A Comparison of the Performance of Exfoliative Cytopathology, Polymerase Chain Reaction (PCR), Culture and ELISA in the Detection of *Brucella canis*

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Abstract: This study was aimed to determine the performance of cytopathology for the diagnosis of *B. canis* on the basis of a comparison with culture, PCR and ELISA results. For this purpose, swabs for cytological, molecular and bacteriological examinations blood serum samples for serological tests were taken from 147 dogs which are suspected to have genital infection.. While the cytological samples were stained with May-Grünwald Giemsa solution, the swab samples were tested by bacteriologically and PCR and the serum samples were assayed by ELISA. According to the results, while ELISA yielded a seropositivity rate of 11.5%, PCR demonstrated a positivity rate of 1.8%. No *B. canis* could be isolated from the samples were taken for bacteriological examinations. At cytological examination, it was observed that the samples contained coccobacilli presented with abundant neutrophil leukocytes and macrophages. Some of these immune cells and epithelial cells were observed to contain round/oval shaped bacteria in their cytoplasm. The results of this study suggest that cytopathology can be used for auxiliary test for the diagnosis of brucellosis.
Keywords: Brucella canis, Cytopathology, PCR, ELISA.

Brucella canis'in Tanısında Eksfolyatif Sitopatoloji Verilerinin Polimeraz Zincir Reaksiyonu (PZR), Kültür ve ELISA Yöntemleri ile Karşılaştırması

Özet: Bu çalışmada, B. canis'in tanısında sitopatolojik yöntemin kullanılabilirliğinin PCR, kültür ve ELISA yöntemleri ile belirlenmesi amaçlandı. Bu amaçla toplam 147 genital sistem enfeksiyonu şüphesi olan köpekten alınan sitolojik, swap ve kan serumu örnekleri incelendi. Sitolojik örneklere May Grünwald Giemsa boyaması yapılırken, mikrobiyolojik incelemeler amacıyla elde edilen swaplar bakteriyolojik kültür ve PCR ile test edildiler. Serum örnekleri ELISA ile test edildi. Yapılan çalışmalar sonucu ELISA yöntemiyle yapılan incelemelerde %11.5 oranında seropozitiflik, PCR yönteminde ise %1.8 oranında pozitiflik tespit edildi. Bakteriyolojik muayene için alınan örneklerde *B. canis* izole edilemedi. Sitolojik incelemelerde kokobasil ile beraber çok sayıda nötrofil lökosit ve makrofajla karşılaşıldı. Yer yer bu hücrelerin ve epitel hücrelerin sitoplazmasında yuvarlak-oval şekilli etkenlerle karşılaşıldı. Yapılan bu çalışmanın sonuçları sitopatolojinin brusellanın tanısında yardımcı bir test olarak kullanılabilirliğini ortaya koymuştur.
Anahtar Kelimeler: Brusella canis, Sitopatoloji, PCR, ELISA.

Introduction

B. canis is a ubiquitous zoonotic bacterial pathogen, which causes abortion and infertility in dogs (Carmichael, 1990; Hollet, 2006). The very few studies conducted to date in humans and animals suggest a serological prevalence ranging between 7.45%-12.7% for infection with this pathogen (Diker et al., 1987; Kustritz, 2005; Oncel et al., 2005; Sayan et al., 2011). Brucellosis is diagnosed based on bacteriological, serological and molecular tests. To the authors' knowledge, to date, only one study has been conducted on the isolation of *B. canis* in Turkey. The definitive diagnosis of *B. canis* infection is made by either bacterial isolation from blood, vaginal secretion, foetal and placental tissues and semen or by molecular techniques (Alton et al., 1988; Corbal et al., 1979). The polymerase chain reaction (PCR) is an ideal diagnostic alternative in that, when compared to bacterial isolation, it produces results within a shorter time period, does not require the use of viable bacteria and offers a practical and affordable option (Keid et al., 2007). Cytopathology has a wide area of use in the diagnosis of neoplasms, monitoring of tumour development, follow-up of tumour treatment, diagnosis of inflammatory lesions, bacterial identification and epidemiological research. However, cytopathological data yield more accurate results when complemented with other diagnostic

methods (Boon et al., 1982; Ghisleni at al., 2006; Watchel, 1964; Wellman, 1990).

This study was aimed at determining both the usability of cytopathology for the diagnosis of brucellosis and the accuracy of this method when supported with culture, ELISA and PCR results.

Material and Methods

Cytological, bacteriological and serum samples taken between the years 2013 and 2016 from 147 sheltered dogs (42 males and 105 females) of different breed, sex and age, which were suspected of having genital infection due to the presence of clinical signs including genital discharge, metritis, posthitis and conception failure, constituted the material of the study. This study was conducted with the approval of the Local Ethics Committee of DOLVET-HADYEK (04.04.2014/2014-21). All proce-dures were carried out in compliance with the relevant national laws relating to the conduct of animal experimentation.

Cytopathological Method: Samples intended for cytopathological examination were taken for the application of exfoliative cytology technique. For this purpose, in the female animals, cell samples were collected by rolling sterile cotton swabs over the caudo-dorsal vaginal mucosa, such that contact with the vulva was avoided. In the male animals, the swabs were rolled over the preputial mucosa. The cotton tips of the swabs were gently placed on glass slides and rubbed over their surface so as to transfer the cells onto the slides. Subsequently, the smears were air-dried and stained with the May-Grünwald Giemsa method. Firstly, the smears were maintained in May-Grünwald solution for 5 minutes. Later, they were washed with distilled water for 30 seconds to remove the excess dye. For cytoplasmic staining, the smears were stained with Giemsa solution for 15 minutes. Then the smears were washed under tap water, air-dried and finally examined by light microscopy.

Bacterial Isolation and Identification: For the isolation of *B. canis,* the vaginal and preputial swabs taken from the dogs were cultured in tryptic soy broth, which contained serum (5-10%) and dextrose (1%) and was supplemented with amphotericin B (1 μg/ml) and vancomycin (20 μg/ml) for selectivity. These selective and enriched media were incubated at 37°C in an atmosphere of 5-10% (v-v) CO_2 for a period of 6 weeks. Each week they were sub-cultured onto solid media. For this purpose, Farrell's medium (Farrel, 1974) and a modified Thayer-Martin selective medium were used. Both media were prepared in accordance with the methods described by the World Organisation for Animal Health (OIE) (OIE., 2009). Farrell's medium was supplemented with Brucella selective supplement (Oxoid, SR0083A) for selectivity. Samples that yielded no bacterial isolation at the end of the 6-week-period were considered to be negative.

Bacterial Strains and Antigen Preparation: In this study, the M-strain of *B. canis,* obtained from the culture collection of the microbiology laboratory, was used for antigen preparation. A loopful of the confirmed M-strain of *B. canis* was inoculated into slanted Sabouraud's dextrose agar (SDA) tubes and incubated at 37 °C for 48 hours. Antigen production was performed as described by Barrouin-Melo et al. (2007). Accordingly, non-viable cultures were filtered through sterile gauze and centrifuged at minimum 3500 xg for 10 minutes at a temperature below 10 °C. After the supernatant was discarded, the cells were suspended in PBS (pH 7.4) and centrifuged twice more as described before. The resulting final pellet was diluted with 10 ml of PBS and autoclaved at 120 °C under 1.5 atmosphere pressure for 20 minutes. Subsequently, the bacteria solutions transferred into sterile Eppendorf tubes were centrifuged at 12000 xg for 20 minutes at 4ºC. The supernatant was harvested and dispensed in small volumes into sterile cryovial tubes so as to be stored at -20 °C to be later used as the ELISA solid-phase antigen.

Positive and Negative Sera: True positive and true negative sera were obtained from the OIE Brucella reference laboratory of the Animal and Plant Health Agency (APHA) in Britain.

Indirect ELISA: For the performance of the indirect ELISA method, a combination of previously conducted techniques was employed (Nielsen et al., 2007; Oliveira et al., 2011). The diluted ELISA solid-phase antigen, prepared at the optimum antigen concentration, and the antigen diluted in 0.05 M sodium carbonate (pH 9.6) antigen-coating buffer solution were distributed in a volume of 100 μl into each well of 96-well flat-bottom polystyrene microtiter plates (NUNC 692620). Subsequently, the antigen-coated plates were incubated at 4 °C for 18-24 hours and were later washed 5 times in PBS containing 0.05% Tween 20 (PBS/T). Next, the positive and negative sera, which were diluted at a proportion of 1/200 with PBS/T, were added twice for each serum sample, in a volume of 100 μl, into the wells of the plates. The plates were covered and incubated at room temperature for 1 hour. After the plates were washed for a further 5 times, a protein A/G conjugated with horseradish peroxidase (HRPO) was diluted with PBS/T at the

proportion indicated in the package insert and distributed into each well at a volume of 100 µl. After being incubated at room temperature for 1 hour, the plates were once again washed with PBS/T 5 times, and added 100 µl of chromogenic substrate (4.0 mM H_2O_2 and 1.0 mM 2,2'-azino-bis (3-ethyllbenzothiazoline-6-sulfonic acid) diammonium salt in 0.05 M citrate buffer pH 4.5). After the plates were maintained at room temperature for 10 to 15 minutes, they were added with 100 µl of 1mM sodium azide to slow down the reaction. Next, the absorbance values of the wells were determined at 450 nm using an automatic ELISA reader (VERSAmax 3.13/B2573). The threshold value was determined as the negative control mean plus 3 standard deviations (SD).

Bacterial DNA Extraction and PCR: For the extraction of *B. canis* DNA from the swab samples, a nucleic acid isolation robot (Kurabo, model Quick Gene 80) and a DNA isolation kit (QuickGene DNA tissue kit S) were used in accordance with the manufacturer's instructions. The extracted DNA was stored at -20 °C until being used. The primers used for PCR and the target area are shown in Table 1. The amplification reaction mixture was prepared in a volume of 50 µl, which contained 200 µM of each dNTP, 50mM KCl, 10mM Tris-HCl (pH 9.0), 1.5mM magnesium chloride, 0.5 µM of each primer, 1.25 U Taq polymerase (Fermentas) and 5 µl of template DNA. The amplification reaction was performed according to the steps described below and by using a Palm-Cycler (CG1-96) device. The PCR protocol was as follows: 1X2 min 95ºC, 40X30 sec 95 °C, 40X30 sec 62 °C, 40X30 sec 72 °C and the final extension 1X5 min 72 °C. While *B. canis* RM6/66 reference DNA was used as a positive control, nuclease-free water was used as a negative control. After the PCR was performed, 10 µl of each amplicon were mixed with 1 µl of loading buffer and loaded into the 2% agarose gel wells. Next, the gel was stained with ethidium bromide (0.5 µg/mL) for the visualisation of the DNA bands under UV light.

Table 1. Primers used for the amplification of the target DNA

Primer	Sequence (5'–3')	Amplikon (bp)	DNA target
ITS66f	5'-ACATAGATCGCAGGCCAGTCA-3'	214	16S-23S rRNA
ITS279r	5' AGATACCGACGCAAACGCTAC-3'		interspace region

Results

Cytopathological Results: Cytopathological examination demonstrated that the cases, confirmed to have been caused by *B. canis* by the use of molecular techniques, were characterized by dense neutrophil leukocyte infiltration and the presence of widely distributed macrophages. These cells were partly necrotic. Part of the neutrophil leukocytes, macrophages and epithelial cells contained many round-oval shaped bacteria in their cytoplasm. In these infected cells, the nucleus was observed to have been pushed aside, and moreover, some of the nuclei were observed to have degenerated. Some of the preparations displayed a large number of neutrophil leukocytes together with the intracytoplasmic and extracellular presence of nonspecific bacteria of varying size and shape (Fig. 1A-B).

The other diagnostic methods employed in this study showed that these cases were negative for B. canis. It was determined that the female cases, which were confirmed to have bacterial infection, were in the oestrus phase of the sexual cycle. In some of the cases, free erythrocytes were also observed.
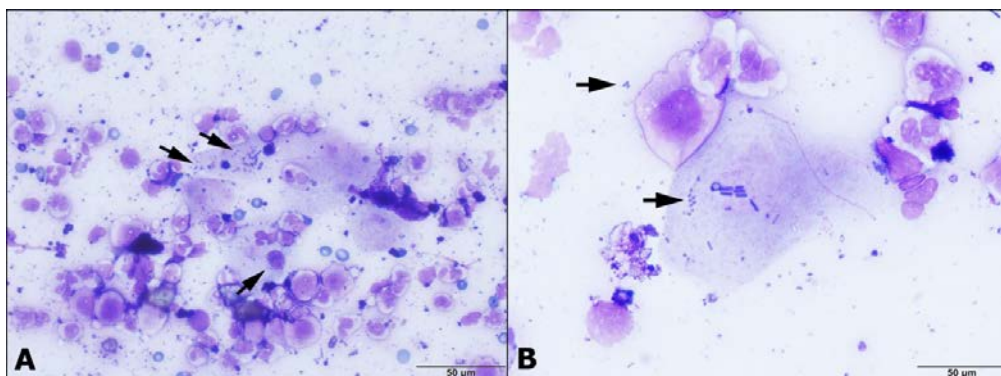

Figure 1A-B. Intracellular and extracellular, small and coccobacilli shape nonspecific bacteria (arrows), May-Grünwald Giemsa, (A), (B).

Bacterial Isolation and Identification: For isolation *B. canis*, the vaginal and preputial swabs taken from the dogs were inoculated into selective and enriched tryptic soy broth containing amphotericin B (1 μg/ml), vancomycin (20 μg/ml), serum (5-10%) and dextrose (1%). These selective and enriched media were incubated at 37 °C in an atmosphere of 5-10% (v-v) CO_2 for a period of 6 weeks. Each week, these cultures were subcultured onto Farrell's medium and a modified Thayer-Martin selective solid medium. However, Brucella spp. were not isolated within or at the end of this time period.

Indirect ELISA: In total, 157 serum samples were tested using a home-made ELISA, in which M-strain of *B. canis* was used as antigen. Thirteen of these serum samples produced a positive reaction. Thus, in result, 11.5% of the serum samples was found to be positive for *B. canis* (Fig. 2).

Figure 2. Home made ELISA results (each sample was tested duplicate).

Bacterial DNA Isolation and PCR: DNA was extracted from the swab samples and was used for the amplification of the 16S-23S rRNA interspace of Brucella spp. by PCR. Out of the 110 swab samples taken from the male and female animals, only 2 (1.8%) (the samples numbered 33 and 38) were found to be positive for Brucella spp (Fig. 3).

Figure 3. PCR results using DNAs from swabs.

Discussion

Cytopathology has a wide range of use in the diagnosis of tumours, the selection of the appropriate treatment method for tumours on the basis of their character, the monitoring of the development of tumours and their post-treatment follow-up, the diagnosis of inflammatory lesions and causative agent identification in such cases, post-mortem examination and epidemiological research (Boon et al., 1982; Ghisleni et al., 2006; Watchel, 1964; Wellman, 1990). Despite its advantages, the cytopathological method is known to produce more accurate results when supported with other diagnostic methods. In the present study, the usability of cytopathology for the diagnosis of *B. canis* infection and the accuracy of this method was assessed on the basis of a comparative evaluation with PCR and ELISA techniques. The results of the present study demonstrated that the cytopathological findings were in agreement with the results of the other diagnostic methods used.

The presence of *B. canis* infection in Turkey has been documented with serological research. In 1983, serum samples taken from 134 healthy dogs in the Ankara province were tested by 2-mercaptoethanol added tube agglutination test (ME-TAT), which yielded a seropositivity rate of 6.7% (Istanbulluoglu and Diker., 1983). In 1987, employed the same test to 222 canine serum samples and obtained similar results (a seropositivity of 6.3%) (Diker et al., 1987). Most recently, in 2005, 362 serum samples collected in the İzmir and İstanbul provinces were tested by TAT, ME-TAT and ELISA techniques, which produced seropositivity rates of 12.7%, 7.73% and 7.45%, respectively (Oncel et al., 2005). Nevertheless, research conducted in this particular area is scarce and there is not enough data to elucidate the current situation of canine brucellosis in Turkey. In the present study, which was carried out in the Şanlıurfa province, the seropositivity rate determined in the tested dogs was 11.5%, whilst the PCR results demonstrated a positivity rate of 1.8%. The results obtained in this study suggest that the prevalence of the disease in Turkey may be higher than the rates previously indicated in literature reports. Further regional studies would contribute to determining the true prevalence of the infection.

Canine brucellosis is generally diagnosed by bacteriological and serological tests. For this purpose, blood, vaginal secretion, foetal and placental tissues and semen are used to isolate the causative agent (Flores-Castro and Carmichael, 1978; Johnson and Walker., 1992; Moore and

Gupta, 1970). More than 50% of infected dogs remain bacteremic for at least 1 year. The isolation of the causative agent being burdensome and time-consuming and isolation not being able to be achieved in the very early and chronic phases of the disease have increased the importance of the serological diagnosis of canine brucellosis (Carmicheal, 1990; Hollett, 2006; Johnson and Walker, 1992). Nevertheless, despite the advancements achieved in diagnostic techniques, it is observed that the diagnostic process has lengthened. On the other hand, there is a need for the availability of more rapid and reliable tests that can be used on the field by veterinary practitioners for the diagnosis of infectious diseases. The results of the present study showed that cytopathology, of which the advantages have been described above, can be used for the early diagnosis of infectious diseases.

In the present study, among the animals included in the sampling frame, the seropositivity rate was determined to be 11.5%, whilst the PCR results demonstrated a positivity rate of 1.8%. To date, only very few studies have been conducted on the serological diagnosis of canine brucellosis in Turkey (Diker et al., 1987; İstanbulluoglu and Diker, 1983; Oncel et al., 2005). The results obtained in the present study do not fully agree with the results reported in previous research (Diker et al., 1987; Oncel et al., 2005), but show partial similarity. The positivity rate determined by ELISA in the present study is two-fold higher than the positivity rates previously reported for canine brucellosis. In study, no B. canis could be isolated. This might be attributed to the vaginal shedding of the agent being intermittent and at a level lower than the number of bacteria found in the aborted foetus and its membranes. Furthermore, serological tests are indirect tests and bear a high risk of producing cross reactions. Thus, high seropositivity rates may partly arise from such cross reactions.

Rapid cytological procedures, which are still being developed and require not much laboratory equipment to be employed, are gaining an increased importance in the diagnosis of diseases. In particular, the use of this method for the diagnosis of breast and cervix cancer in humans has enabled a decrease in the number of cases that would otherwise result in infertility and even death if they had remained undiagnosed. Although the data obtained in the present study is limited, the results obtained suggest that cytopathology could be used for the early diagnosis of canine brucellosis, which is an infectious genital disease of dogs. Different from our results, Madoz et al. (2014) reported that the cytological method proved to be inadequate for the diagnosis of bovine subclinical endometritis. In their study, in which they compared the performance of the cytological and bacteriological methods in the diagnosis of tuberculous lymphadenitis, Abdista et al. (2014) reported that the cytological method offered a high sensitivity but low specificity. In another study, Powers (1998) indicated that the cytological method is not adequate for diagnosis when used alone and needs to be complemented with other diagnostic techniques.

In result, the data obtained in the present study demonstrated the usability of cytopathology as thee auxillary test for diagnosis of *B. canis* infection in domestic animals. However, the number of material used, and thus, the data obtained in this study being limited requires the conduct of further more comprehensive studies. Furthermore, the present study showed that cytology fell short in the identification of nonspecific bacteria, which were detected in some of the cases investigated. This demonstrated that while cytology is highly sensitive, it is lowly specific. Therefore, results obtained with cytology should be complemented with clinical anamnesis data and the results of other diagnostic techniques. The results of the present study are expected to both provide data for veterinary practitioners and contribute to future cytopathological and bacteriological research in this field.

Acknowledgment

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