Determine the prevalence of *Brucella* spp. and *Leptospira* spp. in blood samples by multiplex polymerase chain reaction collected from cattle, sheep and goats in herds located in provinces of Iran

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Abstract

Leptospirosis and brucellosis are common zoonosis that affect many species of mammals mostly causing economical losses. Further, very important fact is huge danger for human and animal health around the world. The purpose of the study is to determine the prevalence of *Brucella* spp. and *Leptospira* spp. using multiplex polymerase chain reaction (mPCR) method, in blood samples collected from cattle, sheep and goats. In this study, a total number of 250 blood samples (5 cc of blood with ethilen diamin tetra asetic acid) were collected randomly from 100 cattle, 80 sheep and 70 goats located on 6 herds in Chaharmahal Va Bakhtiari and Esfahan provinces, Iran. After DNA extraction and setting of mPCR for *Brucella* spp. and *Leptospira* spp. mPCR products were screened. The DNA of these microorganisms was detected by multiplex PCR from 31 and 21 out of 100 cattle, respectively. Four of 70 goat's blood samples from goat breeding farms were positive for *Leptospira* spp. and 11 were positive for *Brucella* spp. Out of 80 sheep blood samples 23 were positive for *Brucella* spp. and 14 for *Leptospira* spp. The results of the present study show ruminant as an important reservoir for transmission of these zoonotic diseases to humans in Iran. mPCR has the ability to concurrently detect both *Brucella* and *Leptospira* species from blood samples of ruminants. The convenience and the possibility of detection of both bacteria at a time, strongly support the use of this mPCR for routine diagnostics.

Introduction

Zoonosis or diseases transmitted from animals to human have been recognized as significant public health concerns for period of one hundred years. Much of the primary history of veterinary science was focused on the control of diseases and many emerging and re-emerging infectious disease problems internationally are zoonotic. Leptospirosis and brucellosis are common zoonosis that affects many species of mammals, causing economical losses. Further, very important fact is huge danger for human and animal health (domestic and wild animals as well as humans) around the world.[1]

Brucellosis is caused by bacteria of the genus *Brucella* spp. It is a facultative intracellular gram-negative aerobic bacterium. Brucellosis is an important global infectious disease. It is characterized by reproductive failure in females and sterility in males. In males it causes a range of symptoms, characterized by undulating fever and it is one of the most ancient described zoonosis.[2] It is particularly common in Middle East, South Europe and North Africa.[3] Brucellosis is the most important infection of cattle causing abortion in Iran, as well.[4,5]

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection of pathogenic motile spirochetes bacterium belonging to the genus *Leptospira* spp. They infect a range of hosts, involving mammals, reptiles, amphibians and birds. They pose an important public health problem of increasing concern and have a great impact on the reproductive efficiency of livestock.[6]

The significance of these diseases is increased by many factors such as fast spreading, time-consuming treatment, difficulty of control and prevention and expensive therapy. These diseases cause dangerous consequences like prevention of trading animals and animal products; moreover these diseases inhibit public and economic development of breeders commonly found on pastoral regions.[7] Both of these bacteria cause abortion in infected ruminants. Moreover, these infections are zoonotic and populations such as veterinary surgeons, farm workers, shepherds and abattoir workers are at high risk. *Leptospira* spp. and *Brucella* spp. may be excreted from affected animals in urine in large amount. This is a base of transmission between animals or from animal to human by a direct contact or through with urine contaminated food and water.[8,9]

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Nowadays, there are many laboratory techniques for diagnosis of leptospirosis and brucellosis in human and animals. *Brucella* spp. and *Leptospira* spp. can be serologically detected,[10,11] nevertheless many factors may cause false positive and negative results and even cross reactions with other factors such as *Salmonella* spp., *Yersinia* spp. and *E. coli.*[12-14] Bacteriological isolation is generally used for diagnosis. Though, the isolation of both pathogens, is usually time-consuming, difficult and laborious.[15-18] Molecular diagnosis based on polymerase chain reaction (PCR) has been successfully described for the detection of *Leptospira* spp. and *Brucella* spp.[19] To overcome these diagnostic problems molecular methods, for example PCR, have been used for increased specificity and sensitivity. Many PCRs have been developed for the detection of *Brucella* spp. and *Leptospira* spp. and it's practical to extract DNA from bacterial cells, tissue samples and blood.[15,16,20-23]

Multiplex PCR (m-PCR) is a precise PCR resulting procedure where multiple targets DNA sequences can be detected in a single reaction.[24,25] Application of this assay to *Brucella* spp. and *Leptospira* spp. can easily detect these organisms at the same time from infected animals. mPCR diagnostics would

help to overcome these difficulties and increase the diagnostic capacity of PCR. mPCR is cost effective and has the potential to save considerable amounts of time.

The purpose of this study is to determine the prevalence of *Brucella* spp. and *Leptospira* spp. in blood samples collected from cattle, sheep and goats in 6 herds located in Chaharmahal Va Bakhtiari and Esfahan provinces (Iran) using mPCR method. mPCR has the ability to concurrently detect both *Brucella* spp. and *Leptospira* spp. from blood samples of ruminants. Understanding of these diseases in the cattle and sheep populations is serious for both, the veterinary and public health services. Practical priorities must be set and controls must be implemented.

Materials and Methods

Samples and DNA extraction

For the purpose of testing, from February 2013 to March 2013, 250 blood samples (jugular vein sampling) were collected randomly from 100 cows, 80 sheep and 70 goats (more than 8 months of age) from 6 herds located in Chaharmahal Va Bakhtiari and Esfahan provinces (Iran). Five cc of blood with EDTA (ethilen diamin tetra asetic acid) was collected from jugular vein of each sheep, cattle and goat. All samples were sent under refrigeration to the Biotechnology Research Centre of Islamic Azad University of Shahrekord. It was stored at -20°C until DNA extraction. DNA was extracted from blood samples by DNA extraction and purification kit (Cinnagen, Tehran, Iran) according to manufacturer's instructions. The extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell.[26]

Primers, DNA amplification and screening of polymerase chain reaction products

In the study oligonucleotide primer sequences were applied, as described by Baily *et al.*[15] and Theodoridis *et al.*[27] before. These oligonucleotide primer sequences were formed in Table 1. The mPCR assay was performed in a last volume of 25 μL mixture containing 50 mmol KCl, 10 mmol Tris-HCl (pH 8.3), 1.5 mmol MgCl2, 0.2 mmol of each deoxynucleotide triphosphate, 0.5 μmol of each primer, 1.25 unit Taq polymerase (Cinnagen, Tehran, Iran) and 5 μlL of DNA template. The expected size of amplicons was 223 bp for *Brucella* spp. and 408 bp for *Leptospira* spp.; the mPCR assay established the novel primers of PCR assays.

Reactions were initiated at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and a last elongation step at 72°C for 5 min, with a last hold at 4°C. The products obtained at the end of mPCR were run by 2% gel electrophoresis and stained with ethidium bromide (120 V/208 mA). Then the products were screened in UV screening systems. 100 bp molecular weight markers (100 bp ladder, Fermentas GmbH, St. Leon-Rot, Germany) were used as standard measure.

Statistical analysis

Data were analyzed by using SPSS (version 15) software.

Results

In this study, DNA extractions from the collected samples were carried out and mPCR were applied for *Brucella* spp. and *Leptospira* spp., then mPCR products were screened. Results of these screenings were detection of *Brucella* spp. positive products observed in the fragments of 223 base pairs and *Leptospira* spp. positive products observed in the fragments of 408 base pairs (Figure 1).

The results of the prevalence of *Brucella*

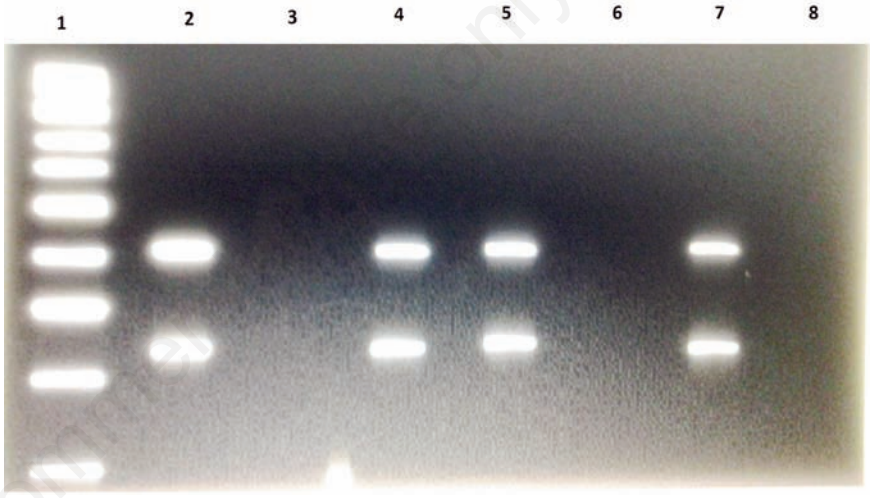

Figure 1. Ethidium bromide-stained agarose gel electrophoresis of Multiplex PCR for the detection of *Brucella* spp. and *Leptospira* spp. in samples after PCR amplification (PCR products of *Brucella* spp.: 223 bp, and PCR products of *Leptospira* spp.: 408 bp). Lane 1: 100 bp DNA ladder (Fermentas, Germany); Lanes 2: are positive control; Lanes 3: Negative control; Lanes 4, 5 and 7: positive samples; Lanes 6 and 8: negative samples.

Table 1. The oligonucleotide primers for *Brucella* spp. and *Leptospira* spp.

Genus/species	Primer set (5' 3')	Target gene	Length of PCR product
Brucella spp.	TGGCTCGGTTGCCAATATCAA CGCGCTTGCCTTTCAAGGTCTG	BCSP31	223 bp
Leptospira spp.	GGCTATCTCCGTTGCACTCTTTG ATCGCCGACATTCTTTCTACACG	LipL41	408 bp

Table 2. The results of the prevalence of *Brucella* spp. and *Leptospira* spp. in blood samples from cattle herds in Esfahan and Chaharmahal Va Bakhtiari province, Iran.

Province	No. samples	Sex (%)		Positive (%) *Brucella* spp.		Positive (%) *Leptospira* spp.	
	Cattle	Male	Female	Male	Female	Male	Female
Esfahan	66	4 (6.07)	62 (93.93)	0	9 (14.51)	1 (25)	6 (9.67)
Chaharmahal Va Bakhtiari	34	2 (5.89)	32 (94.11)	1 (50)	21 (65.62)	1 (50)	13 (40.62)
Total	100	6 (6)	94 (94)	1 (50)	30 (31.91)	2 (33.33)	19 (20.21)

spp. and *Leptospira* spp. in cattle, sheep and goats blood samples are presented in Tables 2-4. The presence of *Brucella* spp. and *Leptospira* spp. DNA were detected by multiplex PCR from 31 and 21 out of 100 cattle, respectively. Four of 70 goat's blood samples from goat breeding farms were positive for *Leptospira* spp. and 11 were positive for *Brucella* spp. Out of 80 sheep blood samples 23 were positive for *Brucella* spp. and 14 for *Leptospira* spp.

The presence of *Brucella* and *Leptospira* species DNA were detected by multiplex PCR from 1 (50%) and 2 (33.33%) out of 6 male cattle, and their presence was 30 (31.91%) and 19 (20.21%) out of 94 female cattle, respectively. Also, multiplex PCR detect DNA of bothmicroorganisms from 4 (22.2%) and 3 (16.66%) out of 18 male sheep; and from 19 (30.64%) and 11 (17.74%) out of 62 female sheep.

Brucella and *Leptospira* species DNA was also detected from 4 (19.04%) and 2 (14.28%) out of 21 male goat, and from 7 (14.28%) and 2 (6.25%) out of 49 female goat, respectively.

Cattle has the highest incidence of *Brucella* spp. and *Leptospira* spp. diseases while, goats have the lowest. In comparison, Esfahan has lower incidence while Chaharmahal Va Bakhtiari has higher incidence of diseases caused by *Brucella spp. and Leptospira spp.*

Discussion and Conclusions

Brucellosis and leptospirosis are diseases of economic importance to any livestock enterprise since they cause abortions in infected animals.[28,29]

Brucella and *Leptospira* species infections usually occur after entering the infected animals to herds. Bacteria from different sources, including infected animals and even ingestion of contaminated food and water, can cause bacteriemia. Target tissue for bacteria during pregnancy is placenta. Abortion occurs as a result of the infection.[5]

Leptospira spp. and many species of *Brucella* are the main factors that cause abortion in the last 2 months of pregnancy in Iran. Leptospirosis has been diagnosed in Iran for many years by MAT and culture methods and the first use of PCR for detection of *Leptospira* spp. in Iran, goes back to 2007.[30-32]

Also, the first report of the isolation of *B. abortus* in Iran as the cause of abortion in cattle dates back to 1944.[33] Since then, this organisms has been isolated on many occasions from bovine fetuses and cow's milk,[4] and now the prevalence rate of brucellosis in sheep and goat is 340/10,000 and in cattle is 56/10,000.[34]

The diagnosis of brucellosis and leptospirosis is typically based on isolation from clinical specimens or serologic evidence of antibodies. The culturing takes days to weeks but has the advantage of detecting the organism directly. For rapid testing of clinical samples or for field surveys, immunological methods were used. However, antigen-antibody interactions can be complicated by nonspecific interactions and false positives from vaccinated animals with high levels of circulating antibodies and can be misdiagnosed as active infections.[5,18]

PCR is a promising option for the diagnosis of various pathogens, and it is a potentially useful method for the detection of *Brucella* and *Leptospira* species from isolated bacteria, blood, semen or highly contaminated tissues.[15,16,18,23,35,36]

Different methods were used in past research.[37] Furthermore, the blood and milk samples obtained from cattle, cheese samples, human blood samples and the samples obtained from naturally infected cows were also used in the research.[7] There are also available studies evaluating the infection agents by PCR in cattle and in sheep with brucellosis cases that ended with abortion.[17]

In Iran, several reports show that the high and low prevalence rate of leptospirosis and brucellosis in different provinces are similar to our study. Reports show that the high prevalence rate of leptospirosis in Iran including Tehran (24.6%),[38] Mashhad (24.24%),[39] Shiraz (32%),[40] Karaj (46.8%),[41] Guilan (22%),[42] Ahvaz (53.73%),[43] and the earliest study on the prevalence of leptospirosis (*L. interrogans*) in Iran indicated a positive rate of 31% in cattle and 17% in sheep,[44] but probably Leptospirosis in Iran is more prevalence than what it seems but since it identified has need to correct laboratory analysis.

Moshkelani *et al*. detected and reported *Brucella* spp. and *Leptospira* spp. by multiplex polymerase chain reaction (PCR) from aborted bovine, ovine and caprine fetuses in Iran. In total of the 276 specimens, 40 (14.4%) and 25 (9.0%) were identified as positive for *Brucella* spp. and *Leptospira* spp., respectively.[5]

According to reports of Parın *et al.* in Turkey, bands related with both *Brucella* spp. and *Leptospira* spp. were detected 24 (31.2%) out of 77 mPCR positive samples. From the remaining 53 (68.8%) samples, 33 samples (62.3%) showed positive bands only related with *Brucella* spp. and 20 samples (37.7%) showed positive bands only related with *Leptospira* spp. The main conclusion of this study was the use of multiplex PCR as a reliable, sensitive and fast technique.[7]

Cetinkaya *et al*. detected 80% sensitivity and 91% specificity in their research conducted by using primer sequences targeted to 16S rRNA in which microbiological cultures obtained from gastric content of aborted lambs were used.[17]

In Turkey, the antibodies against *B. abortus* were detected in serum samples of aborted dairy cattle as 68.1, 65.6, 58.9 and 55.2% by the Competitive Enzyme-Linked Immunosorbent

Table 3. The results of the prevalence of *Brucella* spp. and *Leptospira* spp. in blood samples from sheep herds in Esfahan and Chaharmahal Va Bakhtiari province, Iran.

Province	No. samples	Sex (%)		Positive (%) *Brucella* spp.		Positive (%) *Leptospira* spp.	
	Sheep	Male	Female	Male	Female	Male	Female
Esfahan	26	7 (26.92)	19 (73.08)	1 (14.28)	9 (47.36)	1 (14.28)	3 (15.78)
Chaharmahal Va Bakhtiari	54	11 (20.37)	43 (79.63)	3 (27.27)	10 (23.25)	2 (18.18)	8 (18.60)
Total	80	18 (22.5)	(77.5) 62	4 (22.22)	19 (30.64)	3 (16.66)	11 (17.74)

Table 4. The results of the prevalence of *Brucella* spp. and *Leptospira* spp. in blood samples from goat herds in Esfahan and Chaharmahal Va Bakhtiari province, Iran.

Province	No. samples	Sex (%)		Positive (%) *Brucella* spp.		Positive (%) *Leptospira* spp.	
	Goat	Male	Female	Male	Female	Male	Female
Esfahan	24	7 (29.16)	17 (70.84)	1 (14.28)	0	0	0
Chaharmahal Va Bakhtiari	46	14 (30.43)	32 (69.57)	3 (21.42)	7 (18.75)	2 (14.28)	2 (6.25)
Total	70	21 (30)	49 (70)	4 (19.04)	7 (14.28	2 (14.28)	2 (6.25)

Assay (C-ELISA), Complement Fixation Test (CFT), Rosebengal Plate Test (RBPT) and Serum Agglutination Test (SAT), respectively. The total of 66 (40.5%) of sera were positive for *Leptospira* antigen.[3]

Saad *et al*. reported that PCR method is more specific and sensitive than FAT (Florescense Antibody Test) and MAT (Microscopic Agglutination Test) for detection of *Leptospira* agents in semen, urine and sera of bulls.[45] By evaluating the studies given above, it was evident that *Brucella* and *Leptospira* species were studied separately, and there was not any consideration of investigating these two bacteria together until the multiplex PCR study was conducted by Richtzenhain *et al*. In that study, it was reported that diagnosis of *Brucella* spp. and *Leptospira* spp. could be made in one tube by using mPCR and the sensitivity and specificity of this process was reported as 100% or 92 - 93%, respectively. It was also reported that the diagnosis of these two pathogens should be made more rapidly and more sensitive in routine laboratories.[18]

PCR is applied for detection of various microorganisms, including clinical bacteria and viruses. Sensitivity of PCR is so high, that other methods such as isolation and culture of organisms could not compete with this method anymore. Therefore, this method is a suitable approach to find microorganisms in acute infections and in recent years the PCR technique has increasingly been used as a supplementary method for diagnosis of brucellosis and leptospirosis.[6,37,46-48] By the guidance of these data given above, we decided to detect *Brucella* and *Leptospira* species in cattle, sheep and goats for our research. The goal of our study is presentation of molecular method as faster assay to get more sensitive results. It is also approved that multiplex PCR method is useful in optimal level, not PCR method based upon single locus sequence principle for detection of two bacteria species at the same time. By simultaneously amplifying more than one locus in the same reaction, mPCR has been identified as a rapid and convenient screening assay, with both clinical and research applications. The results show that developed mPCR assay was able to successfully detect *Brucella* spp. and *Leptospira* spp. This method is not a substitute for single PCR, but it can be used to cut the amount of required tests and deliver results more rapidly and cheaply.

The results of the present study showed that the ruminant can be an important reservoir for transmission of these zoonotic diseases to humans in Iran. With thanks to mPCR it is possible to concurrently detect both *Brucella* spp. and *Leptospira* spp. from blood samples of these animals. Several control programs should be performed in Iran on ruminants. The present study shows that molecular methods are right, reliable and rapid assays for detection and identification of *Brucella* spp. and *Leptospira* spp. in samples from ruminants. It seems that this study is the first report of direct detection and segregation of *Brucella* and *Leptospira* species by mPCR assays in ruminant blood samples in Iran. We hope that the mPCR method introduced in this study as a right, safe, fast, sensitive and specific assay for detection and segregation of *Brucella* spp. and *Leptospira* spp. in all samples will be of great benefit in the future.

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