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Molecular prevalence of *Brucella abortus*, *Actinomyces pyogenes* and *Mycobacterium tuberculosis* in reproductive organs of apparently healthy rams slaughtered in Iran

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SUMMARY

Sheep are the most abundant small ruminants in Iranian rural communities. Zoonotic infections and diseases are limiting factors for the fast growth of the sheep industry in Iran, yet surveillance during meat inspection at the slaughter houses is not routinely done. The aim of the study was two folds; 1) to determine the prevalence of *Mycobacterium tuberculosis* (*M. tuberculosis*), *Brucella abortus* (*B. abortus*) and *Actinomyces pyogenes* (*A. pyogenes*) in the testicles of rams from the Isfahan province in central Iran, 2) to determine the predilection sites of these bacteria in the infected testicles. A total of 100 pairs of testicles were randomly collected from different slaughter houses within the Isfahan province, transported to Islamic Azad University, Shahrekord Branch at +4°C until further analyses. IS6110, bcsp31K gene and 16S rDNA of *M. tuberculosis*, *B. abortus* and *A. pyogenes* were detected in PCR reactions respectively. The prevalence of the *M. tuberculosis* was higher (13%) than those of *B. abortus* (8%) and *A. pyogenes* (5%). The present study revealed a higher prevalence of the *M. tuberculosis* than once reported elsewhere in sheep. The findings add to the existing knowledge that sheep may be susceptible to *B. abortus* in addition to *Brucella ovis* and *Brucella melitensis*. The need for proper screening before and after slaughtering is very important for control of these zoonoses.

Keywords: Prevalence, Rams, *Mycobacterium*, *Brucella*, *Actinomyces*, Iran

RÉSUMÉ

Prévalence moléculaire de Brucella abortus, Actinomyces pyogenes et Mycobacterium tuberculosis dans les organes reproducteurs de béliers apparemment sains abattus en Iran.

Les moutons sont les petits ruminants les plus abondantes dans les communautés rurales iraniennes. Les infections et les maladies zoonotiques sont des facteurs limitants pour la croissance rapide de l'industrie du mouton en Iran, mais la surveillance lors de l'inspection de la viande dans les abattoirs n'est pas effectuée régulièrement. L'objectif de l'étude était de déterminer la prévalence de Mycobacterium tuberculosis (M. tuberculosis), Brucella abortus (B. abortus) et Actinomyces pyogenes (A. pyogenes) dans les testicules de béliers de la province d'Ispahan dans le centre de l'Iran, et de déterminer les sites de prédilection de ces bactéries dans les testicules infectés. Un total de 100 paires de testicules ont été prélevés au hasard sur différentes abattoirs dans la province d'Ispahan et transportés à l'Université Azad à + 4 ° C pour analyses. IS6110, bcsp31K et ADNr 16S de M. tuberculosis, B. abortus et A. pyogenes ont été respectivement détectés par PCR. La prévalence de M. tuberculosis était plus élevée (13%) que celles de B. abortus (8%) et A. pyogenes (5%). La prévalence de M. tuberculosis était par ailleurs la plus élevée jamais rapportée chez les moutons. Ces résultats ont également montré que les moutons peuvent être sensibles à B. abortus en plus de B. ovis et B. melitensis.

Mots-clés: Mycobacterium, Brucella, Actinomyces, Bélier, Organes reproducteurs, Iran

Introduction

Iran, a country in the west Asia, is located within of the Middle- East and links the Caspian Sea and Persian Gulf [28]. The sheep population in Iran is about 53 million head. The sheep are mostly kept by rural and peri-urban farmers. Sheep-keeping contributes greatly to the national economy and income of individual rural farmers through the production and sales of meat, milk and wool [25]. However, infectious diseases pose a threat to sheep industry due to reducing productivity of sheep and its products (meat, milk and wool). Zoonotic diseases that infect sheep is also a public concern because of the handling and consumption of sheep meat and milk [14].

Sheep can be infected by bacteria among other etiological agents. *Mycobacterium tuberculosis* complex such as *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Mycobacterium bovis* are the causative agents of tuberculosis in several animals including but not limited to cattle and sheep and since it is a zoonotic [4], human can acquire the disease which is rare in sheep [20]. There are contrasting ideas on the degree of susceptibility of sheep to tuberculosis. The small number of cases of sheep infected by *M. tuberculosis* complex is not consistent with the perception that sheep are susceptible to the infection [2]. The information of infection in sheep by *M. tuberculosis* is scarce and once detected and estimated, the findings are regarded as isolated [20, 27]. According to Allan (1988), prevalence of tuberculosis in sheep is low, only 2 sheep with histological lesions typical

of bovine type tuberculosis (TB) were diagnosed to have the disease out of 9.9 millions of lamb and 1.97 millions of adult sheep. This makes a marked difference in prevalence with other diseases which affect sheep. Following the low number of infected sheep that are detected to contain the bacterium, the assumption that the sensitivity of the methods used in detection of the disease in sheep was questionable as compared to other animals such as cattle [2]. The development of Polymerase Chain Reaction (PCR) and its implementation in TB diagnosis provides a more sensitive way of detecting TB infections even in apparently healthy sheep [11, 18, 26].

Brucella is pathogenic bacteria that cause brucellosis in many animal species including cattle, sheep, goat, camel, buffalo, dog, and horse worldwide [10, 12- 14]. Similarly sheep brucellosis caused by *B. abortus, Brucella melitensis* (*B. melitensis*) and *Brucella ovis* (*B. ovis*), are characterized by abortion in ewes and orchitis and epididymitis in rams with huge economic importance [14]. Brucellosis is spread when infected rams mate ewes that have been previously served by infected rams [7, 13].

Actinomyces pyogenes (*A. pyogenes*), are animal pathogens, they produce hemolytic exotoxin, pyolysin (PLO) and are opportunistic pathogens of economically important livestock such as dairy, beef cattle and sheep. Moreover, *A. pyogenes* is a common inhabitant of the mucous membranes of the urogenital tracts, upper respiratory and gastrointestinal of a many of domestic animal species [23].

Abattoirs and slaughter houses have played an important role in the surveillance of various diseases of human and animal [15]. Zoonotic diseases are more likely to be picked at the slaughterhouses during meat inspection than during the passive clinical surveillance of animals. The objective of the present study was to determine the prevalence of *M. tuberculosis*, *B. abortus* and *A. pyogenes* in the testicles of rams at slaughter and assess their predilection sites in the testicles, in Isfahan province, central of Iran.

Material and Methods

STUDY LOCATION AND STORAGE

Testes with intact epididymis samples from apparently healthy rams were randomly collected after slaughter from different abattoir houses in Isfahan provinces, Central Iran. A total of 100 pairs of testes were collected between January 2013 and July 2013. All collected samples were aseptically placed in cool box (+4°C) and transported to the Biotechnology Research Center of Islamic Azad University, Shahrekord Branch and stored immediately at -20°C until analyzed by PCR for the detection of *B. abortus*, *A. pyogenes* and *M. tuberculosis.* Epididymis was processed separately from the testes in detection of the pathogens.

DNA EXTRACTION

Genomic DNA was extracted directly from each sample using phenol–chloroform method. The quantity of extracted DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [22]. The extracted DNA of each sample was kept frozen at -20°C until further analyses.

PCR AMPLIFICATION OF RESPECTIVE GENE OF EACH BACTERIAL SPECIES

The three Conventional PCR were separately conducted in a thermal cycler (Mastercycler gradient, Eppendrof, Germany) using three different sets of primers (Table I) including the following: amplified bcsp31K gene (223 bp) for detection of *B. abortus* [3], IS6110 insertion (123 bp) for detection of *M. tuberculosis* [6] and 16S rDNA (1600 bp) for detection of *A. pyogenes* [23].

The cycling conditions; denaturation, annealing and extension for *M. tuberculosis*, *B. abortus* and *A. pyogenes* were conducted on the same way as primers set up by Eisenach et al., (1990) [6], Al-Mariri and Haj-Mahmoud (2010) [3] and Shair (2012) [23], respectively and without any modification. PCR products were separated by 2% agarose gel electrophoresis and visualized using ethidium bromide staining.

Results

In the present study, the bcsp31K gene of *B. abortus*, IS6110 gene of *M. tuberculosis* and 16S rDNA of *A. pyogenes* amplified PCR products of 223, 123 and 1600 base pair respectively from testicular samples of sheep from Central Iran (Fig 1 and Fig 2). A total of 13/100 (13%) of the testicular

Agent	Sequence	Bp	References
Brucella abortus	5'-ACGCAGTCAGACGTTGCCTAT-3' 5'-TCCAGCGCACCATCTTTCAGCCTC-3'	223	2
Mycobacterium tuberculosis	5'-CCTGCGAGCGTAGGCGTCGGT-3' 5'-CTCGTCCAGCGCCGCTTCGG-3'	123	5
Actinomyces pyogenes	5'- AGAGTTTGATCCTGGCTCAG-3' 5'- AAGGAGGTGATCCAGCCGCA -3'	1600	20

Table I: The primers used for detection of the selected bacterial species

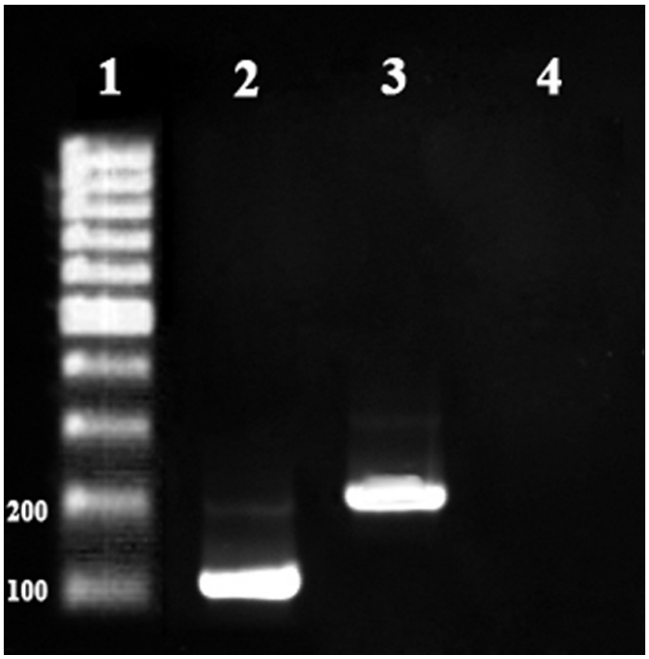

Figure 1: Gel electrophoresis of PCR products for detection of *Brucella abortus* and *Mycobacterium tuberculosis*. Lane 1: DNA ladder (Fermentas, Germany); lane 2: positive samples of *Mycobacterium tuberculosis* (123 bp). Lane 3: positive samples of *Brucella abortus* (223 bp); and lanes 4: negative control.

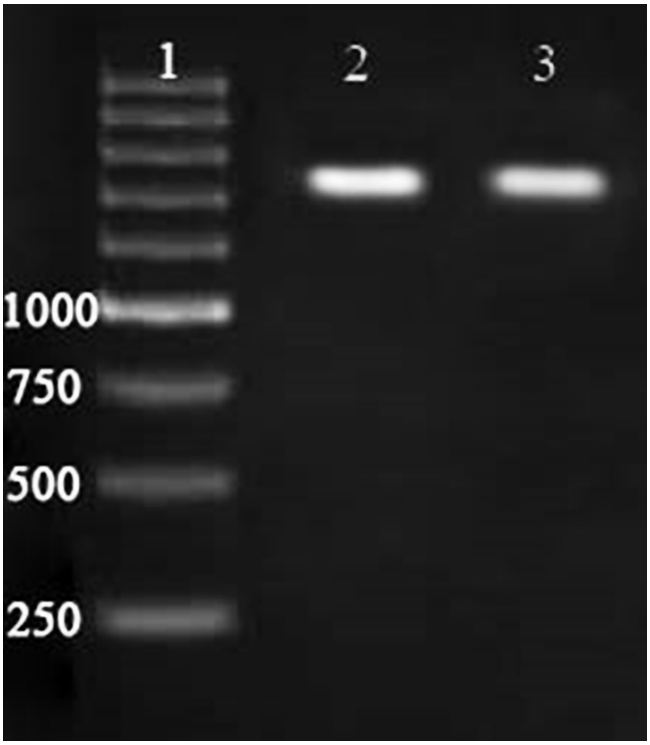

Figure 2: Gel electrophoresis of PCR products for detection of *Actinomyces pyogenes*. Lane 1: DNA ladder (Fermentas, Germany); lane 2 and 3: positive samples of *Actinomyces pyogenes* (1600 bp).

pairs from sheep were positive for *M. tuberculosis*, 8/100 (8%) were positive for *B. abortus* and 5/100 (5%) were positive for *A. pyogenes* (Table II). *M. tuberculosis* was found to exist in both the testicle and the epididymis of one of the testes pair in 10% of the tested samples (n=100). *B. abortus* was detected more in epididymis (5% of the tested samples, n=100) than in testicles. *A. pyogenes* was detected in both the testicles and epididymis in the positive samples (5%) (Table II).

Discussion

The present study has determined the prevalence of *M. tuberculosis*, *B. abortus* and *A. pyogenes* in reproductive organs of apparently healthy rams slaughtered in a Central Iran with public health implications was done using molecular method. Number of assays, such as immunological, molecular and culture methods are available for the detection of bacterial infection; but some assays are laborious, time-consuming, some limitations and requires several days to identify the pathogen. PCR assay is an effective method for the detection of wide range of genes and biological agents. This technique does not have the described limitations in the other methods [3, 6, 11, 14, 23]. Therefore, in our study there was no false positive amplification observed on *M. tuberculosis*, *B. abortus* and *A. pyogenes*, indicating the high specificity of the established PCR assay.

The prevalence of *M. tuberculosis* in Isfahan province was observed to be higher (13%, n=100) than what has been described by several studies conducted elsewhere [2, 5, 9, 19]. Tuberculosis was reported to have low prevalence [2] in many studies that were conducted in sheep either by conventional and or molecular methods. Some of the studies were [19] in Ireland, [9] in Pakistan, [5] in New-Zealand and [27] in Ethiopia. In another study, only two sheep with histological lesions typical of TB were observed in an abattoir

Bacterial species	Origin of positive samples			Total (n= 100)
	Testicles	Epididymis	Both	
Brucella abortus	1	5	2	8 (8%)
Mycobacterium tuberculosis	3	0	10	13 (13%)
Actinomyces pyogenes	0	0	5	5 (5%)
Total	4	5	17	26 (100)

Table II: Number of positive samples (%) and bacterial species detected in epidydimis, testicles and in both positions in studied population of rams

survey of 9.9 million lambs and 1.97 million adult sheep [2, 20]. The explanation of low prevalence by most researchers is the high resistance provided by sheep to *M. tuberculosis* infection [27].

Very few studies have been done so far to detect *M. tuberculosis* in testes bearing in a sense that it is a non targeted organ of the bacterium. According to available literature, this is the first study conducted in Isfahan reporting a high prevalence of *M. tuberculosis* in testes of rams. Despite the fact that the study was not done in other internal organs from the sampled rams for comparison, calls for further investigations as to whether testes may be a good target for molecular detection of *M. tuberculosis* in sheep is required.

Besides *M. tuberculosis*, *B. abortus* is another bacterium investigated in this study. The prevalence of *B. abortus* in the present study (8%, n=100) is numerically the same as what has been reported by other researchers in Iran and elsewhere [17] but is lower compared to that reported previously by Khamesipour et al. (2013) [13] between December 2012 and February 2013 in Isfahan province (38.5%, n=26). In contrast to others the finding in this study adds up to the existing knowledge that sheep may be large susceptible not only to *B. ovis* and *B. melitensis* but also *B. abortus* which is the sole causative agent of brucellosis in cattle. The history of the slaughtered rams on whether they were kept together with cattle was not captured. The findings in the present study support what has been described about sheep infections in south west Asia [7]. Since Iran is one of the countries in this geographical location, its sheep population is highly susceptible to brucellosis and form a reservoir of infection which gives rise to wide spread infections in humans.

M. tuberculosis was detected in both tissues of the testis (testicles and epididymis) in 10 samples (77%) of the positively detected *Mycobacterium* samples (n=13). This shows that the bacterium probably has no predilection site in the testis and hence portrays a normal distribution within the testis. The phenomenon is different in *B. abortus* and *A. pyogenes* where by *B. arbortus* showed to be more abundant in the epididymis than in the testicles. Accordingly Epididymitis caused by *Brucella* spp occurs more commonly than Orchitis in domesticated ruminants and sometimes infection can progress from epididymis to the testicle.

Previous study by Liu et al. (2009) detected and identified gene cassettes of *A. pyogenes* isolates from cows with endometritis by using PCR [16]. Another study by Shair (2011) was to investigate *A. pyogenes* in infected sheep of neighboring village to gazelle breeding center, Riyadh Saud Arabia. In this study primers specific for 16S rDNA in PCR reaction using template DNA from isolates were applied and identified the isolates successfully as *A. pyogenes* [23]. Therefore, Liu et al (2009) and Shair (2011) were able to detect *A. pyogenes* in cows and sheep on different tissues using PCR [16, 23].

It has been observed in this study that all slaughtered rams were healthy and show no any clinical signs of diseases. This supports what has been described earlier that sheep may serve as carriers and reservoirs of etiological agents to other ruminants and man. Furthermore detection of these bacteria which have zoonotic character to apparently health rams is an alarm of increased prevalence of tuberculosis and brucellosis at Isfahan province in human. Previously, it was reported for example that the prevalence of tuberculosis in human was lower in central part and higher in provinces near the country borders [24, 29].

The differences in *Brucella* spp., *Mycobactirium* and *A. pyogenes* prevalence observed in this study compared to other studies may be attributed to the variation in the tests and tissues used. Queipo-Ortuno *et al*., (2005) showed that real-time PCR applied to serum samples was more sensitive than other methods [21]. In addition a study by Gwida *et al*., (2012) confirmed that combination of real-time PCR with one of the conventional serological tests can identify brucellosis in more than 99% of the infected animals [8]. However, Abbas and Agad (2002) revealed that diagnosis and positive cultures depend on the duration, localization of the infection and the type of the infection agent species [1].

In conclusion, the prevalence of *M. tuberculosis* and *B. abortus* in sheep (rams) is higher than *A. pyogenes* in Isfahan province. Since sheep serves as rural man's cow and there is high demand of consuming sheep products such as meat in the province, the need for proper screening before and after slaughtering is of public health significance. Also, control strategies for this pathogens are warranted so as to mitigate the social and economic consequences attributable to natural infections with this infections. Number of assays, are available for the detection of bacterial infection. Moreover, PCR assay is an effective method for the detection of wide range of genes and biological agents. This technique does not have the described limitations in the other methods and was demonstrated to be useful and powerful tools for the rapid detection of bacterial infection.

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Conflict of interest

The authors acknowledge no conflict of interest in this study.

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