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Research Article

A Comparative Study on Detection of *Bartonella henselae* Infection by Culture Followed by PCR, Nested-PCR and IFA [1]

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Abstract

Cats are the main reservoirs of zoonotic *Bartonella henselae* which are the causative agents of Cat Scratch Disease (CSD). The aim of this study is to compare three diagnostic methods including culture followed by PCR from whole blood, nested-PCR from oral swab and whole blood, and IFA from serum samples. The diagnosis of *B. henselae* was compared with the bacteriological methods following conventional PCR and by two separate nested PCR from blood, and oral cavity swabs which were collected from 81 pet and stray cats in Istanbul, Turkey. Also the seroprevalence was determined by indirect fluorescent antibody (IFA) technique in the same animals. *Bartonella* spp. was determined in 26 (32%) of the blood samples by culture. Twenty of them were identified as *B. henselae* and 6 of them were *B. clarridgeiae* by following conventional PCR assay. Of 81 whole blood samples subjected to PCR, 29 (36%) were positive in the nested reaction. Of these, 20 were identified as *B.henselae* and 8 were *B. clarridgeiae.* However, one of the samples was found to be positive for both *B. henselae* and *B. clarridgeiae* DNA by the nested reaction. Of 81oral swab samples subjected to PCR, 25 (31%) were positive in the nested reaction. Of these, 19 were identified as *B. henselae* and 6 were *B. clarridgeiae. B.henselae* IgG antibody seroprevalence was detected as 67% (54/81). Using the combination of blood and oral samples by Nested-PCR simultaneously may increase the sensitivity of the test. Also, the combination of the blood culture with nested- PCR and serology is likely to give the most definitive information in the diagnosis of bartonellosis in cats.

Keywords: *Bartonella, Culture, Nested-PCR, IFA, Oral swab*

Bartonella henselae Infeksiyonunun Saptanmasında Kültür Sonrası PCR, Nested-PCR ve IFA Yöntemlerinin Karşılaştırılması

Özet

Kediler kedi tırmalama hastalığının etkeni olan zoonoz *Bartonella henselae* bakterisinin ana rezervuarıdır. Bu çalışmanın amacı tam kandan kültür sonrası PCR, tam kan ve oral svaptan nested-PCR ve serum örneklerinden IFA yöntemlerini içeren 3 farklı teşhis yöntemini karşılaştırmaktır. *B. henselae* tanısı için İstanbul, Türkiye'de yaşayan 81 ev ve sokak kedilerinden toplanan kan örneklerinden bakteriyolojik kültürü takiben yapılan konvansiyonel PCR, tam kan örnekleri ve ağız boşluğundan alınan svapların 2 farklı nested PCR'I ile karşılaştırıldı. Ayrıca aynı hayvanlarda indirect floresan antikor (IFA) tekniği ile seroprevalance belirlendi. Kültür sonucunda 26 (32%) kan örneğinde *Bartonella* spp saptandı. Konvansiyonel PCR testleri sonucunda bunların 20 adeti *B. henselae,* altı adeti *B. clarridgeiae* olarak identifiye edildi. Nested PCR sonucu, 81 tam kan örneğinin 29'u (36%) pozitifti. Bunların 20 adeti *B. henselae,* sekiz adeti *B. clarridgeiae* olarak identifiye edildi. Aynı zamanda, 1 örnekte hem *B. henselae* hem de *B. clarridgeiae* identifiye edildi. PCR'ı yapılan 81 oral svabın 25'i (31%) nested PCR ile pozitif bulundu. Bunların 19 adeti *B. henselae*, altı adeti *B. clarridgeiae* olarak identifiye edildi. *B.henselae*'ya karşı oluşmuş IgG antikorlarının varlığı incelenen serum örneklerinde seroprevalans %67 (54/81) olarak belirlendi. Sonuç olarak kan ve oral svap örneklerinden Nested-PCR kombinasyonunun kullanımı testlerin sensitivitesini arttırabilmektedir. Ayrıca kan kültürünün nested-PCR ve seroloji ile kombinasyonu kedilerde bartonellosis tanısında muhtemelen en kesin bilgiyi vermektedir.

Anahtar sözcükler: *Bartonella, Kültür, Nested-PCR, IFA, Oral svap*

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INTRODUCTION

Cat scratch disease (CSD) which can be found in many species including humans is a worldwide zoonosis. Agent of the disease, *Bartonella henselae* which is classified in the family Bartonellaceae, is an intraerythrocytic, slightly curved Gram negative bacteria [1]. The confirmatory diagnosis in cats cannot be determined based on clinical signs. Infected cats are usually asymptomatic, but can still present recurrent bacteraemia, which may last from months to years. Currently, the laboratory diagnosis of bartonellosis in cats is based on direct methods (bacterial isolation and PCR) and indirect methods (Serological tests: IFA, ELISA, Western Immunobloot) [2]. Because of their fastidious nature, standard biochemical methods are not convenient for identification [3] and cannot be used in differentiation of the species in the genus, therefore molecular methods are commonly used for this purpose [4]. The aim of this study was to compare three diagnostic methods including culture followed by PCR from whole blood, nested-PCR from oral swab and whole blood, and IFA from serum samples.

MATERIAL and METHODS

Sample Collection

The samples objected in the study were collected from 81 cats which stay or visit private clinics and streets of Istanbul, Turkey. Five of the cats have lesions on their gingiva while 76 of have no lesions.

Blood samples (3 mL) collected by aseptic procedure from the jugular vein of the cats, were placed in serum separator tubes and tubes with EDTA. In the laboratory, the blood with EDTA was divided into two parts. One part was used for blood culture immediately; the other part was stored at -80°C for nested PCR. The sera were stored at -20°C until analysed.

Dry cotton swab was rolled over the gums or oral lesions if exists, and swab specimens were collected into specimen transport media. They were stored in cooling boxes for transport to the laboratory. They were stored at -20°C until tested.

The present study was approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine, Approval no: 2012/182.

Bacterial Isolation

The samples in tubes with EDTA kept at -80°C were thawed at room temperature, vortexed and then centrifuged at 3800 rpm for 70 min. Upon pouring away the supernatant, the pellet was suspended in 125 µL Medium 199 Broth and finally mixed by vortex. 250 µL from the acquired suspensions were inoculated onto Heart Infusion Agar (HIA) supplemented with 5% defibrinated rabbit blood, and the suspension in the medium were diffused on the surface of the medium using their own viscosity. The plates were incubated at 35°C with 5% CO_2 in an incubator for 5 weeks. The presumptive identification of suspected gram negative bacteria was performed by conventional biochemical methods as a genus level [5].

From the isolates, DNAs were extracted by using DNeasy Tissue Kit (Qiagen, Valencia,CA, USA) according to the manufacturer's instructions. The PCR assay was performed as described by Jensen et al.[6]. PCR products were separated on a 3% agarose gel, stained with ethidium bromide and visualized under UV light. The fragments of 172 and 145 bp were evaluated as positive for *B. henselae* and *B. clarridgeiae*, respectively.

Nested-PCR Assay from Whole Blood and Oral Swab Samples

DNAs were extracted from whole blood by using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The oral swabs were immerged for 15 min in 400 mL of PBS and vortexed. Then, DNA was extracted by using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Primary reactions were performed in a 25 µL of volume as follows: 5 µL extracted DNA, 0.2 mM each dNTP, 0.5 mM each primer (P-bhenfa and P-henr1), 3 mM $MgCl_2$, 10X PCR buffer (50 mM KCL, 10 mM Tris-HCL pH 8.8), 0.5 U Taq DNA polymerase. DNA amplification was obtained with pre denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 48.2°C for 30 s and synthesis at 72°C for 30 s. A final extension step at 72°C for 5 min was included at the end of the cycles. The PCR mixtures were cooled at 4°C until using for nested reactions.

Nested reactions were performed in a 25 µL of volume as follows: 1 µL primary amplicon, 0.2 mM each dNTP, 0.5 mM each primer (N-bhenf1a and N-henr), 1.5 mM $MgCl_2$, 10X PCR buffer (50 mM KCL, 10 mM Tris-HCL pH 8.8), 0.5 U Taq DNA polymerase. DNA amplification was obtained with predenaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s and synthesis at 72°C for 30 s. A final extension step at 72°C for 5 min was included at the end of the cycles.

Positive controls, consisting of purified *B. henselae* and *B. clarridgeiae* DNAs, and a negative control (water blank) were included with each run. PCR amplification products were separated on 3% agarose gel, stained with ethidium bromide and visualized under UV light. Bands of 152 and 134 bp were evaluated as positive for *B.henselae* and *B.clarridgeiae*, respectively [7].

IFA

The presence of IgG antibodies against to *B. henselae*

was measured by indirect fluorescence assay (IFA) using commercial slides coated with *B. henselae* (Fuller Laboratories, California, USA). IFA was performed according to the instructions of the manufacturer. For detection of antibodies against to *B. henselae*, the serum samples were initially screened at 1: 64 dilutions in phosphate-buffered saline with goat anti-cat IgG marked with fluorescein isothiocyanate conjugate (Fuller Laboratories, California, USA). Positive and negative controls were run in each test. The intensity of the fluorescence was scored subjectively from 1 to 4, and a fluorescence score of 2 at a dilution of 1:64 was considered to be positive.

Statistical Analyses

The sensitivity, specificity, positive and negative predictive values and diagnostic values for IFA and nested PCR assays were determined for diagnostic effectiveness compared to the culture followed by PCR considered as a gold standard [8].

RESULTS

Culture

Bartonella spp. was determined in 26 (32%) of the blood samples by culture. Twenty of them were identified as *B. henselae* and 6 of them were *B. clarridgeiae* by following conventional PCR assay.

Nested-PCR from Whole Blood

Of 81 whole blood samples subjected to PCR, 29 (36%) were positive in the nested reaction. Of these, 20 were identified as *B. henselae* and 8 were *B. clarridgeiae*. However, one of the samples was found positive for both *B. henselae* and *B. clarridgeiae* DNA by the nested reaction.

Nested-PCR from Oral Swabs

Of 81oral swab samples subjected to PCR, 25 (31%) were positive in the nested reaction. Of these, 19 were identified as *B. henselae* and 6 were *B. clarridgeiae*.

IFA

B. henselae IgG antibody seroprevalence was detected as 67% (54/81).

Number of the positive cats are comparatively summarised according to sampling and test methods on *Table 1*.

Results of the cats with oral lesions are summarized on *Table 2*.

Statistical Analyses

In this study, the specificity and sensitivity of the nested-PCR assay from blood (81.8% and 73.1%, respectively) and oral swab samples (87.3% and 69.2%, respectively) were found relatively high. On the contrary, the sensitivity of the IFA test (80.8%) was found the highest of all when the specificity (40%) was the lowest.

The results are showed on *Table 3*.

Table 1. *Number of the positive cats according to sampling and test methods*

Culture	Nested-PCR from Whole Blood	Nested-PCR from oral samples	IFA	Number of Positives
+	-	-	-	1
-	+	-	-	1
-	-	+	-	0
-	-	-	+	27
+	+	-	-	2
+	-	+	-	1
+	-	-	+	2
-	+	+	-	4
-	+	-	+	3
-	-	+	+	1
+	+	+	-	1
+	+	-	+	3
+	-	+	+	3
-	+	+	+	2
+	+	+	+	13

+ : Bartonella spp positive, - : Bartonella spp negative

Table 2. *Results of the cats with oral lesions according to sampling and test methods*

No of the Cats with Gingivitis	Investigated Methods			
	Culture	Nested-PCR from Whole Blood	Nested-PCR from Oral Samples	IFA
1	-	+	_	+
2	-	-	-	+
3	+	-	-	+
4	+	+	+	-
5	-	-	-	+

+ : Bartonella spp positive, - : Bartonella spp negative

Table 3. *Diagnostic effectiveness for nested-PCR assays and IFA*

Diagnostic Effectiveness	Nested-PCR from Whole Blood	Nested-PCR from Oral Samples	IFA
Sensitivity	73.1%	69.2%	80.8%
Spesifity	81.8%	87.3%	40%
Positive predictivity	65.5%	72%	38.9%
Negative pedictivity	86.5%	85.7%	81.5%
Diagnostic value	79%	81.5%	53.1%

DISCUSSION

Bartonella organisms need special growth medium and they grow very slowly. Some researchers recommended that isolation of the bacterium was the gold standard and they indicated that the most successful method to detect *Bartonella* species from cat blood was culture and characterization of the isolate by PCR [4,9]. But, because of the high prevalence of infection in healthy cats in endemic areas, Pennisi et al.[10] determined that the positive culture was not corroboratory and other compatible diagnoses must be ruled out. Jensen et al.[6] presented a single-step PCR which was suited for the detection of *B. henselae* and *B. clarridgeiae* from culture and blood. However, Rampersad et al.[7] and Engvall et al.[11] reported that this method has questionable sensitivity and show less sensitivity than culture for the detection of *B. henselae* and *B. clarridgeiae* in blood. Therefore, Rampersad et al.[7] recommended enhancement methods such as a nested-PCR from blood.

Nasoiu et al.[2] indicated that sequencing and analysis of bacterial DNA by PCR was a sensitive test to amplify *Bartonella* spp. However, they pointed that because of the bacteria circulates only intermittently, PCR was not offered many advantages over culture. Bai et al.[12] explained the overall low success of culture with the observation of low concentrations of *Bartonella* bacteria in cat blood and they pointed that molecular approach does not provide evidence of viable bacteria in animal samples. In this study, the results of the nested-PCR from blood were 24.6% for *B. henselae* while 11.1% for *B. clarridgeiae*, and the results of the blood culture were 24.6% for *B. henselae* while 7.4% for *B. clarridgeiae*. Our detection rates by Nested-PCR and culture were quite similar. It has been considered that the detection limit of the bacteria in bacteriological and molecular methods might be varied. *Bartonella* species are very fastidious and the culture processes take longer time. In some samples, these bacteria might not survive and could not be cultured; despite of that, the DNA of the bacterium might be detected by the molecular methods. Therefore, nested-PCR from blood may be able to an opportunity for diagnosis of bacteraemia.

Pennisi et al.[13] indicated that the oral swab was an easier procedure than taking blood and testing both blood and oral samples may easily enhance the sensitivity of PCR testing, although their positive results of the nested-PCR from blood more than the rate of the nested-PCR from oral swabs. Furthermore, Kim et al.[14] reported that the results of nested-PCR from saliva (44.1%) were more than the results of nested-PCR from blood (41.8%). In our study, the results of nested-PCR from blood (36%) were higher than the results of nested-PCR from oral swabs (31%). These results have supported the results of Pennisi et al.[13]. Despite the sensitivity of nested-PCR from oral swabs was relatively high, positive results might not show the current infection. In the current study, there have been some cases that positive nested-PCR from oral swabs without positive results for culture and nested-PCR-from blood. It was been thought that, these might be developed due to ingestion of *Bartonella* bacteria in flea dirt when the cats acting grooming behaviour and the infection may be absent. Also, because of the bacteraemia can be intermittent [2], nested-PCR from blood can give negative results.

Quimby et al.[15] described for the first time for presence of microbial DNA from oral swabs collected from cats with and without gingivostomatitis (GS) and they reported that of the five *Bartonella* positive samples, only one was from a cat with GS. Namekata et al.[16] indicated that cats with oral lesions (bacteremic or not) had more frequently PCR positive oral swabs than cats without oral lesions. In our study, only one sample of the five cats with oral lesion was PCR positive. On the other hand, four (two of were culture positive while two of were blood PCR positive) of the five cats with oral lesion had bacteraemia while four cats were seropositive.

The positive results on the serological tests in cats only document exposure to infection [13]. Therefore, the serological test results are not be used to determine the *Bartonella* spp. infection status of individual cats [17]. Park et al.[18] emphasized that serology has only a retrospective value, and the cats might have been infected during some period of their life. Pennisi et al.[10] determined that IFAT is more useful for exclusion than for confirmation of the infection because of the low positive predictive value compared with the good negative predictive value. In our study, the diagnostic value of the IFA test was found that the positive predictive value was only 38.9% and the negative predictive value was 81.5%. These results were in parallel with the results of the authors indicated above.

Serologic tests results do not strictly correlate to PCR analysis and culture. Fabbi et al.[9] indicated the lack of the association between seropositivity and the level of bacteraemia. Lappin and Hawley [19] emphasized that *Bartonella* species serum antibody test results cannot be used to accurately predict bacteraemia in cats as some cats with *Bartonella* species DNA in blood were seronegative and some cats with *Bartonella* species IgG in serum were negative for *Bartonella* species DNA in blood. Beside these, Nasoiu et al.[2] indicated that compared to the bacterial isolation, which lasts between 4 and 6 weeks, the serological tests have the advantage that they are easier to use, and have duration of 1-2 days, while being economic. In this study, 28 cats with serum IgG to *B. henselae* were negative for *B. henselae* DNA in blood while 6 cats with *B.henselae* DNA in blood were seronegative. These results have supported the results of Lappin and Hawley [19] and Nasoiu et al.[2].

It is clear that the clinicians should focus on cats which are reservoir of the CSD and on preventing this zoonotic disease. Using the combination of blood and oral

samples by Nested-PCR simultaneously may be increased the sensitivity of the test. Also, the combination of the blood culture with nested- PCR and serology is likely to give the most definitive information in the diagnosis of bartonellosis in cats.

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