Al-Kufa University Journal for Biology / VOL.9 / NO.2 / Year: 2017
Print ISSN: 2073-8854 & Online ISSN: 2311-6544

Molecular Detection and Genotyping of Babesia species in cattle in Baghdad city.

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Abstract

In cross-sectional study carried in areas surrounding Baghdad province (Dora , Nahrawan, Tajy and Abu Graib)respectively. During June -December 2016, for detection of cattle ***Babesiosis***. One hundred fifty (150) clinically healthy local cattle breeds of different ages, and from both sex were included by **molecular** detection of Babesiosis by **Conventional polymerase chain reaction PCR** . Results revealed that the species of ***Babesia*** during this study were ***Babesia bovis*** and they were detected in 15/150 of cases (10%), **Gene sequencing** and **phylogenetic tree** were done for the isolated species and the results revealed the similarity of our detected species with other species of neighbor countries published in Gene Bank for ***Babesia bovis*** with little differences .infection detected in all parts of the study and included all age groups of cattle. Our isolates showing clear gene Diversity compared with others genes recorded in neighboring countries.

Keywords*: Babesia bovis,* PCR ,phylogenetic tree, Gene Bank.

Introduction:

Bovine Babesiosis, also called red water disease or Piroplasmosis of cattle are protozoan parasitic diseases caused by Genus ***Babesia*** Order ***Piroplasmida*** Phylum ***Apicomplexa.*** (1).
Babesia bigemina and ***Babesia bovis*** are the most widely distributed species affecting cattle in most part of the world except some part of Europe were ***Babesia divergens*** are the most prevalent .(2).
Diagnosis of Babesiosis is same as for other blood protozoan infections were the conventional diagnostic methods are still depend on the demonstration of infective stages in the blood , by Giemsa stained blood smear were it consider the gold standard for diagnosis, beside detection of circulating antibodies in the serum of infected animals by serological test like Enzyme Linked Immunosorbent Assay (ELISA) (3), (4).More over Polymerase chain reaction(PCR) had been widely used for detection of ***Babesia*** parasites owing to their high specificity and sensitivity (5),(6). Because of shortage in studies concerning molecular and genotyping of bovine babesiosis in Iraq and specially in Baghdad province , beside the current study aimed to sight a light on ***Babesia*** species genotype of cattle of Baghdad province

Materials and Methods:

1-Collection of Blood samples

Blood sample were collected from 150 apparently healthy cattle from different parts of Baghdad Province (96 female and 54 male), with ages ranging between 1-10 years, during the period between June 2016 –December 2016.Blood were collected by jugular vein puncture by vacutainer tube , coated with EDTA for PCR technique.

2-Direct Detection of Babesia species in blood sample:

1-Conventional PCR (nested) PCR technique were used according to (5) and manufacturer instructions (Inqaba Biotechnical Industries, Pretoria, South Africa).This procedures includes DNA extraction and primer design according to(Inqaba Biotechnical Industries, Pretoria, South Africa).

2-DNA Extraction:

- The blood sample had mixed for 10 minutes at room temperature.
- A volume of 20 µl of Proteinase K (PK) had dispensed into a 1.5 ml micro-centrifuge tube.
- From each sample, 250 µl of blood was added to the tube containing the Proteinase K (PK) solution, and briefly mixed.
- Cell lysis Buffer (CLD) had added in avolume of 200 µl into a tube. This tube has capped and mixed by vortexing for 10 seconds
- In water bath, tubes had incubated at 56 c˚ (optimal for Proteinase activity) for 10 minutes .
- While the blood sample had incubated , a ReliaPrep™ Binding Column had placed into empty collection tube.
- After tube removing from water bath incubation, a volume of 250 µl of binding Buffer (BBA) had added for each tubes. These tubes had capped and mixed by vortexing for 10 minutes.
- Tubes contents had added to the labeled ReliaPrep ™ Binding Column, there for the column has capped and placed in microcentrifuge.
- Centrifugation had conducted for 1 minute at maximum speed.
- Collection tubes containing the flow through had removed, and the liquid has discarded as hazardous waste.
- Binding columns had placed into a new collection tubes A volume of 500 µl of column wash solution (CWD) has added to the column and centrifuged for 3 minutes at maximum speed through that the flow has excluded.
- Washing steps had repeated twice for three washes.
- Columns had placed in a clean 1.5 ml microcentrifuge tubes.
- Volume of 200 µl of nuclease-free water had added to the column and centrifuged for 1 minute at maximum speed.
- Binding Column has discarded and the eluate has saved at -30 c˚. (7).

3- Determination of DNA Concentration and Purity:

Quantusflorometer used to detect the concentration of the extracted DNA in order to detect the quality of samples for downstream application. For 1 µl of DNA , 199 µl of diluted Quanty flour dye has mixed. There for, after incubation at room temperature , DNA concentration values had detected.(8).

4-Detection of Presence of *B.bigemina* and ***B.bovis*** :

Purified DNA samples had used to assess the specificity of Group 1 and Group 11 primers (Table 1), that described by (5).

Detection the presence of ***B.bigemina*** and ***B.bovis* ,** from study blood samples , using Grpup 1 primer , PCR product has added into the second (nested) PCR mixture compromising similar composition of reagents as the first-round PCR except that the external primers had replaced with the nested PCR primers.

Table (1) : Primers Used for PCR Amplification

Name	Seq.	Tm	Size
B. bigemina PCR-F	CATCTAATTTCTCTCCATACCCCTCC	55	278
B. bigemina PCR-R	CCTCGGCTTCAACTCTGATGCCAAAG		
B. bigemina nPCR-F	CGCAAGCCCAGCACGCCCCGGTGC	55	170
B. bigemina nPCR-R	CCGACCTGGATAGGCTGTGTGATG		
B. bovis PCR-F	CACGAGGAAGGAACTACCGATGTTGA	55	360
B. bovis PCR-R	CCAAGGAGCTTCAACGTACGAGGTCA		
B. bovis nPCR-F	TCAACAAGGTACTCTATATGGCTACC	55	298
B. bovis nPCR-R	CTACCGAGCAGAACCTTCTTCACCAT		

Phylogenetic Analysis:

For phylogenetic study , genomic DNA of randomly selected field sample was amplified with Group11 nested PCR primers (Table 2) targeting gp45 and rap-1 fragments of ***B.bigemina*** and ***B.bovis*** , respectively (5).

Ten bovine samples has selected for ***B.bigemina*** and 10 (ten) for ***B.bovis***.PCR mixture were prepared and randomly cycled, were described in following ,PCR generated fragments of 853 bp for ***B.bigemina*** and 1009 bp for ***B.bovis.***

Table (2) Phylogenetic analysis of *B.bovis* and *B.bigemina*.

Name	Seq	Tm	Size
G2_*B. bigemina* PCR-F	GTGCTGCTTAATCGCACAAAC	55	963
G2_*B. bigemina* PCR-R	AAGATGCCTTCTTCGGTGATG		
G2_*B. bigemina* nPCR-F	CGGATCCTGTTATCGTTCCTG	55	853
G2_*B. bigemina* nPCR-R	GAAGTTACGCCTGGAGTTGG		
G2_*B. bovis* PCR-F	TCAGATTGTTCAAAGAGAGTGCATCC	55	1280
G2_*B. bovis* PCR-R	GTCTTCACCGTTGGAAGTAGTTGAGTC		
G2_*B. bovis* nPCR-F	CACGAGGAAGGAACTACCGATGTTGA	55	1009
G2_*B. bovis* nPCR-R	CCTTTGTAGGTTGGCCAACAGTTTCG		

Detection of Babesia species in purified DNA samples;

For the detection of the presence of **B.bovis** and **B.bigemina** in study DNA samples by using primers in table(1), PCR was performed according to manufacturer instructions (Inqaba Biotechnical Industries). Negative control reactions contained distilled water instead of template DNA.

PCR generated amplicons were analysed by electrophoresis in 1.5 % agarose gels stained by Biotium GelRed Acid Stain (Anatech Instruments,Johannesburg,South Africa) under UV illumination.

GeneRuler 1 kb plus DNA ladder were used as the standard molecular weight marker.

PCR product were sent for sanger sequencing using ABI3730XL, automated DNA sequencer, The results were received and analyzed using genious software.(Macrogen Corporation-Korea .)

Reference Strains:

DNA Sequences of 8 ***Babesia bovis*** isolates were compared with that of *Babesia bovis* isolate vaccine rhoptry associated protein 1 (*rap-1*) gene, partial cds (KM504166.1)] and [*Babesia bovis* isolate T21 rhoptry associated protein 1 (*rap-1*) gene, partial cds (KM504165.1)] (as reference strains) by National Center for Biotechnology Information (NCBI/BLAST) to know the identity between strains.Fasta of ***B.bovis*** isolates.

Phylogenetic Analysis:

Phylogenetic analysis for the sequenced genes was performed using ClustalW in MEGA 6, the neighbor-joining method to identify the genetic similarity and build phylogenetic tree for 8 *Babesia bovis* isolates and the two reference strains.

Computational Studies:

Several computerized programs and databases were used through this study for different purposes, as shown in table (3).

Table (3): Computerized programs and databases.

Programs & Database	Website	Purpose
NCBI Database	http://www.ncbi.nlm.nih.gov	To obtain the publicly available sequences of two reference strains
NCBI/ BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	To do Blasting for sequences to select the Reference strains
Mega V.6	http://www.megasoftware.net/mega.php	To do Multiple Sequence Alignment for phylogenetic analysis

5-Statistical Analysis:

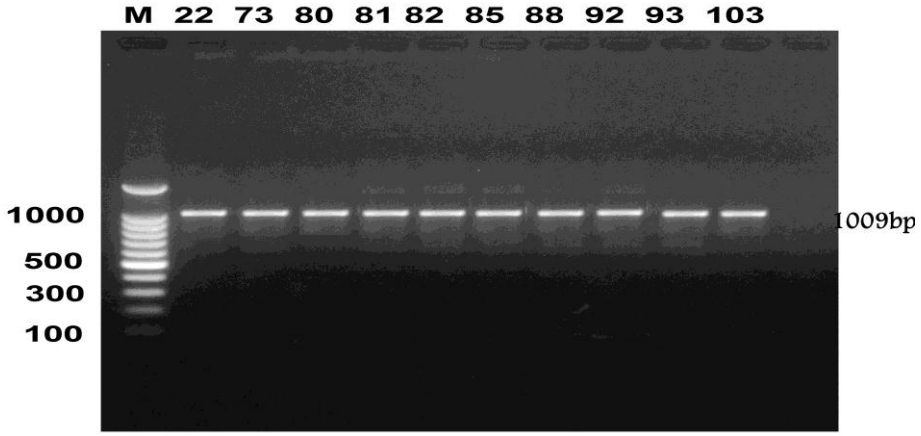
Results statistical analysis were carried using SPSS –version 14,using t-test and chi-square test according to (9).

Results and Discussions:

Among PCR, results of this study revealed that 10% of samples were infected with ***B.bovis*** without recorded any infection with ***B.bigemina*** as shown in table (4) . Figure (1).

Table (4) .Nested PCR results according to species of ***Babesia.***

POLYMERASE CHAIN REACTION TEST		SEX						
		Females		Males		Total		
		No	%	No	%	No	%	P.V
B.bigemina	negative	96	100.00	54	100.00	150	100.00	
	positive	0	.00	0	.00	0	.00	
B.bovis	negative	90	93.75	45	83.33	135	90.00	0.041*
	positive	6	6.25	9	16.67	15	10.00	
	Total	**96**	**100.00**	**54**	**100.00**	**150**	**100.00**	



Figure(1);PCR results showing positive pool samples on gel at 1009 bp for ***B.bovis***. Lane M(100-1000 bp DNA ladder) , Lane (22,73,80,……..103) positive PCR product amplified from study blood samples.

Results were not agreed with most of studies concerning molecular prevalence of cattle Babesiosis , like (10) in Iraq , and(111) in Pakistan who recorded the presence of both ***B.bovis*** and ***B.bigemena*** in their studies, this is may due to difference in study area and study sample or using different genes and different primers design may play important role in this differences.

Among age group of cattle examined by PCR , age group (4-6) years showed the highest rate of infection(73.33%) and the lowest infection rate (6.67%) were recorded in age group(7-9) years with significant difference p=0.016 , table (5).

Table (5): Distribution of cases according to age groups.

AGE	PCR B.bovis					
	Negative		Positive		Total	
	N0	%	N0	%	N0	%
1-3	64	47.41	2	13.33	66	44.00
4-6	43	31.85	11	73.33	54	36.00
7-9	15	11.11	1	6.67	16	10.67
10	13	9.63	1	6.67	14	9.33
Total	**135**	**100.00**	**15**	**100.00**	**150**	**100.00**

χ^2=10.392 df =3 p= 0.016

These results were in agreement with (13) who recorded (83.3%) in animals more than 3 years old, while our results disagree with (14) who declared that were no significantly difference between infection rate in young and older cattle infected with ***Babesia bovis* .**

Distribution of cases by PCR according to study areas showed that Al Tajy and AbuGraib recorded the highest rate of infection (40%) for both areas , followed by Al Doura (13.33%) , and the lowest rate of infection was recorded in AlNahrawan(6.67%) with these results were shown in table(6).These results indicated that Al Tajy and AbuGraib considered as endemic areas with Babesiosis for the availability of the tick vectors.

Table (6): Distribution of case according to study areas.

REGOIN	PCR B.bovis					
	Negative		Positive		Total	
	No	%	No	%	No	%
AL DOURA	46	34.07	2	13.33	48	32.00
AL NAHRAWAN	24	17.73	1	6.67	25	16.67
AL TAJY	39	28.9	6	40.00	45	10.00
ABU GRAIB	26	19.3	6	40.00	32	41.33
Total	**135**	**100.00**	**15**	**100.00**	**150**	**100.00**

χ^2=17.822 df= 3 p=0. 0001*

Among months of the study during 2016 the result of PCR showed that September 2016 showed the highest rate of infection (46.67%) followed by August 2016 and October 2016 with (20.00%) rate of infection for both months. July 2016 showed the lowest rate of infection (2%) . Table (7).these findings were in agreement with (15) and (16).

Table(7): Distribution of cases according to months of the study.

MONTHS	PCR B.bovis					
	Negative		Positive		Total	
	No	%	No	%	No	%
July 2016	37	27.41	2	13.33	39	26.00
August 2016	37	27.41	3	20.00	40	26.67
Sept 2016	22	16.30	7	46.67	29	19.33
Oct 2016	39	28.88	3	20.00	42	28.00
Total	**135**	**100.00**	**15**	**100.00**	**150**	**100.00**

χ^2=8.470 df =2 p=0.076

DNA Sequencing:

DNA Sequences of 18 *Babesia bovis* isolates were compared with that of two reference strains by NCBI/BLAST. The identity of *Babesia bovis* isolate vaccine rhoptry associated protein 1 (*rap-1*) gene, partial cds (KM504166.1) was (99%) and only 1% variability, this return to the diversity between strains, while *Babesia bovis* isolate T21 rhoptry associated protein 1 (*rap-1*) gene, partial cds (KM504165.1) was shown identity (98%).and only 2% variability.

Phylogenetic Analysis:

Multiple sequence alignments were performed using ClustalW in MEGA6 (*Tamura etal.,2013*). Phylogenetic tree was constructed using the neighbor-joining method. Figure (1.1) show the genetic similarity between reference strains *Babesia bovis* isolate vaccine rhoptry associated protein 1 (*rap-1*) gene, partial cds (KM504166.1) and *Babesia bovis* isolate T21 rhoptry associated protein 1 (*rap-1*) gene, partial cds (KM504165.1)] and 8 *Babesia bovis* isolates according (*rap-1) gene*. The variability percentage was (0.01), this meaning there is variability in one nucleotide for each 100 nucleotides.

Figures(2), showed neighbor-joining trees based on rap-1 gene sequences. (KM504166.1) and (KM504165.1). As reference strains.(Mega v.6).

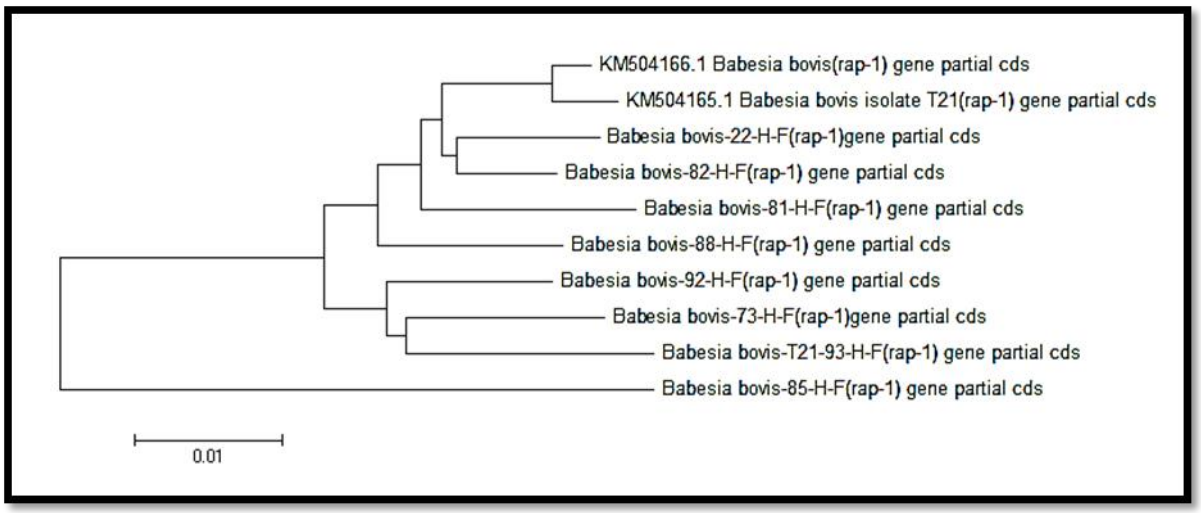


Figure (2): Neighbor-joining trees based on *rap-1* gene sequences. (KM504166.1) and (KM504165.1) (Reference strains), (Mega v.6).

Al-Kufa University Journal for Biology / VOL.9 / NO.2 / Year: 2017
Print ISSN: 2073-8854 & Online ISSN: 2311-6544

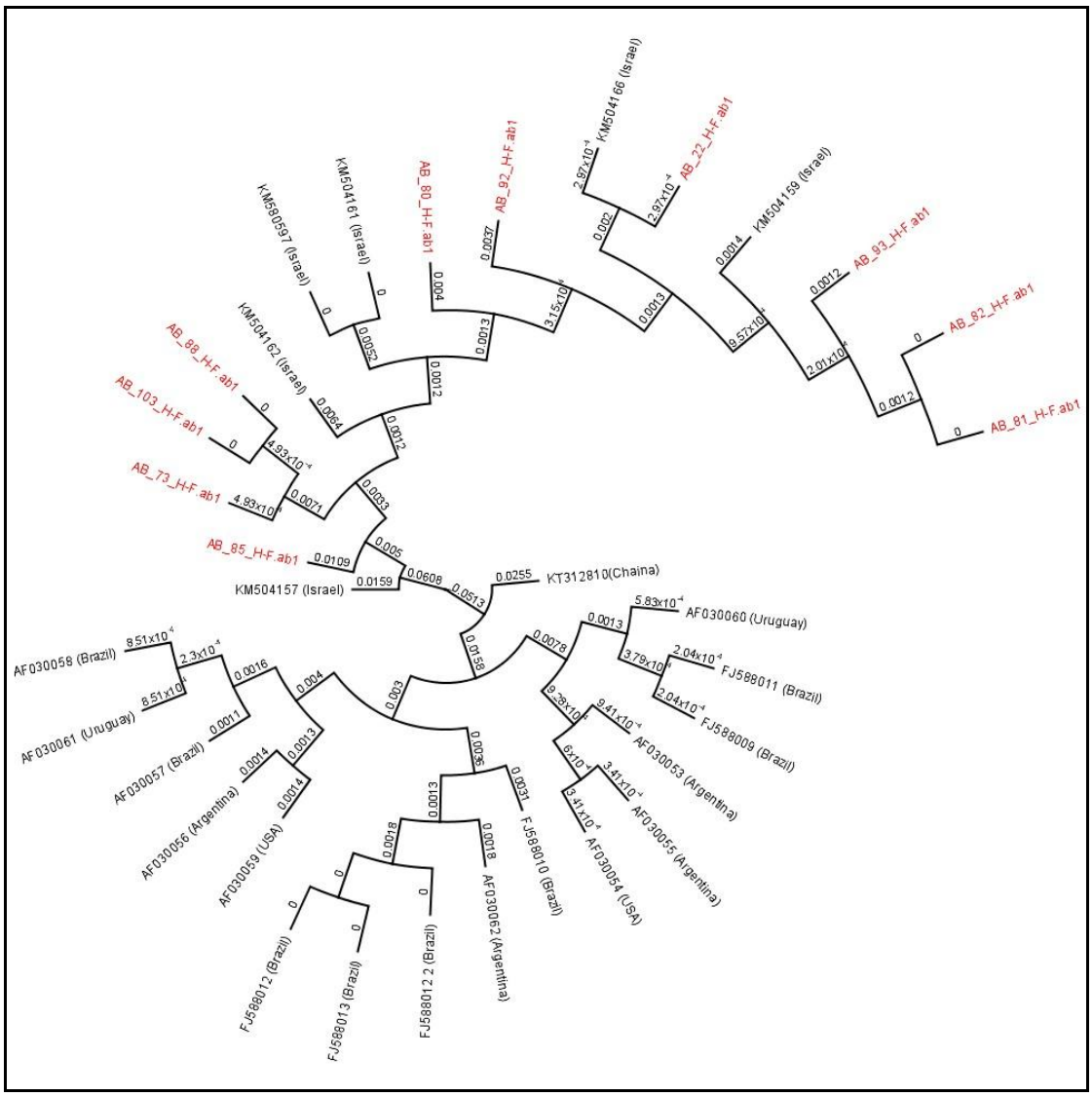


Figure.(3)Unrooted phylogenetic tree of *Babesia bovis* rap-1 gene. The tree was constructed with the maximum likelihood method using Gneious program.The study sequence are shown in red font.

	KT312...	FJ5880...	FJ5880...	FJ5880...	AF030...	FJ5880...	AF030...	AF030...	AF030...	AF030...	AF030...	FJ5880...	FJ5880...	AF030...	AF030...	AF030...	AF030...
KT312810(Chaina)		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
FJ588013 (Brazil)	0.05		0	0	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
FJ588012 (Brazil)	0.05	0		0	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
FJ588012 2 (Brazil)	0.05	0	0		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
AF030062 (Argentina)	0.05	0.00	0.00	0.00		0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
FJ588010 (Brazil)	0.05	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
AF030061 (Uruguay)	0.05	0.01	0.01	0.01	0.01	0.01		0.00	0.00	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
AF030058 (Brazil)	0.05	0.01	0.01	0.01	0.01	0.01	0.00		0.00	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
AF030057 (Brazil)	0.05	0.01	0.01	0.01	0.01	0.01	0.00	0.00		0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
AF030059 (USA)	0.05	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.00	0.02	0.02	0.02	0.02	0.02	0.02
AF030056 (Argentina)	0.05	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00		0.02	0.02	0.02	0.02	0.02	0.02
FJ588011 (Brazil)	0.05	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02		0.00	0.00	0.00	0.00	0.00
FJ588009 (Brazil)	0.05	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00		0.00	0.00	0.00	0.00
AF030060 (Uruguay)	0.05	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00		0.00	0.00	0.00
AF030055 (Argentina)	0.05	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00		0.00	0.00
AF030054 (USA)	0.05	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00		0.00
AF030053 (Argentina)	0.05	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.00	
KM580597 (Israel)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
KM504161 (Israel)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
KM504166 (Israel)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_22_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
KM504159 (Israel)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_93_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_82_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_81_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_92_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_80_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
KM504162 (Israel)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_103_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_88_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_73_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
AB_85_H-F.ab1	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
KM504157 (Israel)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

Figure (4) : Distance matrix of differences between ten local isolates of ***B.bovis*** and standard strain with Accession number of each strain.

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