rôle important dans la résistance et la sensibilté des souches de souris (Foote *et al*., 1997 ; Fortin *et al*., 2001 ; Burt *et al*., 2002). Bien que les travaux sur la résistance génétique des bovins à *B. bovis* soient moins développés que ceux sur *Plasmodium* spp., les 1er résultats montrent que *Bos indicus* est plus résistant que *Bos taurus* (Bock *et al*., 1997, 1999 ; Jonsson *et al*., 2008) et qu'au sein de l'espèce *Bos taurus*, des variations individuelles de résistance sont observées. Peu de données sont disponibles concernant la résistance génétique des moutons à *Babesia* spp. Malandrin *et al*. (2009) et Moreau *et al*. (2009) ont démontré que *B. divergens* peut expérimentalement infecter des moutons non splénectomisés si leurs érythrocytes sont sensibles *in vitro* c'est à dire s'ils sont capables de maintenir la multiplication du parasite *in vitro*. Ceci suggère que la caractérisation de la sensibilité *in vitro* des globules rouges à *Babesia* spp. est un moyen d'évaluer la sensibilité des animaux *in vivo*. Dans notre étude, le test de mesure de la sensibilité des érythrocytes *in vitro* a été utilisé pour évaluer la

résistance génétique de différentes races de moutons (mouton Vendéen = mouton français et mouton Tan = mouton chinois). Les érythrocytes de moutons chinois semblent plus sensibles à *Babesia sp*. BQ1 (Lintan) (67 % de sensibles) et à *B. divergens* (58 % de sensibles) que ceux de moutons français (32 et 5 % respectivement). Ainsi, de façon similaire à ce qui a été décrit chez les bovins et *B. bovis*, le génotype des moutons joue un rôle important dans leur sensibilité à *Babesia* spp. De plus, ces résultats pourraient expliquer en partie la spécificité d'hôte des babésies : les moutons pourraient être un hôte naturel de *Babesia sp*. BQ1 (Lintan) puisque 67 % des moutons chinois ont des globules rouges sensibles alors que pour *B. divergens*, bien que ce parasite puisse infecter les moutons expériementalement, le faible pourcentage (5 %) de moutons Vendéens ayant des érythrocytes sensibles pourraient expliquer que le mouton n'est pas un hôte naturel de *B. divergens*. De plus, une variation individuelle de la sensibilité des érythrocytes de moutons à *Babesia* spp est mise en évidence, ce qui coincide avec les résultats de Benavides et Sacco (2007) obtenus à partir de troupeaux de bovins infectés par *B. bovis*. La résistance naturelle des mammifères à *Babesia* spp varie non seulement entre les espèces et les races animales mais aussi entre les individus. Les mécanismes et les molécules associés à cette résistance génétique restent encore à explorer.

Notre étude a également montré que les globules rouges d'un même individu peuvent exprimer différentes sensibilités à *B. divergens* et à *Babesia sp*. BQ1 (Lintan). D'après les recherches sur *Plasmodium*, la base moléculaire de l'interaction sélective ligand – récepteur impliquée dans la liaison à la cellule et dans l'invasion peut expliquer en partie cette différence de sensibilité (Gaur *et al*., 2004; Baum *et al*., 2005). Alkhalil et al. (2006) rapportent que *Plasmodium falciparum* et *B. divergens*, 2 apicomplexes parasites des érythrocytes, utilisent différents mécanismes pour augmenter la perméabilité des érythrocytes humaines infectés. Les mécanismes moléculaires impliqués dans l'adhésion, l'invasion et le développement du parasite dans les globules rouges pourraient donc avoir un rôle important dans la sensibilité de l'hôte à *Babesia* spp. Les interactions moléculaires entre les babésies et leur hôtes ne sont pas, à ce jour, entièrement comprises mais récemment, les interactions entre les ligands des *Babesia* et les récepteurs des globules rouges de l'hôte ont été discutées. Plusieurs ligands clés pour les globules rouges, présents à la surface des mérozoïtes, ont été décrits : la famille des VMSA (variable merozoite surface antigens) et notamment MSA-1, MSA-2a_1, -2a_2, -2b et 2c, RAP-1 (rhoptry associated protein-1), l'homologue d'AMA-1 (apical membrane antigen-1), l'homologue de TRAP (thrombospondin-related anonymous protein), les protéines SBP-1, -2 et –3 (spherical body protein) (Yokoyama *et al*., 2006). Trois récepteurs érythrocytaires pour les babésies ont été actuellement identifiés : les résidus à acide sialique, les protéines sensibles à la trypsine ou à l'alpha-chymotrypsine et le glycosaminoglycan sulphaté (Yokoyama *et al*., 2006). Kania et al. (1995) ont montré que le pré-traitement des globules rouges de bovins avec la trypsine pouvait diminuer l'invasion des érythrocytes par *B. bigemina* mais que l'alpha-chymotrypsine a peu d'effets. Au contraire, le traitement à l'alpha-chymotrypsine pourrait affecter l'invasion des globules rouges par *B. divergens* mais pas celui

à la trypsine. Ces résultats indiquent que dans le processus d'invasion des érythrocytes, les différentes espèces de babésies pourraient utiliser différents récepteurs d'hôtes, ligands parasitaires et mécanismes, expliquant les différences de sensibilité observés dans notre étude. Le rôle précis de ces molécules dans le processus d'invasion n'est cependant pas encore déterminé. Des études complémentaires sont donc nécessaires pour comprendre au niveau moléculaire les mécanismes de la spécificité d'hôtes *de Babesia* spp.

Chez les animaux sensibles, la réponse immunitaire des moutons pourraient expliquer les différences de virulence entre *Babesia sp*. BQ1 (Lintan) et *B. divergens* que nous avons observées. Notre étude confirme les travaux précédents réalisés sur *B. bovis* (Brown *et al*., 2006b), *B. divergens* (Moreau *et al*., 2009) ou *Plasmodium* spp (Stanisic *et al*., 2009) montrant que les anticorps ne sont pas impliqués dans l'élimination du parasite mais sont associées à la protection contre des fortes parasitémies et la maladie. Ainsi, la cinétique de la production d'anticorps chez les moutons infectés par *Babesia sp*. BQ1 (Lintan) est similaire à celle observée chez les animaux infectés par les autres babésies ovines (*B. ovis, B. motasi, B. crassa*) (Uilenberg *et al*., 1980 ; Christensson et Thunegard, 1981 ; Hashemi-Fesharki et Uilenberg, 1981 ; Habela *et al*. 1990) et lorsque la réponse humorale atteint son maximum, aucun parasite ne peut être détecté sur les étalements sanguins réalisés avec le sang capillaire prélevé à l'oreille. En revanche, nous n'avons pas mis en évidence de relation entre la réponse humorale des animaux et la virulence des parasites. Au contraire, la réponse cellulaire et notamment l'IFNγ et l'IL10 ont un rôle important dans l'évolution de l'infection à *Babesia sp*. BQ1 (Lintan) et à *B. divergens* chez le mouton : l'IFNγ, à la différence de l'IL10, semble être associé à une protection contre le parasite et à une virulence faible. Ceci est en accord avec les mécanismes immunitaires décrits pour *B. bovis* (Goff *et al*., 2001 ; Brown *et al*., 2006b). Les réponses cellulaires protectrices des bovins contre *B. bovis* impliquent des mécanismes faisant intervenir les phagocytes mononucléés, régulés par la réponse de type 1 : l'IFNγ induit la production de NO par les macrophages et la production d'IgG2 opsonisantes. L'induction de la production d'IL10 dans la rate est suivie par la réduction de l'expression d'IL12, d'IFNγ et de TNFα. De plus, la production d'IL10 est associée à la plus forte sensibilité des bovins adultes à *B. bovis* comparé à celle des veaux.

En bilan, une résistance génétique des hôtes à *Babesia sp*. BQ1 (Lintan) et à *B. divergens* a été décrite. La sensibilité des animaux est en partie liée à la capacité de leurs hématies à être envahie et à multiplier le parasite *in vitro*. Les mécanismes moléculaires impliqués restent toutefois à expliquer. Chez les animaux sensibles, la réponse immunitaire, et plus particulièrement la réponse immunitaire cellulaire, a un rôle clé dans le pouvoir pathogène de *Babesia sp*. BQ1 (Lintan) et de *B. divergens* : l'IFNγ a un effet protecteur contrairement à l'IL10. Des travaux sont maintenant nécessaires pour explorer les mécanismes régulateurs de la production de ces cytokines et les molécules parasitaires impliquées

3. Développement d'outils de diagnostic

En Chine, la seule technique disponible pour le diagnostic de la babésiose ovine est l'examen microscopique d'étalements sanguins réalisés avec le sang capillaire prélevé à l'oreille. Cet outil a ses avantages tels que la facilité de réalisation avec un équipement peu coûteux et la visualisation de la forme parasitaire. En revanche, ses défauts ne sont pas à négliger : la sensibilité est faible, des techniciens expérimentés sont nécessaires pour reconnaître les caractères morphologiques des babésies et il est difficile de différencier les différentes espèces morphologiquement. Nous avons développé au cours de notre étude différentes techniques pour détecter les infections par *Babesia* spp infectant les petits Ruminants en Chine**.**

La culture *in vitro* de *B. bovis* a été mise au point pour la 1ère fois par Levy et Ristic (1980) et depuis, elle a été fréquemment utilisée pour multiplier *in vitro* différentes espèces dans le but de diagnostiquer une infection ou d'isoler le parasite. Nous avons, au cours de ce travail, développé un système de culture *in vitro* de *Babesia sp.* BQ1 (Lintan) qui a été utilisé pour détecter les infections par ce parasite. La sensibilité est similaire à celle décrite par Malandrin et al. (2004a) pour *B. divergens* : 1 globule rouge parasité dans 10 µl de globules rouges ce qui correspond à une parasitémie de 10^{-9} (1 globule rouge parasité pour 10^{9} globules rouges). Le temps de génération de *Babesia sp.* BQ1 (Lintan) est plus long que celui de *B. divergens* (une vingtaine d'heures pour *Babesia sp*. BQ1 (Lintan) contre 8 h pour *B. divergens* (Malandrin *et al*., 2004b), ce qui entraîne un délai souvent long pour obtenir les résultats. Le parasite peut être détecté au cours des phases aiguës et chroniques de l'infection. Cependant, cette technique est consommatrice en temps et peu d'échantillons peuvent être traités en même temps. Elle n'est donc pas adaptée pour le diagnostic des infections cliniques qui nécessitent une réponse rapide ou pour des études épidémiologiques. Cependant, c'est la méthode de choix pour isoler le parasite vivant.

La détection du génome de *Babesia* spp. est considérée comme une approche prometteuse pour identifier l'infection à la fois chez l'hôte vertébré et chez le vecteur car c'est une technique rapide, sensible et spécifique. Actuellement, les techniques développées et utilisées sont les sondes d'ADN, la PCR et le RLB (Böse *et al*., 1995; Gubbels *et al.*, 1999) mais elles ont pour inconvénients notamment leur complexité et leur nécessité de matériels couteux. Dans cette étude nous avons développé **la technique LAMP** pour détecter *Babesia* sp. BQ1 (Lintan) et *Babesia* sp. Xinjiang, sur la base du gène ARNr 18S. En effet, la LAMP est une technique qui permet d'amplifier dans des conditions isothermiques, de façon sensible et rapidement l'ADN et la révélation de l'amplification est facile, par observation de la turbidité ou de la fluorescence dans le milieu de réaction (Notomi *et al*., 2000). Cette méthode permet de discriminer les infections à *Babesia sp*. BQ1 (Lintan) des infections à *Babesia* sp. Xinjiang. La sensibilité permet de mettre en évidence 0,02 à 0,2 pg d'ADN génomique, ce qui

correspond à environ 50 µl de globules rouges avec une parasitémie de 10^{-8} à 10^{-7} (Mclaughlin *et al*., 1986) et est meilleure que la PCR nichée (2 pg d'ADN) (Sun *et al*., 2008). Aucune réaction croisée n'est observée avec *T. luwenshuni* et *T. uilenbergi*. La LAMP permet de détecter, chez des animaux non splénectomisés infectés expérimentalement, *Babesia* sp. BQ1 (Lintan) entre 3 et 63 jours post-infection (pi) et *Babesia* sp. Xinjiang entre 14 et 21 jours pi. Des enquêtes épidémiologiques utilisant la LAMP montrent que 14,3 % des moutons sont infectés par *Babesia* sp. BQ1 (Lintan) dans la région du Gannan de la province du Gansu et que 3,5 % des moutons sont infectés par *Babesia sp*. Xinjiang dans la région du Yning de la province du Xinjiang. La technique LAMP a donc une bonne spécificité et une bonne sensibilité et peut être utilisée pour des enquêtes épidémiologiques, notamment pour détecter les infections précoces, pour identifier les vecteurs et pour évaluer le taux de tiques infectés par ces 2 parasites.

Enfin, les techniques sérologiques sont les outils essentiels pour les études épidémiologiques et pour l'évaluation des mesures de prévention telles que la vaccination, la prémunition ou le contrôle des tiques. Les techniques les plus utilisées en matière de babésiose est le test de fixation du complément (CFT), l'immunofluorescence indirecte (IFAT) et l'ELISA. Le CFT est utilisée que pour le diagnostic de la babésiose équine mais est actuellement déconseillée par l'OIE du fait de sa faible sensibilité et spécificité (Tenter et Friedhoff, 1986 ; Weiland, 1986 ; Holman *et al*., 1993 ; Böse et Peymann, 1994 ; Kumar *et al*., 1997). L'IFAT, qui est encore largement utilisée pour mettre en évidence les infections à *Babesia* spp., est difficile à standardiser du fait de la subjectivité de la lecture et est consommatrice en temps (Brüning, 1996). **L'ELISA** en revanche est quantitative, sensible et automatisable. Elle a été mise au point pour le diagnostic des infections dues à différentes espèces de *Babesia* spp. (Purnell *et al*., 1976 ; Molloy *et al*., 1998 ; Ikadai *et al*., 2000 ; Goff *et al*., 2003). Cependant, peu d'études sont ciblées sur le développement de techniques sérologiques simples et rapides permettant de diagnostiquer la babésiose ovine. La détermination de la séro-prévalence est principalement basée sur l'utilisation de l'IFAT (Lewis *et al*., 1981b ; Habela *et al*., 1990 ; Kjemtrup *et al*., 1995 ; Papadopoulos *et al*., 1996 ; Ferrer *et al*. 1998). Aucun ELISA n'est disponible pour la babésiose ovine, excepté celui développé par Duzgun et al. (1991) utilisant un antigène de *B. bovis* pour détecter *B. ovis*. En Chine, aucun test sérologique n'a été mis au point pour détecter les isolats infectant les petits Ruminants. Dans notre étude, nous avons développé un ELISA pour rechercher les infections à *Babesia* sp. BQ1 (Lintan) en utilisant des antigènes solubles de mérozoïtes obtenus par culture *in vitro* (BQMA). La spécificité calculée est de 95,5 ± 0,97 % avec un seuil de positivité de 30 %. Aucune réaction croisée n'est observée avec les sérums de moutons infectés par les principaux hémoparasites présents en Chine (*Babesia* sp. BQ1 (Ningxian), *Babesia* sp. Hebei, *Babesia* sp. Xinjiang, *T. luwenshuni*, *T. uilenbergi* ou *A. ovis)*, excepté avec *Babesia* sp. Tianzhu. La production des anticorps détectés est précoce (à partir du 7ème

jour pi) et persiste pendant au moins 8 mois, ce qui est similaire à la production d'anticorps dirigés contre *B. ovis*, *B. crassa* et *B. motasi* (Uilenberg *et al*., 1980 ; Christensson et Thunegard, 1981 ; Hashemi-Fesharki et Uilenberg, 1981 ; Habela *et al*. 1990). Nous avons utilisé cet ELISA pour évaluer la séro-prévalence de l'infection à *Babesia* sp. BQ1 (Lintan) chez des moutons de la région autonome du Tibet Gannan où *H. qinghaiensis* est la tique prédominante : 66,84% des animaux testés sont séropositifs. Dans la même région, 14,3 % des animaux testés étaient positifs par la technique LAMP. Nous supposons que les différences de prévalence mesurée sont liées aux différences de paramètres détectés par ces 2 techniques. La LAMP permet de détecter directement le parasite pendant 2 mois pi chez les moutons infectés expérimentalement alors que, du fait de leur persistance, l'ELISA permet de détecter les anticorps anti-*Babesia* sp. BQ1 (Lintan) pendant au moins 8 mois. La LAMP devrait donc être utilisée pour détecter les infections récentes à *Babesia sp*. BQ1 (Lintan) alors que l'ELISA permet de détecter les infections récentes et latentes.

Afin d'augmenter la spécificité de l'ELISA utilisant BQMA et limiter les réactions croisées avec d'autres isolats de babésies chinoises, nous avons identifiés différents antigènes – candidats en criblant une banque d'ADNc de mérozoïte de *Babesia* sp. BQ1 (Lintan) et en analysant BQMA par spectrométrie de masse. Dans la banque d'ADNc, 8 fragments EST avec une forte identité avec ceux de *B. bovis* ou *B. bigemina* et 2 gènes de protéines hypothétiques ont été révélés par immuno-criblage de la banque, incluant une Rab1b, une Hsp90, une « gliding-associated protein » P45 (GAP45), une cyclophiline, une protéine membranaire, une RNA « recognition motif containing protein », p200, une protéine d'histone H2A et les protéines hypothétique 1 et 2 (tableau 5). Mis à part les protéines p200 et la protéine d'histone H2A, les séquences entières des autres ont été amplifiées par RACE-PCR. De plus, par spectrométrie de masse, 4 autres antigènes et notamment l'Hsp70 et l'énolase, ont été identifiées. Nous avons étudié, dans ce travail, plus particulièrement l'Hsp90 et la protéine hypothétique 1. Un gène Hsp90 a été cloné et caractérisé pour la 1ère fois chez une babésie ovine, BQHsp90. La protéine recombinante rBQHsp90 n'est pas reconnue par les sérums de moutons prélevés avant infection par *Babesia* sp. BQ1 (Lintan). En revanche, la production d'anticorps anti-rBQHsp90 augmente fortement après infection. Se pose le problème des réactions croisées entre rBQHsp90 et les anticorps dirigés contre les Hsp90 d'autres hémoparasites circulant en Chine, étude qui doit être menée dans le futur. En effet les protéines Hsp90 sont des protéines fortement conservées. Cependant De Andrade *et al*. (1992) a montré que la protéine recombinante Hsp90 de *Leishmania donovani donovani* ne réagit pas avec les sérums de patients infectés par *Trypanozoma cruzi* ou *Toxoplasma gondii.* De la même façon, Hsp90 de *Trichinella spiralis* est spécifique et ne réagit pas avec les sérums de rats contenant des anticorps anti-bactériens (Martinez *et al*., 2001).

Tableau 2. caractéristiques des gènes amplifiés à partir de *Babesia* sp. BQ1 (Lintan)

Clone N°	RACE	longueur (bp)	Longueur des ORF (bp)	Homologie	% d'identité
BLT01	+	758	669	*B. bovis* Rab1b (AY324135)	75%
BLT02	+	2392	2154	*B. bovis* Hsp90 (AF136649)	81%
BLT03	-	643*	/	*B. bigemina* p200 (AF142406)	76%
BLT04	-	322*	/	*B. bovis* Histone H2A protein (XM 001609662)	77%
BLT05	+	1025	567	*B. bovis* gliding- associated protein 45 (XM 001610016)	79%
BLT06	+	749	624	*B. bigemina* cyp gene for cyclophilin (AB510030)	91%
BLT07	+	1140	936	Hypothetic protein 1	/
BLT08	+	901	648	*B. bovis* membrane protein (XM 001611713)	70%
BLT09	+	1359	1125	*B. bovis* RNA recognition motif containing protein (XM 001610542)	70%
BLT10	+	714	636	Hypothetic protein 2	/

*= séquence partielle

Le gène de la protéine hypothétique 1 a été cloné et nommé BQP35 car il code pour une protéine de 35 kDa. Cette protéine a été identifiée comme étant une IUP par SDS-PAGE et par des études *in silico*. Ce serait la 1ère IUP décrite dans le genre *Babesia*. La protéine recombinante rBQ35 a une bonne spécificité sur la base de l'étude des réactions croisées avec d'autres hémoprotozoaires ovins chinois. Des études complémentaires sont cependant nécessaires afin d'estimer la potentialité de cette protéine en tant qu'antigène de diagnostic telles que la cinétique de production des anticorps anti-rBQP35 chez des moutons infectés expérimentalement par *Babesia* sp. BQ1 (Lintan) et l'estimation de la spécificité et de la sensibilité de l'ELISA utilisant cet antigène.

Enfin, parmi les autres protéines identifiées de *Babesia* sp. BQ1 (Lintan), l'Hsp70 pourrait être un bon candidat pour le séro-diagnostic. En effet, par western blot, nous avons mis en évidence qu'elle induit une réponse humorale précoce et tardive qu'elle n'est pas reconnue par les sérums de moutons non infectés par *Babesia* sp. BQ1 (Lintan) ni par les sera de moutons infectés par des hémoparasites chinois autres que *Babesia* sp. Tianzhu. Les Hsp70 sont présentés comme de bons candidats pour détecter d'autres Apicomplexes tels que *B. orientalis* (He *et al*., 2009b), *Theileria* sp. (China) (Miranda *et al*., 2006), *Plasmodium vivax* (Na *et al*., 2007), et *Neospora caninum* (Shin *et al*., 2004). Cette étude préliminaire par spectrométrie de masse a fourni des informations sur la séquence protéique permettant de trouver les gènes correspondants et de produire la protéine recombinante, afin d'estimer par la suite l'ELISA utilisant l'Hsp70 de *Babesia sp.* BQ1 (Lintan). La protéine p200 pourrait être également un antigène intéressant. Il a été utilisé pour la détection de *B. bigemina*, sans réaction croisée avec *B. bovis* (Altangerel *et al*., 2009). Dans le futur, ces antigènes seront la cible de nos investigations pour développer des méthodes de diagnostic des infections à

Babesia spp. en Chine.

En conclusion, nous avons développé 3 méthodes de détection pour les infections à *Babesia* sp. BQ1 (Lintan). La culture *in vitro* est le choix optimal pour isoler les différents isolats géographiques ou pour confirmer l'infection, notamment les infections latentes mais n'est pas utilisable pour le diagnostic ou les enquêtes épidémiologiques. La LAMP est essentielle pour confirmer les infections récentes, pour effectuer des enquêtes épidémiologiques et pour détecter le parasite dans les tiques pour identifier un vecteur ou pour étudier la distribution de ces vecteurs. Enfin, l'ELISA est l'outil de choix pour mettre en évidence les infections persistantes et pour les enquêtes épidémiologiques.

CONCLUSIONS ET PERSPECTIVES

Notre étude avait pour but de clarifier la phylogénie des babésies ovines chinoises, *Babesia sp.* BQ1 (Lintan) et *Babesia sp*. Xinjiang, et de développer des moyens de contrôle de la babésiose en Chine. Sur la base de la séquence du gène BQHsp90, de la morphologie et des réactions croisées étudiées par sérologie, les babésies ovines chinoises peuvent être divisées en 2 groupes, le groupe *B. motasi*-like (*Babesia* sp. BQ1 (Lintan), *Babesia sp*. BQ1 (Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Hebei) et le groupe *Babesia* sp. Xinjiang, ce qui est en accord avec les résultats obtenus avec les gènes ARNr 18S et ITS. *Babesia sp*. Xinjiang est une grande *Babesia*, ne ressemble pas morphologiquement à aucune autre babésie ovine, est peu virulente pour les moutons non splénectomisés et est transmis par *Hyalomma anatolicum anatolicum.* Les caractéristiques moléculaires et biologiques de *Babesia sp.* Xinjiang révèlent qu'il serait une nouvelle espèce infectant les petits ruminants et il est essentiel de lui donner un nom dans le futur. *B. motasi*-like a des caractéristiques morphologiques similaires à *B. motasi* Europe et est transmis par *Haemaphysalis* spp. (*H. qinghaiensis* et *H. longicornis* pour *Babesia* sp. BQ1 (Lintan) et *H. longicornis* pour *Babesia* sp. BQ1 (Ningxian)). Cependant, il est essentiel maintenant de déterminer la capacité de transmission de ces parasites par *H. punctata* car c'est la seule tique vectrice décrite pour *B. motasi*. Au sein du groupe *B. motasi*-like en Chine, il y a au moins 2 espèces ou sous-espèces. L'un contient des babésies faiblement virulentes pour les petits Ruminants (*Babesia sp*. BQ1 (Lintan), *Babesia sp.* Tianzhu ou *Babesia sp.* Madang) et l'autre des babésies fortement virulentes (*Babesia sp.* BQ1 (Ningxian) et *Babesia sp.* Hebei). Cependant, pour clarifier totalement la classification de ces parasites, des informations supplémentaires sur les caractéristiques biologiques de *Babesia* sp. Tianzhu, *Babesia* sp. Madang et *Babesia* sp. Hebei sont nécessaires, telles que la tique vectrice, la virulence et même l'infectivité pour l'Homme.

La résistance génétique des moutons joue un rôle important contre les infections à *Babesia* spp. La spécificité d'hôte des babésies et la sensibilité d'un individu peuvent être en partie expliquées par la sensibilité *in vitro* des érythrocytes. La sélection de race d'animaux ayant une résistance génétique naturelle contre la babésiose pourrait être un moyen intéressant de lutte contre la babésiose en Chine. Pour cela, le(s) gène(s) impliqué(s) doit(doivent) être identifié(s). Chez les hôtes sensibles, la réponse immunitaire, et en particulier la réponse immunitaire cellulaire, peut expliquer en partie la virulence du parasite : la production d'IL10 est associée à une virulence importante de *Babesia* spp alors que les niveaux élevés d'IFNγ produits sont liées à leur faible pathogénicité. Le développement de vaccins capables de stimuler une réponse immunitaire cellulaire protectrice pourrait être une stratégie de contrôle des infections à *Babesia* de forte virulence en Chine. Pour cela, les molécules cibles impliquées dans la production d'IFNγ et/ou l'invasion des érythrocytes doivent être identifiées. Les prochaines études devront être ciblées notamment sur l'exploration des molécules et les mécanismes impliqués dans le processus d'invasion des érythrocytes par

Babesia spp. et dans la modulation de la réponse immunitaire de l'hôte.

Enfin, afin d'évaluer la prévalence de la babésiose ovine en Chine, 3 méthodes de détection des infections à *Babesia sp*. BQ1 (Lintan) et *Babesia sp.* Xinjiang ont été développées. La technique de culture *in vitro* de *Babesia sp*. BQ1 (Lintan) est essentielle pour confirmer une infection ou pour isoler le parasite. Cette technique est probablement utilisable pour détecter d'autres babésies chinoises car nous avons réussi à cultiver avec le même système *Babesia sp*. Xinjiang (résultats non montrés). La sensibilité de cette méthode doit être déterminée, même si elle semble ne pas poser de problème puisque des lignées monoclonales par dilution limite ont été obtenues. Cette technique permettra d'obtenir des parasites en grande quantité sans qu'il y ait besoin d'infecter des animaux et d'étudier les interactions entre le parasite et les érythrocytes de l'hôte. Elle pourrait être utilisée également pour tester *in vitro* de nouvelles molécules thérapeutiques anti-*Babesia* spp. La technique LAMP est une méthode simple et rapide pour détecter *Babesia sp.* BQ1 (Lintan) et *Babesia sp.* Xinjiang dans le sang de moutons infectés. Elle a une forte sensibilité et spécificité et pourra être utilisée lors d'enquête épidémiologique de la babésios ovine en Chine. Elle peut permettre également la détection du parasite dans les tiques vectrices afin d'évaluer le taux d'arthropodes infectés ou d'identifier d'autres tiques vectrices. La technique ELISA est un choix optimal pour détecter les infections à *Babesia sp.* BQ1 (Lintan) lors d'enquête épidémiologique. Elle est de forte spécificité et elle permet de détecter le parasite durant la phase aiguë et durant la phase chronique de l'infection. Différents antigènes potentiels de sérodiagnostic ont été identifiés. L'utilisation en serodiagnostic des protéines rBQHsp90 et rBQP35 doit être précisée, mais notre étude montre que ce sont probablement des antigènes intéressants. Pour les autres antigènes tels que Hsp70, p200, protéine membranaire…, les protéines recombinantes correspondantes doivent être produites afin d'évaluer leur potentiel en sérodiagnostic.

En conclusion, les outils développés au cours de notre étude permettront à plus long terme de développer de nouvelles stratégies de contrôle de la babésiose ovine en Chine et d'en évaluer leur efficacité. Ils permettront également d'augmenter les connaissances sur les isolats de babésies ovines présentes en Chine, notamment leurs caractéristiques biologiques mais aussi les effets des interactions entre les isolats sur leur transmissibilité ou sur leur infectivité lors d'éventuelles co-infections et co-transmissions.

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ANNEXES

Annexe 1. Alignement des séquences nucléotidiques du gène Hsp90 de *Babesia sp.* BQ1 (Lintan) et *B. bovis*.

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Babesia sp. BQ1 (Lintan)   ACAACAAAAA GTATCTACCC AGCGACATCT TTCTTGCGGA TTTATTATTG CAGCTATCTC ATTAAAAATG GCGACGGAGA GTCAGGAGAC CTACGCGTTC  [ 100]
Babesia bovis AF136649     ---------- -------GAG ..A..G.GAA C.AG.C.AA. .AA....AA. ...TATAT.T T..C..... ..A..A.CAC AA..A..A.. .........T  [ 100]
Babesia bovis XM_001611504 ---------- ---------- ---------- ---G.C.AA. .AA....AA. ...TATAT.T T..C..... ..A..A.CAC AA..A..A.. .........T  [ 100]

Babesia sp. BQ1 (Lintan)   AACGCAGACA TTTCTCAGTT GTTGAGTTTG ATTATCAACG CCTTCTACAG CAACAAGGAG ATTTTCCTCC GTGAACTTAT CAGTAACGCC AGTGATGCCT  [ 200]
Babesia bovis AF136649     ........T. .C..C..... .......C.C .......... .T........ T..T..A..A ..C.....T. ....G..C.. .......... ..C.....T.  [ 200]
Babesia bovis XM_001611504 ........T. .C..C..... .......C.C .......... .T........ T..T..A..A ..C.....T. ....G..C.. .......... ..C.....T.  [ 200]

Babesia sp. BQ1 (Lintan)   TGGAAAAGAT CCGTTATGAG GCCATCAAGG ACCCGAAGCA AGTGGAGGAC TTCCCCGAGT ACCAGATCAG TCTGTCCGTC GACAAGGCTA ACAAGACCCT  [ 300]
Babesia bovis AF136649     .......A.. .A.A..... ..T....... ....A..... G..C..... .......... .......... C..A....CT ......A.C. ........T.  [ 300]
Babesia bovis XM_001611504 .......A.. .A.A..... ..T....... ....A..... G..C..... .......... .......... C..A....CT ......A.C. ........T.  [ 300]

Babesia sp. BQ1 (Lintan)   CATTATTGAG GACACTGGTA TCGGAATGAC CAAAGCTGAT TTGATCAACA ACTTGGGTAC CATCGCTAAG TCTGGCACCA AGGCCTTCAT GGAGGCCATT  [ 400]
Babesia bovis AF136649     G.CC..C..A .......... .T..T..... ....A.C... .......... .......... ......C... ..C..A.... ....T..... ...A......  [ 400]
Babesia bovis XM_001611504 G.CC..C..A .......... .T..T..... ....A.C... .......... .......... ......C... ..C..A.... ....T..... ...A......  [ 400]

Babesia sp. BQ1 (Lintan)   CAGGCTGGTG CCGACATGTC TATGATCGGT CAGTTCGGTG TCGGTTTCTA CTCGGCTTAC CTGGTTGCCG ACAAAGTGAC TGTTGTGTCT AAGAACAACA  [ 500]
Babesia bovis AF136649     ..A..C.... .T........ .......... .......... .G........ ...A..C... ..C.....T. ....G..C.. C..C..A..A ..........  [ 500]
Babesia bovis XM_001611504 ..A..C.... .T........ .......... .......... .G........ ...A..C... ..C.....T. ....G..C.. C..C..A..A ..........  [ 500]

Babesia sp. BQ1_(Lintan)   ACGATGACCA ATACATGTGG GAATCCAGTG CCAGTGGCCA CTTCACCGTT ACCAAGGATG AGTCTGGCGA GCAGTTGAAG CGTGGTACCC GTCTCATCCT  [ 600]
Babesia bovis AF136649     .......... G..TG..... ..G..T.AC. .......... .......... ........C. ......AA.. T...C.T..A ........A. ....G..AT.  [ 600]
Babesia bovis XM_001611504 .......... G..TG..... ..G..T.AC. .......... .......... ........C. ......AA.. T...C.T..A ........A. ....G..AT.  [ 600]

Babesia sp. BQ1_(Lintan)   TCACTTGAAG GACGACCAGA GTGAGTACCT CGAGGAGCGC CGTCTCAAGG ACCTCGTGAA GAAGCACAGC GAGTTCATCT CGTTCCCCAT TCGTCTGTCC  [ 700]
Babesia bovis AF136649     G...C.T... .......... .......... T.....A..T ..CT.G.... .A.....C.. A........T ..A....... .C........ CA.A.....T  [ 700]
Babesia bovis XM_001611504 G...C.T... .......... .......... T.....A..T ..CT.G.... .A.....C.. A........T ..A....... .C........ CA.A.....T  [ 700]

Babesia sp. BQ1 (Lintan)   GTTGAGAAGA CGACGGAGAC TGAAGTCACC GACGACGAGG CGGAGGCTAC TACCGCGTCC GAATCCAAGG ACGAGGAGAA GATCAAGGAT GTCACTGATG  [ 800]
Babesia bovis AF136649     .....A.... .T..C..A.. C..G..T... .......... .T..AC.C.. .GAA..---. ..G......C CT.....A.. .....CT..C ..A.....A.  [ 800]
Babesia bovis XM_001611504 .....A.... .T..C..A.. C..G..T... .......... .T..AC.C.. .GAA..---. ..G......C CT.....A.. .....CT..C ..A.....A.  [ 800]

Babesia sp. BQ1 (Lintan)   AGACTGAGAA TGAGGGTGAG GATGCCAAAG AAGGTGAAGA AAAGGAGGGT GAGAAAACCG CTGAGAAGAA GAAGCGCAAG GTCACCTCCG TGACCCGTGA  [ 900]
Babesia bovis AF136649     .AGAA...G. AA...AAA.. ..A..AG..A .G.A..G... .G.AA..A-- ---------- .G..A..... ...A..... ..T...AAT. .C........  [ 900]
Babesia bovis XM_001611504 .AGAA...G. AA...AAA.. ..A..AG..A .G.A..G... .G.AA..A-- ---------- .G..A..... ...A..... ..T...AAT. .C........  [ 900]

Babesia sp. BQ1 (Lintan)   GTGGGAGATG TTGAACAAGC AGAAACCCAT TTGGATGCGT CTGCCCACTG AGGTTACTCA CGAGGAGTAC GCAAGCTTCT ACAAGAATTT GTGTAACGAC  [1000]
Babesia bovis AF136649     A.....A... C.T....... .A..G..T.. .......A.G T.......C. ....C..CA. ...A..... ..C....... .......CC. ..CC..T..T  [1000]
Babesia bovis XM_001611504 A.....A... C.T....... .A..G..T.. .......A.G T.......C. ....C..CA. ...A..... ..C....... .......CC. ..CC..T..T  [1000]
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Babesia sp. BQ1 (Lintan)   TGGGAAGATC ACTTGGCTGT GAAGCACTTC AGTGTTGAGG GTCAGCTTGA GCTCAAGGCG TTGCTTTTCA TCCCGAAGCG CGCCCCGTTC GACATGTTCG  [1100]
Babesia bovis AF136649     ........C. .......A.. C......... .....A..A. .A..A..... AT.......C A.C......G .A..C..A.. T..A..A... ..........  [1100]
Babesia bovis XM_001611504 ........C. .......A.. C......... .....A..A. .A..A..... AT.......C A.C......G .A..C..A.. T..A..A... ..........  [1100]

Babesia sp. BQ1 (Lintan)   AAAGCCGCAA GAAGAAGAAC AACATCAAGT TGTATGTGCG TCGTGTGTTC ATTATGGACG ACTGCGAAGA GTTGATTCCG GAATGGCTGG GTTTCATGAA  [1200]
Babesia bovis AF136649     ...A...... A......... .......... ....C..A.. C......... ..C....... ....T..T.. .C....C..C ..G.....T. .....G.A..  [1200]
Babesia bovis XM_001611504 ...A...... A......... .......... ....C..A.. C......... ..C....... ....T..T.. .C....C..C ..G.....T. .....G.A..  [1200]

Babesia sp. BQ1 (Lintan)   GGGTGTCGTT GACTCCGAGG ACCTCCCTCT GAACATCAGT CGTGAAATTC TTCAGCAGAA CAAAATTCTT AAGGTCATTC GCAAGAACCT CGTTAAGAAG  [1300]
Babesia bovis AF136649     ......T..C .....T.... ....T..... T.....T..C A.A...G.G. .C.....A.. ...G..C... .....T.... ........T. G..A.....A  [1300]
Babesia bovis XM_001611504 ......T..C .....T.... ....T..... T.....T..C A.A...G.G. .C.....A.. ...G..C... .....T.... ........T. G..A.....A  [1300]

Babesia sp. BQ1 (Lintan)   TGCCTTGAGT TGTTCTCCGA ACTCACTGAG AAGAAGGAGG ACTTCAAGAA GTTCTACGAG CAGTTCAGCA AGAACTTGAA ATTGGGTATC CACGAAGACA  [1400]
Babesia bovis AF136649     .....G..AC .C.....T.. GT.G...... .......... .......... .........A .......... .....C.T.. G......... .....G....  [1400]
Babesia bovis XM_001611504 .....G..AC .C.....T.. GT.G...... .......... .......... .........A .......... .....C.T.. G......... .....G....  [1400]

Babesia sp. BQ1 (Lintan)   ACGCTAACCG CACCAAGATC GCCGAGCTGT TGCGTTACGA GACCAGCAAG AGCGGTGATG AGGCCATCAG CCTAAAGGAG TACGTTGACC GCATGAAGCC  [1500]
Babesia bovis AF136649     ..A.....A. A.A......T T.T..AT... .A........ A...TCA... ........C. .......... .T.G.....A .....C..T. ..........  [1500]
Babesia bovis XM_001611504 ..A.....A. A.A......T T.T..AT... .A........ A...TCA... ........C. .......... .T.G.....A .....C..T. ..........  [1500]

Babesia sp. BQ1 (Lintan)   CGACCAGAAG TACATCTACT ACATCACTGG CGAGTCAAAG CAGAGCGTTG CCAACTCTCC CTTCCTTGAG GTCCTGCGTT CTAAGGGCAT CGAGGTCATC  [1600]
Babesia bovis AF136649     ...G...... .....T.... ....T..... T..A..T... .....T.... .......... A......... TG.T....C. .C.G...... T.........  [1600]
Babesia bovis XM_001611504 ...G...... .....T.... ....T..... T..A..T... .....T.... .......... A......... TG.T....C. .C.G...... T.........  [1600]

Babesia sp. BQ1 (Lintan)   TACATGACTG ACCCCATTGA CGAGTACGCC GTCCAACAGA TCAAGGAGTT CGAGGGCAAA AAGCTCAAGT GCTGCACTAA GGAGAACCTT GAGTTGGAGG  [1700]
Babesia bovis AF136649     .......... ....A..... T..A.....T ..T..G.... .......... T........G ..A..T.... ....T..... ...A..T..C ..AC.C....  [1700]
Babesia bovis XM_001611504 .......... ....A..... T..A.....T ..T..G.... .......... T........G ..A..T.... ....T..... ...A..T..C ..AC.C....  [1700]

Babesia sp. BQ1 (Lintan)   ACACTGAGGA AGAGCGCAAG AGTTTCGAGA CCCTGCAGAA GGAGATGGAA CCTCTTTGCC GTGTGATTAA GGAGATTCTC CACGACAAGG TTGAGAAGGT  [1800]
Babesia bovis AF136649     .......A.. ...A...... .AC....... ..T..G.A.. ...A...... .....A.... .CC.C..C.. ...A.....T .......... .C..A.....  [1800]
Babesia bovis XM_001611504 .......A.. ...A...... .AC....... ..T..G.A.. ...A...... .....A.... .CC.C..C.. ...A.....T .......... .C..A.....  [1800]

Babesia sp. BQ1 (Lintan)   CGTTTGCGGC AAGCGCTTCA CCGAGAGCCC ATGTGCTTTG GTCACCAGCG AATTCGGATG GTCCGCTAAC ATGGAGCGTA TCATGAAGGC TCAGGCCTTG  [1900]
Babesia bovis AF136649     T.....T... ..A..T..T. ....A..... T......... ..A....... .......... .......... .....A.... .T........ C.....AC.A  [1900]
Babesia bovis XM_001611504 T.....T... ..A..T..T. ....A..... T......... ..A....... .......... .......... .....A.... .T........ C.....AC.A  [1900]

Babesia sp. BQ1 (Lintan)   CGCGACAACA ACTTTGGGAG TTTCATGATC AGCAAGAAGA CTATGGAGCT CAACCCCCAC CACAGCATCA TGAAGGAGTT GAAGCAGCGT GCTGAGGCAG  [2000]
Babesia bovis AF136649     ..T....G.. G......T.. C..T.....A .......... .......A.. T.....T... ........T. .......AC. C.G....... .....AA.C.  [2000]
Babesia bovis XM_001611504 ..T....G.. G......T.. C..T.....A .......... .......A.. T.....T... ........T. .......AC. C.G....... .....AA.C.  [2000]
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Babesia sp. BQ1 (Lintan)   ACAAGAGCGA CAAGACCTTG AAGGACCTCG TATGGCTGTT GTACGACACT GCCATTTTGA CCTCCGGCTT CAACCTTGAC GACCCTACTC AGTTCGGCGG  [2100]
Babesia bovis AF136649     .......... ...A..TC.C .....T..G. .......T.. ......T..C .....G.... .......T.. ....T.G..T ........C. .......T..  [2100]
Babesia bovis XM_001611504 .......... ...A..TC.C .....T..G. .......T.. ......T..C .....G.... .......T.. ....T.G..T ........C. .......T..  [2100]

Babesia sp. BQ1 (Lintan)   TCGAATATAC CGCATGATTA AGTTGGGTCT GTCATTAGAT GACGACGCTG CGGTTGAGGA TGTAGAAATT CCATCTCTGG ACGAAGTCGT GGTCGACCCT  [2200]
Babesia bovis AF136649     A..C..C... ..T.....C. .A........ A..CC.C..C ..T..AC..A .T.G...A.. C..C..TC.C ...C.AT... .......T.. ...T......  [2200]
Babesia bovis XM_001611504 A..C..C... ..T.....C. .A........ A..CC.C..C ..T..AC..A .T.G...A.. C..C..TC.C ...C.AT... .......T.. ...T......  [2200]

Babesia sp. BQ1 (Lintan)   AAGATGGAGG AGGTGGACTG AGCG---TTG CGCCATCCGC GTTAGC---- GTTTTTCACC ATCCGG-TAG AAAAGTGTAT CACTCCACTA ATTTAGTAGC  [2300]
Babesia bovis AF136649     ........A. .A..T....A ....CGC..T TA.TG.TATG .G....CACT .CA..G..G. GG..ATC..C .G.C..A.G. ...GTA.A.. ....TAG.A.  [2300]
Babesia bovis XM_001611504 ........A. .A..T....A ....CGC..T TA.TG.TATG .G....CACT .CA..G..G. GG..ATC..C .G.C..A.G. ...GTA.A.. ....TAG.A.  [2300]

Babesia sp. BQ1 (Lintan)   ATCTTTAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA  [2400]
Babesia bovis AF136649     GA.AA..T.. TT.T.T.TTT CTC....... .......... .---------- ---------- ---------- ---------- ---------- ----------  [2400]
Babesia bovis XM_001611504 GA.AA..T.. TT.T.T.TTT CTC.T----- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [2400]
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Annexe 2. Alignement des séquences nucléotidiques (cDNA ou gDNA) du gène Hsp90 de *Babesia* spp. ovine de Chine

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Babesia sp. BQ1 (Lintan)-cDNA   ACAACAAAAA GTATCTACCC AGCGACATCT TTCTTGCGGA TTTATTATTG CAGCTATCTC ATTAAAAATG GCGACGGAGA GTCAGGAGAC CTACGCGTTC  [ 100]
Babesia sp. BQ1 (Lintan)-gDNA   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 100]
Babesia sp. BQ1 (Ningxian)-gDNA ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 100]
Babesia sp. Hebei-gDNA          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 100]
Babesia sp. Tianzhu-gDNA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 100]
Babesia sp. Xinjiang-gDNA       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 100]

Babesia sp. BQ1 (Lintan)-cDNA   AACGCAGACA TTTCTCAGTT GTTGAGTTTG ATTATCAACG CCTTCTACAG CAACAAGGAG ATTTTCCTCC GTGAACTTAT CAGTAACGCC AGTGATGCCT  [ 200]
Babesia sp. BQ1 (Lintan)-gDNA   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 200]
Babesia sp. BQ1 (Ningxian)-gDNA ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 200]
Babesia sp. Hebei-gDNA          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 200]
Babesia sp. Tianzhu-gDNA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 200]
Babesia sp. Xinjiang-gDNA       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 200]

Babesia sp. BQ1 (Lintan)-cDNA   TGGAAAAGAT CCGTTATGAG GCCATCAAGG ACCCGAAGCA AGTGGAGGAC TTCCCCGAGT ACCAGATCAG TCTGTCCGTC GACAAGGCTA ACAAGACCCT  [ 300]
Babesia sp. BQ1 (Lintan)-gDNA   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 300]
Babesia sp. BQ1 (Ningxian)-gDNA ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 300]
Babesia sp. Hebei-gDNA          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 300]
Babesia sp. Tianzhu-gDNA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 300]
Babesia sp. Xinjiang-gDNA       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 300]

Babesia sp. BQ1 (Lintan)-cDNA   CATTATTGAG GACACTGGTA TCGGAATGAC CAAAGCTGAT TTGATCAACA ACTTGGGTAC CATCGCTAAG TCTGGCACCA AGGCCTTCAT GGAGGCCATT  [ 400]
Babesia sp. BQ1 (Lintan)-gDNA   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 400]
Babesia sp. BQ1 (Ningxian)-gDNA ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 400]
Babesia sp. Hebei-gDNA          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 400]
Babesia sp. Tianzhu-gDNA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 400]
Babesia sp. Xinjiang-gDNA       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [ 400]

Babesia sp. BQ1 (Lintan)-cDNA   CAGGCTGGTG CCGACATGTC TATGATCGGT CAGTTCGGTG TCGGTTTCTA CTCGGCTTAC CTGGTTGCCG ACAAAGTGAC TGTTGTGTCT AAGAACAACA  [ 500]
Babesia sp. BQ1 (Lintan)-gDNA   ---------- ---------- ---------- -----..... .......... .......... .......... .......... ..........  [ 500]
Babesia sp. BQ1 (Ningxian)-gDNA ---------- ---------- ---------- -----..... .......... .......... .......... .......... ...C..C... ..A.......  [ 500]
Babesia sp. Hebei-gDNA          ---------- ---------- ---------- -----..... .......... .......... .......... .......... ...C..C... ..A.......  [ 500]
Babesia sp. Tianzhu-gDNA        ---------- ---------- ---------- -----..... .......... .......... .......... .......... .......... ..........  [ 500]
Babesia sp. Xinjiang-gDNA       ---------- ---------- ---------- -----..... .......... .......... ..T..A..G. ....G..A.. A...A.C..C ..AC.....G  [ 500]

Babesia sp. BQ1 (Lintan)-cDNA   ACGATGACCA ATACATGTGG GAATCCAGTG CCAGTGGCCA CTTCACCGTT ACCAAGGATG AGTCTGGCGA GCAGTTGAAG CGTGGTACCC GTCTCATCCT  [ 600]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
Babesia sp. BQ1 (Ningxian)-gDNA ....C..T.. G......... .......... .......T.. .......... ..T.....C. ....C..... .......... .......... ..........  [ 600]
Babesia sp. Hebei-gDNA          ....C..T.. G......... .......... .......T.. .......... ..T.....C. ....C..... .......... .......... ..........  [ 600]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]
Babesia sp. Xinjiang-gDNA       .T..C..... ...TG.A... .....A.A.. .......A.. T.....TA.C .....A..C. ....C..A.. A..AC.T..A .....A..G. .C...G.A..  [ 600]
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Babesia sp. BQ1 (Lintan)-cDNA   TCACTTGAAG GACGACCAGA GTGAGTACCT CGAGGAGCGC CGTCTCAAGG ACCTCGTGAA GAAGCACAGC GAGTTCATCT CGTTCCCCAT TCGTCTGTCC  [ 700]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
Babesia sp. BQ1 (Ningxian)-gDNA .......... .......... .C........ .......... .....G.... ....T..... .......... .......... .......... ..........  [ 700]
Babesia sp. Hebei-gDNA          .......... .........G .C........ .......... .....G.... ....T..... .......... .......... .......... ..........  [ 700]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 700]
Babesia sp. Xinjiang-gDNA       ....C.T..A ..A..T.... .C..A..... G..A..A... ...T.G.... .A..T..T.. ...A.....T ..A..... .......A.. A..C..T..A  [ 700]

Babesia sp. BQ1 (Lintan)-cDNA   GTTGAGAAGA CGACGGAGAC TGAAGTCACC GACGACGAGG CGGAGGCTAC TACCGCGTCC GAATCCAAGG ACGAGGAGAA GATCAAGGAT GTCACTGATG  [ 800]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... ......T... .......... ..........  [ 800]
Babesia sp. BQ1 (Ningxian)-gDNA .......... ....A..... ...G..G... .....T.... .T......C. ...TC.CG.. ........A. .T.....A.. ...T...... ..........  [ 800]
Babesia sp. Hebei-gDNA          .......... ....A..... ...G..G... .....T.... .T......C. ...TC.CG.. ........A. .T.....A.. ...T...... ..........  [ 800]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 800]
Babesia sp. Xinjiang-gDNA       ..A..A.... .......A.. A.....G..A .....T.... .C......T. GGAAA.TGGA ...---..AC CT........ ...A.CC..C .....C..C.  [ 800]

Babesia sp. BQ1_(Lintan)-cDNA   AGACTGAGAA TGAGGGTGAG GATGCCAAAG AAGGTGAAGA AAAGGAGGGT GAGAAAA--- CCGCTGAGAA GAAGAAGCGC AAGGTCACCT CCGTGACCCG  [ 900]
Babesia sp. BQ1_(Lintan)-gDNA   .......... .......... .......... .......... .......... .......--- .......... .......... .......... ..........  [ 900]
Babesia sp. BQ1_(Ningxian)-gDNA ..G....A.. G........A ..G....... ....C..... C.....A... ..C....--- .......... .......... .......... ..........  [ 900]
Babesia sp. Hebei-gDNA          ..G....A.. G........A ..G....... ....C..... C.....A... ..C....--- .......... .......... .......... ..........  [ 900]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......--- .......... .......... .......... ..........  [ 900]
Babesia sp. Xinjiang-gDNA       .AGTA..C.. A..A.AG.G. ..G.A...G. .T.AC...A. .C.A..T.AC A.CG...AGG ...A.A.... ......A... .....T..T. .T..C..A..  [ 900]

Babesia sp. BQ1 (Lintan)-cDNA   TGAGTGGGAG ATGTTGAACA AGCAGAAACC CATTTGGATG CGTCTGCCCA CTGAGGTTAC TCACGAGGAG TACGCAAGCT TCTACAAGAA TTTGTGTAAC  [1000]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
Babesia sp. BQ1 (Ningxian)-gDNA .......... .......... .......G.. A......... ........T. .......... .......... .....C.... .......A.. CC.C..C...  [1000]
Babesia sp. Hebei-gDNA          .......... .......... .......G.. A......... ........T. .......... .......... .....C.... .......A.. CC.C..C...  [1000]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1000]
Babesia sp. Xinjiang-gDNA       ...A.....A ...C.A.... .......... A......... A.A..T..A. G......... .A....A... .......... .......A.. C.........  [1000]

Babesia sp. BQ1 (Lintan)-cDNA   GACTGGGAAG ATCACTTGGC TGTGAAGCAC TTCAGTGTTG AGGGTCAGCT TGAGCTCAAG GCGTTGCTTT TCATCCCGAA GCGCGCCCCG TTCGACATGT  [1100]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... ....T..... .......... .......... .......... ..........  [1100]
Babesia sp. BQ1 (Ningxian)-gDNA .......... .C........ .......... .......... .......... ....T..... .......... .......C.. .......... ..........  [1100]
Babesia sp. Hebei-gDNA          .......... .C........ .......... .......... .......... ....T..... .......... .......C.. .......... ..........  [1100]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... ....T..... .......... .......... .......... ..........  [1100]
Babesia sp. Xinjiang-gDNA       ..T....... .C..T..... ...C..A... ..T.....A. .A.....AT. G..AT..... ..TC.T.... ..G.A..A.. ...T..A..A ..........  [1100]

Babesia sp. BQ1 (Lintan)-cDNA   TCGAAAGCCG CAAGAAGAAG AACAACATCA AGTTGTATGT GCGTCGTGTG TTCATTATGG ACGACTGCGA AGAGTTGATT CCGGAATGGC TGGGTTTCAT  [1200]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ........G.  [1200]
Babesia sp. BQ1 (Ningxian)-gDNA ....G..... .......... .......... .......C.. A......... .......... .......T.. .........C ..T....... ........G.  [1200]
Babesia sp. Hebei-gDNA          ....G..... .......... .......... .......C.. A......... .......... .......T.. .........C ..T....... ........G.  [1200]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... .......... .......... .......... ........G.  [1200]
Babesia sp. Xinjiang-gDNA       .......... .........A .......... .AC.T..C.. .A.A...... .......... .......T.. ...AC.C..A ..A..G.... .TA.C.....  [1200]
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Babesia sp. BQ1 (Lintan)-cDNA   GAAGGGTGTC GTTGACTCCG AGGACCTCCC TCTGAACATC AGTCGTGAAA TTCTTCAGCA GAACAAAATT CTTAAGGTCA TTCGCAAGAA CCTCGTTAAG  [1300]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1300]
Babesia sp. BQ1 (Ningxian)-gDNA T........G ..C....... .......T.. .......... ..C....... .......... ......G..C ........T. .......... ...T......  [1300]
Babesia sp. Hebei-gDNA          T........G ..C....... .......T.. .......... ..C....... .......... ......G..C ........T. .......... ...T......  [1300]
Babesia sp. Tianzhu-gDNA        .......... .......... .......T.. .......... .......... .......... .......... .......... .......... ..........  [1300]
Babesia sp. Xinjiang-gDNA       C.......CT ..C..T.... .A..T..A.. AT........ ..CA.A..GG ....A..A.. A.....G..C ........T. .C........ ...T..C...  [1300]

Babesia sp. BQ1 (Lintan)-cDNA   AAGTGCCTTG AGTTGTTCTC CGAACTCACT GAGAAGAAGG AGGACTTCAA GAAGTTCTAC GAGCAGTTCA GCAAGAACTT GAAATTGGGT ATCCACGAAG  [1400]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1400]
Babesia sp. BQ1 (Ningxian)-gDNA .......... .......... ......G... .......... .......... .......... .......... .......... ...G...... ..........  [1400]
Babesia sp. Hebei-gDNA          .......... .......... ......G... .......... .......... .......... .......... .......... ...G...... ..........  [1400]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1400]
Babesia sp. Xinjiang-gDNA       ..A.....A. ..C.A..... ......T..A ..A....... .........G ...A...... ..C..A.... ....A...C. C..G...... ..........  [1400]

Babesia sp. BQ1 (Lintan)-cDNA   ACAACGCTAA CCGCACCAAG ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1500]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... GTAGGTTCAG TGGCAATAAA ATGATTCATG TATGGATGTG GTAAATCGTG TGTTTGCGGC GAGGACTTGG TCGCTGAATT  [1500]
Babesia sp. BQ1 (Ningxian)-gDNA .......... .......... GTATGCTCAG TAGCAGTAGA ATGATTCATG TATGGATGTG ATAAACTGTG TGTTTGCGTC TAAAATTTGG TCGCTGGGTT  [1500]
Babesia sp. Hebei-gDNA          .......... .......... GTATGTTCAG TAGCAGTAGA ATGATTCATG TATGGATGTG ATAAACTGTG TGTTTGCGTC TAAAATTTGG TCGCTGGGTT  [1500]
Babesia sp. Tianzhu-gDNA        .......... .......... GTAGGTTCAG AGGCAATAAA ATGATTCATG TATGGATGTG GTAAATCGTG TGTTTGCGGC GAGGACTTGG TCGCTGGGTT  [1500]
Babesia sp. Xinjiang-gDNA       .......A.. ...T.TG... GTAGGTT--- --ACATTATA TTTAGTTACA CAC------- ACAAAC---- ---------- --------GC TCGGTGAAT-  [1500]

Babesia sp. BQ1 (Lintan)-cDNA   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1600]
Babesia sp. BQ1 (Lintan)-gDNA   TTT--GGCGT TTCCGGTCGC GAAATATCGC TTCCAGCGAA TCTACGTCGG AGGCCTTTTC CGGCTGGAAT AACAATTCAT CGTCCTTAAG GATGCCTTAT  [1600]
Babesia sp. BQ1 (Ningxian)-gDNA TTTT-GGCGT TTCCGGTCGC GAAATATCGC TTCCAGCGAA TGTACGTCGG AGGCCTTTTC CGGCTGGGAT AACAATTCAT CGTCCTTAAG GATGCCTCAC  [1600]
Babesia sp. Hebei-gDNA          TTTTTGGCGT TTCCGGTCGC GAAATATCGC TTCCAGCGAA TGTACGTCGG AGGCCTTTTC CGGCTGGGAT AACAATTCAT CGTCCTTAAG GATGCCTCAT  [1600]
Babesia sp. Tianzhu-gDNA        TTT--GGCGT TTCCGGTCGC GAAATATCGC TTCCAGCGAA TCTACGTCGG AGGCCTTTTC CGGCTGGAAT AACAATTCAT CGTCCTTAAG GATGCCTTAT  [1600]
Babesia sp. Xinjiang-gDNA       ---------- --CCGGCCGT GGAGCAGTGC CTTTAACGTT GACACGTTCG AGGCATTT-C AGGCTGGTAT ATCAATTCAT CGTCCTTAAG GATGCCTAAC  [1600]

Babesia sp. BQ1 (Lintan)-cDNA   ---------- ---A------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1700]
Babesia sp. BQ1 (Lintan)-gDNA   CGGTGTCCGA CTC.GCGGTG GTACATTTTC GTTATTGCTT CGTAAATTTA TGTGTAATAT GCGGGTGCGT CTCCTTATTT GAATTGCAAC GCAATTGCAT  [1700]
Babesia sp. BQ1 (Ningxian)-gDNA CAGTGTCCGA CTC.GCGGTG GTACATTTTC GTCCTTGCTT TCTAAATTTA TGTGTGATAT GTGGATGCGT TTTCCTATTT AAATTGCAAC GCAATTGCGT  [1700]
Babesia sp. Hebei-gDNA          CAGTGTCCGA CTC.GCGGTG GTACACTTTC GTCCTTGCTT TGTAAATTTA TGTGTGATAT GTGGATGCGT TTGCTTATTT AAATTGCAAC ACAATTGCGT  [1700]
Babesia sp. Tianzhu-gDNA        CGGTGTCCGA CTC.GCGGTG GTACATTTTC GTTATTGCTT TGTAAATTTA TGTGTAATAT GCGGGTGCGT ATCCTTATTT GAATTGCAAC GCAATTGCAT  [1700]
Babesia sp. Xinjiang-gDNA       CCGTGTCCGA ATC.ACGGTG ACACACT--- ---------- ---------- -GTATCAT-- ---------- ---------- ---------- -----TCC--  [1700]

Babesia sp. BQ1 (Lintan)-cDNA   ---------- ---------- ---------- ---------- ---------- ---------- --T------- ---------- ---------- ----------  [1800]
Babesia sp. BQ1 (Lintan)-gDNA   TGTGGTACC- ---------- AGTTCGTGAT GCCGACGCTT ATATGGGCGT ATTTACTACA TG.GCTTTCC ATGGCGTTAA GTGGATTCGG CTTTGTTCAC  [1800]
Babesia sp. BQ1 (Ningxian)-gDNA TGTTGTACAT TAATTGTACA ATTTTGTAAT GCCCACGCTT ATATGGGCGT ATTTTCTGCA TT.GCCTTAC ATGGTGTTAG ATGGATTCGT ATT--TCCAC  [1800]
Babesia sp. Hebei-gDNA          GGTTGTACAT TAATTGTACA ATTTCGTAAT GCCCACGCTT ACATGGGCGT ATTTTATGCA TT.GTGTTAC ATGGTGTTAA ATGGATTCGT ATT--TCCAC  [1800]
Babesia sp. Tianzhu-gDNA        TGTTGTACA- ---------- AGTTCGTAAT GCCAACGCTT ATATGGGCGT ATTTACTACA TG.GCTTTCC ATGGCGTTAA GTGGATTCGG CTTTGTTCAC  [1800]
Babesia sp. Xinjiang-gDNA       ---------- ---------- ----CGTAAC ACCACCGTTT ---------T GTTTTTTTGA TA.----TAT GCGCTTTTAC ATGCATACGC ----------  [1800]
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Babesia sp. BQ1 (Lintan)-cDNA   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -AGTTTCATG ATCAGCAAGA  [2500]
Babesia sp. BQ1 (Lintan)-gDNA   CCAACGGGT- TTACCCTGTG CCGAAATTGC ACCGTATTT- -CTGATTTTG CGCTGCATTA CCCACTTTAA CGTGTGTTCA G......... ..........  [2500]
Babesia sp. BQ1 (Ningxian)-gDNA CCAACGGGT- ATACCCTGTG CCGAAAGTGC ACCGTGTTT- -CTGATAT-G CGCTGCTTTA CCCACGTTAA CGTGTGTTCA G......... ..A.......  [2500]
Babesia sp. Hebei-gDNA          CCAACGGGT- ATACCCTGTG CCGAAAGTGC ACCGTGTTT- -CTGATAT-G CGCTGCTTTA CCCACGTTAA CGTGTGTTCA G......... ..........  [2500]
Babesia sp. Tianzhu-gDNA        CCAACGGGT- TTACCCTGTG CCGAAATTGC ACCGTATTT- -CTGATTT-G CGCTGCATTA CCCACTTTAA CGTGTGTTCA G......... ..........  [2500]
Babesia sp. Xinjiang-gDNA       CCAATGGGTT ATTCCATGTG ACGAAAGTGC ACTTTTATTG CCTGACTT-G CGCCATTTAT TATGATTTAA CAATTTCTCA G.A....... G.G.......  [2500]

Babesia sp. BQ1 (Lintan)-cDNA   AGACTATGGA GCTCAACCCC CACCACAGCA TCATGAAGGA GTTGAAGCAG CGTGCTGAGG CAGACAAGAG CGACAAGACC TTGAAGGACC TCGTATGGCT  [2600]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [2600]
Babesia sp. BQ1 (Ningxian)-gDNA .......... .........T .......... .......... .......... .......... .......... .........T .......... ..........  [2600]
Babesia sp. Hebei-gDNA          .......... .........T .......... .......... A......... .......... .......... .........T .......... ..........  [2600]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [2600]
Babesia sp. Xinjiang-gDNA       ....C..... .T.G.....A .......... .......... .........A ........A. .T........ T......... C.T.....TT .G........  [2600]

Babesia sp. BQ1 (Lintan)-cDNA   GTTGTACGAC ACTGCCATTT TGACCTCCGG CTTCAACCTT GACGACCCTA CTCAGTTCGG CGGTCGAATA TACCGCATGA TTAAGTTGGG TCTGTCATTA  [2700]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [2700]
Babesia sp. BQ1 (Ningxian)-gDNA .......... .......... .......... A......... .......... ....A..... ......C... .......... .....C.... ....C.....  [2700]
Babesia sp. Hebei-gDNA          .......... .......... .......... A......... .......... ....A..... ......C... .......... .....C.... ..........  [2700]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... ......C... .......... .......... ..........  [2700]
Babesia sp. Xinjiang-gDNA       TC.T...... ..A.....G. ....A..T.. .........G ..G....... .C........ T..A..T..T .......... .C...C.C.. .T.....C..  [2700]

Babesia sp. BQ1 (Lintan)-cDNA   GATGACGACG CTGCGGTTGA GGATGTAGAA ATTCCATCTC TGGACGAAGT CGTGGTCGAC CCTAAGATGG AGGAGGTGGA CTGAGCGTTG CGCCATCCGC  [2800]
Babesia sp. BQ1 (Lintan)-gDNA   .......... .......... .......... .......... .......... .......... .......... .......... .......--- ----------  [2800]
Babesia sp. BQ1 (Ningxian)-gDNA .......... .......... .......... .......... .......G.. .......... .......... .......... .......--- ----------  [2800]
Babesia sp. Hebei-gDNA          .......... .......... .......... .......... .......G.. .......... .......... .......... .......--- ----------  [2800]
Babesia sp. Tianzhu-gDNA        .......... .......... .......... .......... .......... .......... .......... .......... .......--- ----------  [2800]
Babesia sp. Xinjiang-gDNA       ..C.....GC ...T..CC.. ......C..G .....C..GT ....T..G.. ...A..A... .......... .......... .......--- ----------  [2800]

Babesia sp. BQ1 (Lintan)-cDNA   GTTAGCGTTT TTCACCATCC GGTAGAAAAG TGTATCACTC CACTAATTTA GTAGCATCTT TAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA  [2900]
Babesia sp. BQ1 (Lintan)-gDNA   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [2900]
Babesia sp. BQ1 (Ningxian)-gDNA ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [2900]
Babesia sp. Hebei-gDNA          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [2900]
Babesia sp. Tianzhu-gDNA        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [2900]
Babesia sp. Xinjiang-gDNA       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [2900]

Babesia sp. BQ1 (Lintan)-cDNA   AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAA [2955]
Babesia sp. BQ1 (Lintan)-gDNA   ---------- ---------- ---------- ---------- ---------- ----- [2955]
Babesia sp. BQ1 (Ningxian)-gDNA ---------- ---------- ---------- ---------- ---------- ----- [2955]
Babesia sp. Hebei-gDNA          ---------- ---------- ---------- ---------- ---------- ----- [2955]
Babesia sp. Tianzhu-gDNA        ---------- ---------- ---------- ---------- ---------- ----- [2955]
Babesia sp. Xinjiang-gDNA       ---------- ---------- ---------- ---------- ---------- ----- [2955]
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Annexe 3. Alignement des séquences en acides aminés de la protéine Hsp90 de *Babesia* spp. ovine en Chine

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Babesia sp.BQ1(Lintan)   MATESQETYA FNADISQLLS LIINAFYSNK EIFLRELISN ASDALEKIRY EAIKDPKQVE  [ 60]
Babesia sp.Tianzhu       ---------- ---------- ---------- ---------- ---------- ----------  [ 60]
Babesia sp.BQ1(Ningxian) ---------- ---------- ---------- ---------- ---------- ----------  [ 60]
Babesia sp.Hebei         ---------- ---------- ---------- ---------- ---------- ----------  [ 60]
Babesia sp.Xinjiang      ---------- ---------- ---------- ---------- ---------- ----------  [ 60]

Babesia sp.BQ1(Lintan)   DFPEYQISLS VDKANKTLII EDTGIGMTKA DLINNLGTIA KSGTKAFMEA IQAGADMSMI  [120]
Babesia sp.Tianzhu       ---------- ---------- ---------- ---------- ---------- ----------  [120]
Babesia sp.BQ1(Ningxian) ---------- ---------- ---------- ---------- ---------- ----------  [120]
Babesia sp.Hebei         ---------- ---------- ---------- ---------- ---------- ----------  [120]
Babesia sp.Xinjiang      ---------- ---------- ---------- ---------- ---------- ----------  [120]

Babesia sp.BQ1(Lintan)   GQFGVGFYSA YLVADKVTVV SKNNNDDQYM WESSASGHFT VTKDESGEQL KRGTRLILHL  [180]
Babesia sp.Tianzhu       ---....... .......... .......... .......... .......... ..........  [180]
Babesia sp.BQ1(Ningxian) ---....... .......... .......... .......... .......... ..........  [180]
Babesia sp.Hebei         ---....... .......... .......... .......... .......... ..........  [180]
Babesia sp.Xinjiang      ---....... .........I ..H.D....V ...N...... I......... ......V...  [180]

Babesia sp.BQ1(Lintan)   KDDQSEYLEE RRLKDLVKKH SEFISFPIRL SVEKTTETEV TDDEAEATTA SESKDEEKIK  [240]
Babesia sp.Tianzhu       .......... .......... .......... .......... .......... ..........  [240]
Babesia sp.BQ1(Ningxian) .......... .......... .......... .......... .......P.P A.........  [240]
Babesia sp.Hebei         ....G..... .......... .......... .......... .......P.P A.........  [240]
Babesia sp.Xinjiang      .E........ ....E..... .......... .......... .......SET G.-.P....T  [240]

Babesia sp.BQ1(Lintan)   DVTDETENE- GEDAKEGEEK EGEKTAEKKK RKVTSVTREW EMLNKQKPIW MRLPTEVTHE  [300]
Babesia sp.Tianzhu       .........- .......... .......... .......... .......... ..........  [300]
Babesia sp.BQ1(Ningxian) .....A.K.- ..E.....D. ..D....... .......... .......... ..........  [300]
Babesia sp.Hebei         .....A.K.- ..E.....D. ..D....... .......... .......... ..........  [300]
Babesia sp.Xinjiang      .....VDK.E ...KDDEKQD DN..ADK... .......... .......... ....S...N.  [300]

Babesia sp.BQ1(Lintan)   EYASFYKNLC NDWEDHLAVK HFSVEGQLEF KALLFIPKRA PFDMFESRKK KNNIKLYVRR  [360]
Babesia sp.Tianzhu       .......... .......... .......... .......... .......... ..........  [360]
Babesia sp.BQ1(Ningxian) .......... .......... .......... .......... .......... ..........  [360]
Babesia sp.Hebei         .......... .......... .......... .......... .......... ..........  [360]
Babesia sp.Xinjiang      .......... .......... .......... .....V.... .......... ..........  [360]

Babesia sp.BQ1(Lintan)   VFIMDDCEEL IPEWLGFVKG VVDSEDLPLN ISREILQQNK ILKVIRKNLV KKCLELFSEL  [420]
Babesia sp.Tianzhu       .......... .......... .......... .......... .......... ..........  [420]
Babesia sp.BQ1(Ningxian) .......... .......... .......... .......... .......... ..........  [420]
Babesia sp.Hebei         .......... .......... .......... .......... .......... ..........  [420]
Babesia sp.Xinjiang      .......... .....S.I.. A......... ....V..... .......... ..........  [420]

Babesia sp.BQ1(Lintan)   TEKKEDFKKF YEQFSKNLKL GIHEDNANRT KIAELLRYET SKSGDEAISL KEYVDRMKPD  [480]
Babesia sp.Tianzhu       .......... .......... .......... .......... .......... ..........  [480]
Babesia sp.BQ1(Ningxian) .......... .......... .......... .......... .......... ..........  [480]
Babesia sp.Hebei         .......... .......... .......... .......... .......... ..........  [480]
Babesia sp.Xinjiang      .......R.. .D........ .........M .......... ......L... .........E  [480]

Babesia sp.BQ1(Lintan)   QKYIYYITGE SKQSVANSPF LEVLRSKGIE VIYMTDPIDE YAVQQIKEFE GKKLKCCTKE  [540]
Babesia sp.Tianzhu       .......... .......... .......... .......... .......... ..........  [540]
Babesia sp.BQ1(Ningxian) .......... .......... .......... .......... .......... ..........  [540]
Babesia sp.Hebei         .......... .......... .......... .......... .......... ..........  [540]
Babesia sp.Xinjiang      .......... ...T...... ..G..T.... .......... .......... ..........  [540]

Babesia sp.BQ1(Lintan)   NLELEDTEEE RKSFETLQKE MEPLCRVIKE ILHDKVEEVV CGKRFTESPC ALVTSEFGWS  [600]
Babesia sp.Tianzhu       .......... .......... .......... .......K.. .......... .......E..  [600]
Babesia sp.BQ1(Ningxian) .......... .......... .......... .......K.. .......... ..........  [600]
Babesia sp.Hebei         .......... .......... .......... .......K.. .......... ..........  [600]
Babesia sp.Xinjiang      ..D....... .....E.E.. .....HL... .......K.I .......... ..........  [600]

Babesia sp.BQ1(Lintan)   ANMERIMKAQ ALRDNNFGSF MISKKTMELN PHHSIMKELK QRAEADKSDK TLKDLVWLLY  [660]
Babesia sp.Tianzhu       .......... .......... .......... .......... .......... ..........  [660]
Babesia sp.BQ1(Ningxian) .........K .......... .......... .......... .......... ..........  [660]
Babesia sp.Hebei         .......... .......... .......... .......... .......... ..........  [660]
Babesia sp.Xinjiang      .......... .....S..N. .V........ .......... .......... ..........  [660]

Babesia sp.BQ1(Lintan)   DTAILTSGFN LDDPTQFGGR IYRMIKLGLS LDDDAAVEDV EIPSLDEVVV DPKMEEVD [718]
Babesia sp.Tianzhu       .......... .......... .......... .......... .......... ........ [718]
Babesia sp.BQ1(Ningxian) .......... .......... .........P .......... .......... ........ [718]
Babesia sp.Hebei         .......... .......... .......... .......... .......... ........ [718]
Babesia sp.Xinjiang      ...M...... .E........ .......... ...EPVA... .......... ........ [718]
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Annexe 4. Alignement de la séquence du gène ARNr de *Babesia* spp. et de *Theileria* spp. infectant les petits Ruminants

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Babesia ovis            AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT AAGTACAAGC TTTT-TACGG TGAAACTG
Babesia motasi          .......... .......... .......... .......... .......... .......... .......... ..G.-..... ........
Babesia sp.BQ1 (lintan) .......... .......... .......... .......... .......... .......... .......... ....-..... ........
Babesia sp. Xinjiang    .......... .......... .......... .......... .......... .......... .......... ....-..... ........
Theileria sp. China1    .......... .......... .......... .......... .......... .......... .....T.... ....A..T.. ........
Theileria sp. China2    .......... .......... .......... .......... ...C...... .......... .....T.... ....A..T.. ........

Babesia ovis            TACAACAGTT ATAGTTTATT TGGTATTCGT GTT--CCATG GATAACCGTG CTAATTGTAG GGCTAATACA AGTTCGATGC CTCT--GG
Babesia motasi          .......... .......C.. ...AG..... T..--..... .......... .......... .......... .......... ..T.T-..
Babesia sp.BQ1 (lintan) .......... .......C.. ...AG..... T..--..... .......... .......... .......... .......... ..T.T-..
Babesia sp. Xinjiang    .......... .......C.. .......... T..TT..... .......... .......... .......... .......... ..T.TT..
Theileria sp. China1    .......... .......... ..A.G..... T..T-A.... .......... .......... .......... T......GA. ..TCG-..
Theileria sp. China2    ..T....... .......... ..A.G..... T.CT-A.... .......... .......... .......... T......GA. ..TCG-..

Babesia ovis            GCTGTA-AAC CAACCTCGGT -------TCG GTGATTCATA GTAAACTTGC GAATCGC-TG CTT--GCGGC GATGGCCCAT TCAAGTTT
Babesia motasi          ...T.TA... ...TT.G--- --------.T. .......... A......... ..........--- T..----T.. ....TT.... ........
Babesia sp.BQ1 (lintan) ...T.TA... ...TT.G--- --------.T. .......... A......... ..........--- T..----A.. ....TT.... ........
Babesia sp. Xinjiang    .T.T.TT..A .CT.T.TTT. -------... .......... A......... ........A.. ...TT..... .......... ........
Theileria sp. China1    .ACC..A... ...A.CGCT. GCGGTTAA.. .......... A......... ........-A. ...TT..... ....TAT... ........
Theileria sp. China2    .ACC..A... ...A.CGCT. GCGGTGTC.. .......... A......... ........-A. ...CG..C.. ....TAT... ........

Babesia ovis            CTT--GACGG TAGGGTATTG GCCTACCGAG GCAGCAACGG GTAACGGGGA ATTAGGGTTC GATTCCGGAG AGGGAGCCTG AGAAACGG
Babesia motasi          ...--..... .......... .......... .......... .......... .......... .......... .......... ........
Babesia sp.BQ1 (lintan) ...--..... .......... .......... .......... .......... .......... .......... .......... ........
Babesia sp. Xinjiang    ...--..... .......... .......... .......... .......... .......... .......... .......... ........
Theileria sp. China1    ...--..... .......... ......T... ...A.G.... .......... .......... .......... .......... ........
Theileria sp. China2    ...TG..... .......... ........G. ...A.G.... .......... .......... .......... .......... ........

Babesia ovis            AGGAAGGCAG CAGGCGCGCA AATTACCCAA TCCTGACACA GGGAGGTAGT GACAAGAAAT AACAATACAG GGCT-ACTGT CTTGTAAT
Babesia motasi          .......... .......... .......... .......... .......... .......... .......... ...A-TA... ........
Babesia sp.BQ1 (lintan) .......... .......... .......... .......... .......... .......... .......... ...A-TA... ........
Babesia sp. Xinjiang    .......... .......... .......... .......... .......... .......... .......... ....-.T... ........
Theileria sp. China1    .......... .......... .......... .......... .......... .......... ........G. ....TTA... ........
Theileria sp. China2    .......... .......... .......... .......... .......... .......... ........G. ....T.AC.. ........

Babesia ovis            GACCCAAACC CTCGCCAGAG TACCAATTGG AGGGCAAGTC TGGTGCCAGC AGCCGCGGTA ATTCCAGCTC CAATAGCGTA TATTAAAC
Babesia motasi          ...TT..... ....G..... .......... .......... .......... .......... .......... .......... ........
Babesia sp.BQ1 (lintan) ...TT..... ..G.G..... .......... .......... .......... .......... .......... .......... ........
Babesia sp. Xinjiang    ...TT..... ...A...... .......... .......... .......... .......... .......... .......... ........
Theileria sp. China1    A.TTT..... TCTT...... ..T....... .......... .......... .......... .......... .......... .......A
Theileria sp. China2    A.T.T..... TCTT...... ..T....... .......... .......... .......... .......... .......... .......A
```

Annexe 5. Alignement des séquences du gène de l'ARNr 18S *Babesia* sp. BQ1 (Lintan) et *Babesia* sp. Xinjiang

```
Babesia sp. BQ1 (lintan) AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT AAGTACAAGC TTTTTACGGT GAAACTGCGA ATGGCTCATT  [ 100]
Babesia sp. Xinjiang     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 100]

Babesia sp. BQ1 (lintan) ACAACAGTTA TAGTTTCTTT GGAGTTCGTT TT--CCATGG ATAACCGTGC TAATTGTAGG GCTAATACAA GTTCGATGCC TTTT-GGCGG CGTTTATTAG  [ 200]
Babesia sp. Xinjiang     .......... .......... ..TA...... ..TT...... .......... .......... .......... .......... ....T..... ..........  [ 200]

Babesia sp. BQ1 (lintan) CTTTT-AAAC CAATTTGTT- -GGTGATTCA TAATAAACTT GCGAATCGCT T-------TA GCGATGTTCC ATTCAAGTTT CTGCCCCATC AGCTTGACGG  [ 300]
Babesia sp. Xinjiang     T....T.... .TC...T..T C......... .......... .........A .GCTTTTGCG ......GC.. .......... ...A...... ..........  [ 300]

Babesia sp. BQ1 (lintan) TAGGGTATTG GCCTACCGAG GCAGCAACGG GTAACGGGGA ATTAGGGTTC GATTCCGGAG AGGGAGCCTG AGAAACGGCT ACCACATCTA AGGAAGGCAG  [ 400]
Babesia sp. Xinjiang     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 400]

Babesia sp. BQ1 (lintan) CAGGCGCGCA AATTACCCAA TCCTGACACA GGGAGGTAGT GACAAGAAAT AACAATACAG GGCATATGTC TTGTAATTGG AATGATGCCG ACTTAAACCC  [ 500]
Babesia sp. Xinjiang     .......... .......... .......... .......... .......... .......... ...TAT.... .......... .......GT. ..........  [ 500]

Babesia sp. BQ1 (lintan) TGGGCAGAGT ACCAATTGGA GGGCAAGTCT GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC AATAGCGTAT ATTAAACTTG TTGCAGTTAA AAAGCTCGTA  [ 600]
Babesia sp. Xinjiang     .CAC...... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 600]

Babesia sp. BQ1 (lintan) GTTGAATTTG AGC-----TC GCGTGTGCGC CTTTTG-GCG AATCGCGTGC TT---TTGGT TTT----ACT TTGAGAAAAT TAGAGTGTTT CAAGCAGACT  [ 700]
Babesia sp. Xinjiang     .........C ...GTTGT.. T.....T.C. T.....C.G. TT...TC.A. ..CGC..T.. ...TATT... .......... .......... .......G..  [ 700]

Babesia sp. BQ1 (lintan) TTTGTCTTGA ATACTTCAGC ATGGAATAAT AGAGTAGGAC CTTGGTTCTA TTTTGTTGGT TTTGA--GCC TTGGTAATGG TTAATAGGAA CGGTTGGGGG  [ 800]
Babesia sp. Xinjiang     .......... ......G... .......... .......... .........T C......... ...TGTTA.. .......... .......... ..........  [ 800]

Babesia sp. BQ1 (lintan) CATTCGTATT TAACTGTCAG AGGTGAAATT CTTAGATTTG TTAAAGACGA ACTAGTGCGA AAGCATTTGC CAAGGACGTT TTCATTAATC AAGAACGAAA  [ 900]
Babesia sp. Xinjiang     .......... .......... .......... .......... .......... ....C..... .......... .......... .C........ ..........  [ 900]

Babesia sp. BQ1 (lintan) GTTAGGGGAT CGAAGACGAT CAGATACCGT CGTAGTCCTA ACCATAAACT ATGCCGACTA GGGATTGAAG GTCGTCATTT TTG---GACT CCTTCAGCAC  [1000]
Babesia sp. Xinjiang     .......... .......... .......... .......... .......... .......... .......G.. .......... ..CTTC.... ..........  [1000]

Babesia sp. BQ1 (lintan) CTTGAGAGAA ATCAAAGTCT TTGGGTTCTG GGGGGAGTAT GGTCGCAAGT CTGAAACTTA AAGGAATTGA CGGAAGGGCA CCACCAGGCG TGGAGCCTGC  [1100]
Babesia sp. Xinjiang     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  [1100]

Babesia sp. BQ1 (lintan) GGCTTAATTT GACTCAACAC GGGGAACCTC ACCAGGTCCA GACAGAGTAA GGATTGACAG ATTGATAGCT CTTTCTTGAT TCTTTGGGTG GTGGTGCATG  [1200]
Babesia sp. Xinjiang     .......... .......... .......... .......... ....A...T. .......... .......... .......... .........A ..........  [1200]
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Annexe 6. Alignement des séquences nucléotidiques des gènes BQP35 de *Babesia sp*. BQ1 (Lintan) et de la protéine hypothétique (XM_001610973) de *B. bovis*

```
BQP35                                GGAAAAGCAG TTTTGCTGCC GGTTGGTGGG TTAATTATAT TGGCTAAATT GACTGAGTGT TGCTGGAAAA AAGAGCACCA CTCACGTAAC GGAGTATCTG  [ 100]
B.bovis hypothetic protein XM_001610973 ---------- ---------- ---------- ---------- ---------- ------A..C C-T.C...C. ..AGCT.TGT T..CAAG.G. .A..A.AA.C  [ 100]

BQP35                                GACTTGTCGC TAACCGAGAT GAAGAAACGC GAGTTCAACA ACAACCAGCA GGATGCAATC CCAAGCGACG CTACCCCTGC TTCTAATATT GCATCTCAAA  [ 200]
B.bovis hypothetic protein XM_001610973 .-.GC...A. AGCAT.GT.. ......G..T .GAA.G...C GT---..... ...GT.GTC. .A..AT.GAA TACAT.A..A .CACTC.GC. AGTAT.A...  [ 200]

BQP35                                AAGTGGATGC GGCAGCGAGC GGGAAACCGG AGCGAAAAAA CGATGTGAAG GCAGCGCCGC GCAGCGACGA GCGTTCAAAT GCGAAAACAG AATCGAAGCA  [ 300]
B.bovis hypothetic protein XM_001610973 ..TCC...T. A.GCT.CTA. TCA..GT.A. .-TA.G..GC T..CC----. ...AAT.AT. .GT.GTTG.G .TA.CT..GC CT..GGTA.A ..CT.TGTT.  [ 300]

BQP35                                GGAATTAAAT GCAGAA--AA TACAA-ACGA TGGC--GCTG ATGCTACTTC ACTACCGGTT AATGGGCACA GGAACGTCGT CAACAAGATT GACGCGCTGC  [ 400]
B.bovis hypothetic protein XM_001610973 CC....C.CC .TCCG.CTGT ..T.GC.TAT .CA.AG.... CAAGC.G.C. .ACT..ACAG .GGC.T.C.. .C.T.AAT.C ...GGCAGC. TC.C.T.CC.  [ 400]

BQP35                                GCATGAGCTC GTATGCATCA GTTGCCAAAG GTGCAGCGAG CTTTCAGGAT GGGCAGAATT TCAGAGGGT- -ATTACCGT- AACGACCAGC ACCGTCGACG  [ 500]
B.bovis hypothetic protein XM_001610973 .....GA... C......... ........G. ..T.TA.TTC TAC.ACACC. .A..CCGCCC CTCC.C.ACC A.CA.TA..G .....A.G.. CT.....T..  [ 500]

BQP35                                TCCCGATAAC GATGCTGAGG AGGATGATGT GGTGAAGACT ATGATGAGGC TGTATCGCAT GAGGTATAAG GAGTTTAGGA TGCTTA---- -TCGAGAATC  [ 600]
B.bovis hypothetic protein XM_001610973 CATG..GC.T ..AC....T. .TA.G..CC. ...CCGT.T. ........AA ..C....... ...A.TCCGT ..T..C.A.. GAAACGAGGA T.T..AT.A.  [ 600]

BQP35                                CGTACCGCGA ACGCGATGCG GCAAA----C TCGCAGGAGC AGCG---CGC ATCACGCTCG GAACAAATCC ATAACTCGCT G-ACGTTTTT CTCT-GCATC  [ 700]
B.bovis hypothetic protein XM_001610973 AAC.G.AG.G T.ATATCA.A .....GCAGT .TAA...... .A.AAAAT.A ...T.AGAA. .....G.AAA C.G.AA...C AC.T..GAA. G.TAA.G...  [ 700]

BQP35                                TTCG-AGCAA CGATTCCGTG GTGGTACCGA CATCGAAGGA GGAT-CGGTT TCCATCCGGA GGTGATAGTG A--CGAGCTG CAGATCG--C GAGTGCACCC  [ 800]
B.bovis hypothetic protein XM_001610973 AG..T.ATG. ..TAG..... AAT.CG.-A. T.C..TTCTG CTT.ATC.CA G...C.TAA. C..TC.TTCA .TT.C..TAC TGAG..AAA. CT.A.T..TA  [ 800]

BQP35                                GATCT--GCC GAATTCTACA CTATCTGATG GTGTATCTGT CGACGTAGAC GAGCACAAC- --GAGGCTGC CGGCGATGA- --ATCCAACA CGTATGGAGC  [ 900]
B.bovis hypothetic protein XM_001610973 A....CA.GA ..G.GGA.A. GC.GG..T.. .G......AA ..GGCGT... A..GCTGGTT CA..AA...T TTCT.....G CA.C.G.CT. .TACCACCA.  [ 900]

BQP35                                AAGGCAGCGA CGTCACGATG GGGTACGCAT TAATTATTTC GACCGCAGAC GCAGGAATTT CAACGGACGA TTTCCAGCCG ATGGCGAAGA TCGCGGTTTC  [1000]
B.bovis hypothetic protein XM_001610973 .C.T.....G ...GCG..A. CCAGT..A.A CC.C.T.GCA TCTA....G. .T.AAGC.AC .GGT.AC..G CAGG..AA.. .G.AG..T.. A..GAAC.--  [1000]

BQP35                                AGCAGCTACG GCAGGTACAA AAATAAATAC AACGGTCATA ACAACACCAA ACGTGACTTT AAAAAAGATG AA-AAGCCAC AAGAGGCTTG ACATCATCCA  [1100]
B.bovis hypothetic protein XM_001610973 -AT.AT.... ...AA...CG T...CG.GTG C..CC..C.. .TGTA..A.. G..C..A..C .....G..A. .GCG..TTG. TCA..CT.GA .T.AAT..TT  [1100]

BQP35                                ACACGTGGAA TGTTAATAAT TCAATAGTAC ATTTGGCTGG GATTTCTCTT CAGCCTGTGA CAGAAAAAAA AAAAAAA [1177]
B.bovis hypothetic protein XM_001610973 TT.AC.AAT. ..A.------ ---------- ---------- ---------- ---------- ---------- ------- [1177]
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Annexe 7. Alignement des séquences en acides aminés de la protéine BQP35 de *Babesia sp*. BQ1 (Lintan) et de la protéine hypothétique (XP_001611023) de *B. bovis*

```
BQP35                                ---------- ---------- MKKREFNNNQ QDAIPSDATP ASNIASQKVD AAASGKPERK NDVKAAPRSD ERSNAKTESK QELNAENTND GADATSLPVN  [100]
B. bovis hypothetical protein XP_001611 MPSNKSYVSK SEENRASQHG ....GM.R-. .ESSQNGIHH DHSASIK.S. SGSYS.SDK. LTG.SSVVGV SKPEV..VLP IHRPTVIAYS Q.ASSPT.QR  [100]

BQP35                                GHRNVVNKID ALRMSSYASV AKGAASFQ-D GQNFRGYYRN DQHRRRPDND AEEDDVVKTM MRLYRMRYKE FR--MLIENP YRERDAANSQ EQRASRSEQI  [200]
B. bovis hypothetical protein XP_001611 RPSINAKAAS PP..D..... ...ST.TTPE PAPP.PTIV. ERP...MEHE PDDK.L.RI. ..MH...FRD .KRNEDLN.N S.VISQQS.L KEQQNE.QKE  [200]

BQP35                                HNSLTFFSAS SSNDSVVVPT SKEDRFPSGG DSDELQIASA PDLPNSTLSD G-VSVDVDEH NEAAG----- -DESNTYGAR QRRHDGVRIN YFDRRRRNFN  [300]
B. bovis hypothetical protein XP_001611 QKTE.PHVNV KDQRND.AVN AQYRSAL.QP PKRSFNSSTE SN.STKSQES .KAG.G.SNG RDK..SETVS DEQPT.TTT. ...AEAS.NH FASS..KATG  [300]

BQP35                                GRFPADGEDR GFSSYGRYKN KYNGHNNTKR DFKKDEKPQE A [341]
B. bovis hypothetical protein XP_001611 D.QANEEDE. NYN-..K.R. RVHPP.V... E...E.RVAQ . [341]
```

Annexe 8. Liste de publications de Guiquan Guan

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Annexe 9 : Article : Transmission of *Babesia sp*. to sheep with field-collected *Haemaphysalis qinghaiensis*. **Guan G.Q**., Yin H, Luo J.X., Lu W.S., Zhang Q.C., Gao Y.L., Lu B.Y. Parasitol. Res. (2002), 88:S22-S24.

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ORIGINAL PAPER

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Transmission of *Babesia* sp to sheep with field-collected *Haemaphysalis qinghaiensis*

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Abstract *Haemaphysalis qinghaiensis* ticks collected in the Gannan Tibet Autonomous Region were infested onto a sheep from a *Babesia*-free area. A strain of small *Babesia* (1.8–2.1 μm in length) was isolated from the sheep. Most of the *Babesia* in erythrocytes were round, oval, single pyriform, double pyriform, budding or elongated in form. Measurements were made of 100 single sides of the double-pyriform *Babesia* and compared with those for *B. motasi* and *B. ovis* from Holland, using Student's *t*-test. The Gannan small *Babesia* was similar to the *B. ovis* from Holland, but differed significantly from the Dutch *B. motasi*.

Introduction

Since *Babesia ovis* was first reported by Babes as the causative agent of ovine babesiosis in 1892, other *Babesia* species have been found to occur in sheep, like *B. crassa*, *B. foliata*, *B. motasi*, *B. ovis* and *B. taylori*, etc. (Levine 1985). Ovine babesiosis occurs over a wide geographic area, ranging from China in the east to Algeria in the west (Lewis et al. 1981). In China, ovine babesiosis has been reported in Sichuan and Heilongjiang Provinces (Chen 1982; Zhao et al. 1986) and in the eastern Gansu Province (Lian et al. 1997; Yin et al. 1996, 1997). The latter authors isolated and identified two strains as *B. motasi* and *B. ovis*. In the present study, we analysed the morphology, pathogenicity and transmission of *Babesia* sp by *Haemophysalis qinghaiensis*.

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Materials and methods

Animals

Sheep (5–7 months old) and goats (1.0–1.5 years old) were purchased from a *Babesia*-free area. They were splenectomized 6 months before experiments. Microscopic examination of blood films for *Babesia* showed them to be negative.

Ticks

Haemaphysalis qinghaiensis adults were collected in the field in Lintan County, Gannan Tibet Autonomous Region, in April 2000.

Transmission test

A total of 45 male and 15 female *H. qinghaiensis* collected in the field in the Gannan Tibet Autonomous Region were fed on an intact sheep (no. 2002). Every day after tick infestation, the rectal temperature was taken and blood smears were examined microscopically for the presence of haemoprotozoa.

Infection of animals

Aliquots of 15 ml of blood containing *Babesia* sp. (preserved in liquid nitrogen) were inoculated into splenectomized goats (nos. 9945, 9946). After inoculation, splenectomized goat 9946 was injected with dexamethasone at a dosage of 2 mg/day, for 3 days.

Morphological observation and morphometric analysis

When parasitaemia began to increase, blood smears from the ear vein were prepared, fixed in methanol, stained with Giemsa and examined microscopically. Measurements were made of 100 single sides of double-pyriform specimens of the Gannan *Babesia* sp., as illustrated by Uilenberg et al. (1980) and compared with *B. motasi* and *B. ovis* originating from Holland. The mean length of each strain was calculated and an analysis of variance was performed on the data, using Student's *t*-test.

Observation of pathogenicity

Every day after inoculation, the rectal temperature of each animal was taken. The erythrocyte level and haemoglobin level were also measured daily, using blood collected from the ear vein. Clinical symptoms were also observed daily.

Results

Transmission test

At 22 days after tick infestation, double-pyriform parasites were seen in the blood smears. When *Babesia* parasitaemia reached 10 parasites/100 viewing fields, blood was collected from sheep 2002 with anti-clotting agents and inoculated intravenously into an intact sheep (no. 2013). Two days after inoculation, parasites of *Babesia* sp. were seen in the blood smears. When parasitaemia reached 12 parasites/100 viewing fields, blood was collected with anti- clotting agents and preserved in liquid nitrogen.

Infection of animals

After blood containing *Babesia* sp. was inoculated into splenectomized goats 9945 and 9946, blood smears showed *Babesia* sp. on day 10 post-infestation and on day 8 post-inoculation, respectively. The parasitaemia of goat 9945 rose slowly, reached 1 parasite/3 viewing fields on day 14 post-inoculation and later reduced slowly. On day 19 post-inoculation, the parasitaemia was 0 parasites/100 viewing fields. The parasitaemia of goat 9946 increased rapidly and reached 1.5% on day 11 post-inoculation, when the goat was injected with Dexamethasone. On day 15 post-inoculation, parasitaemia reached 85% in goat 9946.

Morphological observation

The parasite described here was a small *Babesia* sp. During the early days of infestation, the most common forms were round, oval and single pyriform. Double pyriform, budding and elongated forms increased as parasitaemia increased. Various forms were described, as follows.

Double pyriform

The piroplasms measured 1.8–2.1 μm × 0.9–1.7 μm. The narrow end of each piroplasm linked them together. The angle between them was generally obtuse. The piroplasms was almost round, because the commonest form was wide. Either the nucleus was located in the narrow end of the piroplasm or there were nuclei at each end. The ratio was about 24%.

Single pyriform

The size of the piroplasms was 1.9–3.0 μm × 0.9–2.1 μm; and they were narrow and long. Most piroplasms had a two-lobed nucleus. The ratio was about 25%.

Round and oval

This form was thick and stained blue at the margin; and the central part was transparent. The piroplasms were 2.0–3.2 μm × 1.0–1.8 μm. The ratio was 27%.

Budding form

The central part is occupied by the nucleus. Two major leaves and a branching leaf extended in three orientations. This form was characteristic of *Babesia* sp. The ratio was 7%.

Elongated

When the parasitaemia was high, many elongated parasites appeared, shaped like an amoeba. The ratio was 17%.

Morphometric analysis

Morphometric analysis revealed significant differences in the long axis and short axis between double merozoites of *B. motasi* from Holland and *Babesia* sp. from Gannan, but *B. ovis* from Holland and *Babesia* sp. were similar.

Pathogenicity

The results concerning the pathogenicity of this parasite are presented in Table 1. It is evident that this strain was almost non-pathogenic for small ruminants. No tested animals showed the typical symptoms of babesiosis, except for one goat which was injected with Dexamethasone.

Discussion

According to data in the literature, the known vectors of *Babesia ovis* are *Dermacentor marginatus*, *Haemaphysalis punctata*, *Hyalomma anatolicum excavatum*, *Rhipicephalus bursa*, *R. turanicus*, etc. (Friedhoff 1997). However, none of these vector ticks of *B. ovis* has yet been identified in Gannan, China. In contrast, there are several reports indicating that the main ticks species are *D. silvarum, Haemaphysalis longicornis* and *H. qinghaiensis*, etc. in regions where ovine babesiosis occurs. It is clear from the results of the present study that *H. qinghaiensis* is the vector species of *Babesoa* sp in the Gannan Tibet Autonomous Region, China.

The morphological observations on *Babesia* sp from Gannan were in agreement with *B. ovis* described by Lian et al. (1997) and Yin et al. (1997) for strains isolated in eastern Gansu, China. The size agreed with the

Table 1 Reactions of intact sheep and splenectomized goats to infection with *Babesia* sp. (Gannan strain). Animals 2002 and 2013 were intact sheep, animals 9945 and 9946 were splenectomized goats. *Hb* Haemoglobin, *RBC* red blood cells

Animal no.	Maximum parasitaemia (%)	Prepatent period (Days)	Body temperature (°C)	Haematological counts			
				RBC		Hb	
				($\times 10^{-4}$/l)		(mg/l)	
				Low	High	Low	High
2002	10	1	40.0	1032	1082	800	1020
2013	12	1	40.2	950	1115	820	920
9945	33	2	40.2	–	–	–	–
9946	85	5	41.2	382	701	410	720

ranges of *B. ovis* given by Ristic and Kreier (1981; 1.0–2.5 μm) and Papadopoulos et al. (1996; 1.4–2.2 μm), but differed a little from the size given by Habela et al. (1990; 1.12 μm × 0.23 μm). Budding-form parasites similar to *B. motasi* (Lewis et al. 1981; Alani and Herbert 1988) and *B. ovis* (Lian et al. 1997; Yin et al. 1997) were observed. No cross forms were found, as described by Yeruham et al. (1998). Although four pyriform parasites were seen in blood smears, those were more similar to two pairs of splitting double pyriforms. Using Student's *t*-test, morphometric analysis of double pyriforms (length 1.8–2.1 μm, width 0.9–1.7 μm) in smears showed they were similar to *B. ovis* from Holland, but significantly different from *B. motasi* from Holland. However, the identity of the *Babesia* sp described here needs to be further studied as very little information has been known about it.

B. ovis is considered to be highly pathogenic (Ristic and Kreier 1981; Ristic 1988; Friedhoff 1997). The mortality rates in susceptible sheep ranged from 30% to 50% after experimental infection or field infection (Friedhoff 1997). The results of the laboratory experiment demonstrated that *B. ovis* from Ganan, like that isolated from Somalia (Edelstein 1975), is not pathogenic for intact sheep and goats. Nevertheless, it is possible that strains from different regions differ in their virulence. In the Gannan Tibet Autonomous Region, ovine babesiosis never occurs naturally in sheep and goats; and *Babesia* spp have not been detected by local veterinarians in small ruminants yet. This confirms that the strain of *B. ovis* described here is non-pathogenic for sheep and goats.

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CARACTÉRISATION BIOLOGIQUE ET MOLÉCULAIRE DE *BABESIA SPP.* INFECTANT LES PETITS RUMINANTS EN CHINE

La babésiose ovine est une maladie transmise par les tiques, due à un hémoprotozoaire du genre *Babesia*.. Dans cette étude, les isolats chinois de *Babesia* ovines, *Babesia sp.* BQ1 (Lintan) et *Babesia sp*. Xinjiang ont été caractérisés d'un point de vue biologique et moléculaire. Les séquences des gènes Hsp90 et les réactions croisées montrent que ces babésies peuvent être séparées en 2 groupes : le groupe *Babesia sp*. Xinjiang et le groupe *B. motasi*-like, celui-ci étant lui-même divisé en 2 sous-groupes, le groupe "pathogène" et le groupe "non pathogène". Les expériences de transmission ont montré que les tiques vectrices de *Babesia* sp. BQ1 (Lintan) sont *Haemaphysalis qinghaiensis* et *H. longicornis* et celle de *Babesia* sp. Xinjiang est *Hyalomma anatolicum anatolicum.* D'après les données biologiques et moléculaires, *Babesia sp*. Xinjiang peut être considéré comme une nouvelle espèce de babésie ovine. Les études de la sensibilité de 2 races de moutons à *Babesia* sp. BQ1 (Lintan) et à *B. divergens* ont montré que la résistance génétique des hôtes et la sensibilité des individus sont liées à la capacité des érythrocytes à multiplier le parasite. L'étude de la réponse immunitaire révèle que l'IFNγ est associé à la faible infectivité de *B. divergens* et l'IL10 à la capacité plus importante de *Babesia sp*. BQ1 (Lintan) à infecter les moutons. Trois méthodes de détection des infections à *Babesia* sp. BQ1 (Lintan) ou à *Babesia* sp. Xinjiang ont été développées, chacune apportant des renseignements différents sur le stade de l'infection. Des antigènes potentiels identifiés, comme BQHsp90, BQp35, Hsp70, p200, sont prometteurs pour le développement de sero-diagnostic dans le futur.

BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF *BABESIA* SPP. INFECTIVE TO SHEEP AND GOATS IN CHINA

Ovine Babesiosis caused by genus *Babesia*, is a tick-born haemoprotozoan disease. In this study, Chinese ovine *Babesia* strains, especially *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang have been characterized biologically and molecularly. Based on Hsp90 gene sequences and cross-reactions, these *Babesias* could be separated into 2 groups, *Babesi*a sp. Xinjiang group and *B. motasi*-like group which could be further divided into two subgroups, pathogenic subgroup and non-pathogenic subgroup. Experimental transmissions verify that vector ticks of *Babesia* sp. BQ1 (Lintan) are *Haemaphysalis qinghaiensis* and *H. longicornis* and that of *Babesia* sp. Xinjiang is *Hyalomma anatolicum anatolicum*. On the basis of evidence from molecular phylogeny and biological characteristics, *Babesia sp*. Xinjiang should be a novel *Babesia* species. Studies of susceptibility of two sheep breeds to each *Babesia* sp. BQ1 (Lintan) and *B. divergens* indicated that host genetic resistance and individual susceptibility to *Babesia* spp. have relationship with ability of erythrocytes to sustain multiplication of the parasites. Test of immune responses reveals IFNγ is associated with lower infectivity of *B. divergens* and IL10 with higher infectivity of *Babesia* sp. BQ1 (Lintan) to sheep. Three detection methods for *Babesia* sp. BQ1 (Lintan) or *Babesia* sp. Xinjiang infection have been developed, in vitro culture, Loop-mediated Isothermal Amplification (LAMP) and indirect enzyme-linked immunosorbent assay (ELISA) that could provide different information about the infection. Potentially diagnostic antigens identified, such as BQHsp90, BQp35, Hsp70, p200, are promising in application of serological diagnosis in future.