

## Screening Feline Blood Donors for *Bartonella henselae* Infection: Comparison between Indirect Immunofluorescent Antibody Test (IFAT) and Polymerase Chain Reaction (PCR) Results

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### Abstract

In order to minimize pathogen transmission, all blood donors should be appropriately screened for infectious agents. Screening for *Bartonella* spp. infection in feline blood donors is a recommended practice in veterinary blood banks across the world.

The aim of this study was to compare results of an indirect immunofluorescence antibody test (IFAT) in identifying *Bartonella henselae* antibodies with the results of PCR amplification of *Bartonella* spp. DNA to establish the best IFAT cut off to identify non-bacteremic cats. A secondary aim of this study was to evaluate demographic and clinicopathologic factors that may be associated with *Bartonella henselae* infection status. From a population of stray cats in Milan city, 82 serum samples were evaluated by IFAT for *Bartonella henselae* antibodies and PCR was performed on 90 whole blood samples for amplification of *Bartonella* spp. DNA. A total of 14/82 (17.1%) samples were seropositive with an IFAT titer  $\geq 1:64$  (cut-off for infection). *Bartonella* spp. DNA was identified in 11/90 (12.2%) samples by PCR.

Overall 20/90 (22.2%) infected cats were identified by either IFAT  $\geq 1:64$  and/or PCR-positive results. Hyperbetaglobulinemia ( $P=0.02$ ) and originating from zone 2 of Milan city ( $P=0.03$ ) were statistically associated with positive *Bartonella* infection status.

The overall IFAT sensitivity was 50.0%, specificity 87.5%, positive predictive value 35.7% and negative predictive value was 92.65%. The ROC analysis showed that the area under the curve was 0.747 ( $P=0.0032$ ) and that an IFAT cut off  $< 1:32$  had the highest sensitivity in identifying *Bartonella* PCR-negative cats. When feline blood donors undergo serological screening for *Bartonella henselae* infection an IFAT cut off  $< 1:32$  has the highest sensitivity for identifying non-bacteremic cats. However some serologically negative cats could be bacteremic and therefore screening of a feline blood donor using a combination of IFAT and PCR is recommended. Protein electrophoresis should be performed in all potential donor cats.

### Introduction

Feline transfusion medicine is a new and rapidly growing specialization in veterinary medicine and many cats are now included as blood donors in blood donation programs. The quality of feline blood collected for transfusion depends on many factors. In addition to immune-mediated reactions caused by infusion of allogeneic cells or proteins, adverse transfusion events can be caused by infectious agents from transfusion of contaminated blood obtained from an infected donor. Therefore, to minimize pathogen transmission, all blood donors should be appropriately screened for infectious agents. Testing is recommended for pathogens which meet at least three of the following criteria: (1) the pathogen has been documented to cause clinical infection in recipients after blood transmission, (2) the pathogen is capable of causing subclinical infection such that carriers might inadvertently be identified as healthy blood donors, (3) the pathogen can be detected using culture or molecular methods from the blood of an infected animal, and (4) the resultant infection in the recipient has the potential to cause life-threatening illness and be difficult to eliminate with antimicrobial drugs [1,2].

*Bartonella henselae* infection in cats meets all these criteria. *Bartonella* can induce chronic and persistent infection following blood transfusion. Infection can be latent and induce unpredictable recurrence of bacteremia [3-5] and cause signs such as transient lethargy, fever, neurologic dysfunction, reproductive failure, and mild anemia [3-6]. Infected cats, may be highly bacteremic for several months with no significant clinical signs [7]. Although *Bartonella* infection is usually asymptomatic, or characterized by mild clinical signs in otherwise healthy cats, infection is a significant problem in immunocompromized individuals (such as recipients of blood transfusion), who get the most serious forms of bartonellosis. In addition, it has been shown in man that blood storage does not inactivate *B. henselae* which can remain viable in RBC units for up to 35 days at 4°C [8]. Finally no antibacterial therapy is available to clear the pathogen from cats [9,10]. For all these reasons screening for *Bartonella* spp infection in feline blood donors is a recommended practice in veterinary blood banks around the world [1,2].

Detection of *B. henselae* in blood is the gold standard in diagnosis of *Bartonella* infection in cats [9,10] and the best method to identify bacteremic cats. However there are some limitations in the identification of *Bartonella henselae* in blood by blood culture: a prolonged incubation period (4 to 6 weeks) [3,11], requirement for a specialized laboratory, possibility of false negative results because cultivation on media is very difficult (particularly when a low number of bacteria are present in peripheral blood at time of collection) or because the organism died in transport to the laboratory. In addition cats can be intermittently bacteremic showing wide fluctuations in the levels of bacteremia over time with intermittent negative culture [5,6]. To reduce false-negative test results, repeated blood cultures are required. All these factors limit the utility of bacterial culture as a diagnostic tool for routine screening of blood donors.

Molecular biology methods such as Polymerase Chain Reaction (PCR) are more suitable for screening feline blood donors for *Bartonella* spp. infection. PCR is a specific and sensitive method and rapidly detects the presence of *Bartonellae* in feline blood samples. However PCR assays require specialized laboratories, stringent quality control to avoid both false-positive and false-negative results, can be expensive to perform, and are currently not standardized among laboratories [9]. In addition, whilst true positive PCR assay results document the presence of microbial DNA, they do not prove the organism was alive and therefore capable of inducing infection in blood recipients. False-negative PCR assay results could occur because of intermittent bacteremia, previous use of antibiotics, lack of microbial DNA in the sample tested, or inhibitory or interfering substances in biologic specimens [9]. In addition, Economic factors in veterinary medicine often limit the testing of blood donor animals. Some tests such as PCR could be cost prohibitive for some blood donor programs.

The use of an Indirect Immunofluorescence Antibody Test (IFAT) as an alternate to PCR testing for *Bartonella henselae* infection in feline blood donors has been included in the guidelines provided by European Advisory Board on Cat diseases (ABCD) group for minimizing risks of iatrogenic infectious complications in feline blood transfusion [2]. Serum antibody tests can be performed more quickly than blood culture and are inexpensive when compared to PCR.

The aim of this study was to compare results of IFAT in identifying *Bartonella henselae* antibodies and the results of PCR in amplifying *Bartonella* spp. DNA in order to identify the best IFAT cut off for identification of non-bacteremic cats. In addition, as clinical diagnosis of bartonellosis is complicated by poor understanding of the clinical spectrum of *Bartonella* infection, the second aim of this study was to evaluate demographic and clinicopathologic factors that may be associated with *Bartonella henselae* infection status.

## Abbreviation

AUC: Area Under Curve; BCS: Body Condition Score; CBC: Complete Blood Counts; CI: Confidence Interval; IFAT: Indirect Immunofluorescent Antibody Test; NLR: Negative Likelihood Ratio; NPV: Negative Predictive Value; PCR: Polymerase Chain Reaction; PLR: Positive Likelihood Ratio; PPV: Positive Predictive Value; ROC: Receiving Operator Characteristic; TNR: Trap, Neuter and Release

## Materials and Methods

### Animals and Blood Samples

Frozen serum and whole blood samples from previously identified seropositive cats [12] were used for this study. These samples were collected from cats between June and December 2014 during a survey on stray cats from Milan city in Northern Italy (Latitude: 45°27'51"N, Longitude: 9°11'22"E). The study population comprised 90 stray cats captured from courtyards in urban areas of Milan for a trap, neuter, and release (TNR) sterilization program performed as part of a national program to control stray pet populations under Italian National Law (law no. 281/1991). Interventions for the prevention, diagnosis, therapy, and control of diseases in stray feline populations are allowed under Lombardy regional law no. 33/2009; therefore, approval of the study design by an ethics committee was not necessary. In the previous study [12] serum samples were screened with an IFAT for *Bartonella henselae* IgG antibody with the highest screened antibody titer of 1:128. In the present study the previous IFAT positive serum samples were analyzed for the end point IFAT titer for *B. henselae*. In addition whole blood samples of these 90 cats were analyzed by PCR to amplify *Bartonella* DNA. Data available for each cat were age (adult or young cats, based on dentition), gender, body condition score (BCS), colony of origin, clinical disorders diagnosed in the cats at the time of sample collection, complete blood counts (CBC), total serum protein and protein electrophoresis (albumin, alpha-, beta-, and gamma-globulin concentrations).

## Serological analysis

*Bartonella henselae* IgG antibodies were detected using a commercially available IFAT kit (Biopronix Product Line, Agrolabo Spa, Italy) using slides prepared with fixed *B. henselae*-infected Hep-2 cells. All sera were diluted 1:32 in phosphate-buffered saline (PBS, pH 7.2) and incubated on wells of the slides at 37°C in a humidity chamber for 30 min. The slides were rinsed twice in PBS, once in distilled water and air-dried. Each well of the slides was probed with fluorescein isothiocyanate-conjugated sheep anti-Cat IgG (Biopronix-Agrolabo Spa, Italy) diluted 1:50 in Evans Blue solution and incubated at 37°C in a humidity chamber for 30 min. The slides were washed and dried as described above and examined with a fluorescence microscope. Sera were screened starting from 1:32 dilution, and any serum sample positive at that dilution was titrated in serial twofold dilutions to the endpoint titer. Cut-off value for *B. henselae* infection was  $\geq 1:64$  [11,13,14]. Negative and positive control samples were included on each slide. Slides were examined by fluorescence microscopy at 40 $\times$  magnification (Axioskop, Zeiss, Germany), as previously described [6].

## DNA extraction and PCR

DNA was extracted from feline blood samples using the High Pure Template Preparation Kit (EZNA tissue DNA kit, VWR-Omega Bio-Tek, USA), following the manufacturer's instructions. The DNA samples were stored at -20°C until used as template in PCR protocols. The primers p24E and p12B, previously described [15], were used in this protocol to amplify a 296 bp fragment of the *Bartonella* 16S rRNA gene. The PCR amplification was performed in 25  $\mu$ l of reaction mixtures containing 0.5  $\mu$ l of deoxynucleoside triphosphates (GeneAmp dNTP Mix with dTTP, Applied Biosystems, Life Technologies, UK), 0.5  $\mu$ l of each primer, 0.125  $\mu$ l of Taq polymerase and 5  $\mu$ l of PCR buffer (GoTaq G2 Polymerase, Promega), 16.375  $\mu$ l of Nuclease free water and 2  $\mu$ l of extracted DNA. PCR amplifications were performed in an automated thermal cycler (Thermal Cycler 2720, Applied Biosystems, UK) for 55 cycles. Each cycle consisted of denaturation at 94 °C for 15 seconds, annealing at 66°C for 15 s, extension at 72 °C for 15 s; an initial denaturation of 2 min at 95 °C and a final extension of 1 min at 72°C. PCR controls included a known positive DNA extract and a reagent blank. PCR products were analyzed by electrophoresis on 1.5% agarose (EURx) gel at 100 V for 45 min; the gel was stained with ethidium bromide and examined. The DNA molecular weight marker was 100 bp DNA Ladder (Promega, USA).

## Statistical analysis

Cats were divided into 2 groups, *Bartonella henselae* infected and non-infected with an IFAT titer  $\geq 1:64$  and/or PCR-positive result and an IFAT titer  $< 1:64$  and/or PCR-negative result, respectively. Titers below 1:32 were considered as zero in the analysis. For univariate analyses, nonparametric tests (Chi-square or Fisher's exact test) were used to test for associations between infective and non-infective status and putative explanatory factors or risk factor for infection status, such as age, gender, BCS, colony of origin, clinical, hematological and electrophoretic alterations.

Associations between serological results and PCR status were tested with a Fisher exact test. For comparison of IFAT titers in PCR-positive and negative cats, non-parametric Mann-Whitney rank sum test was used. Calculation of sensitivity (Se), specificity (Sp), positive likelihood ratio (PLR), negative likelihood ratio (NLR), positive predictive value (PPV), negative predictive value (NPV), was performed for evaluation of IFAT results in comparison of PCR status as reference standard. To assess performance of the IFAT test at different cut offs, Se, Sp, NLR, PLR, NPV and PPV were calculated generating a receiving operator characteristic (ROC) curve using PCR as criterion-reference standard and a prevalence of *Bartonella henselae* infection in stray cats of Milan city of 16% [13]. The performance of the IFAT test was analyzed by comparing the area under the curve (AUC), with 1 indicating a perfect test and 0.5 indicating results similar to chance. The area under the ROC curve provides a single numerical estimate of overall accuracy that can be interpreted as the average probability that an infected animal will have a positive test value compared to a non-infected animal. All statistical analysis were performed using MedCalc Statistical Software version 16.8.4 (MedCalc Software bvba, Ostend, Belgium; <https://www.medcalc.org>; 2016).

## Results

### Serology

The IFAT detected 23/82 (28.1%) serum samples with six different end point antibody-titers against *B. henselae* (Table 1). There were 14/82 (17.1%) seropositive samples with an IFAT titer  $\geq 1:64$  (cut-off for infection).

### Polymerase chain reaction

Amplification of *Bartonella* spp. DNA by PCR was possible in 11/90 (12.2%) samples.

Overall 20/90 (22.2%) cats were infected (i.e. IFAT  $\geq 1:64$  and/or PCR-positive results).

Endpoint IFAT antibody titer	No. of cats (%)	No. of <i>Bartonella henselae</i> PCR-positive cats (%)	P-value (Fisher's exact test)
<1:32	59 (71.9)	3 (30.0)	<b>0.004</b>
1:32	9 (11.0)	2 (20.0)	0.301
1:64	3 (3.7)	1 (10.0)	0.326
1:128	3 (3.7)	2 (20.0)	<b>0.037</b>
1:256	5 (6.1)	2 (20.0)	0.110
1:512	2 (2.4)	0 (0.0)	1.000
1:1024	1 (1.2)	0 (0.0)	1.000
Total	82 (100)	10 (100)	-

**Table 1.** *Bartonella henselae* IFAT end point titers and comparison with PCR results for *Bartonella* spp. in 82 stray cat of Milan city, northern Italy.

P value in bold are statistically significant as  $\leq 0.05$

## Risk factor study

Summary statistics relating to CBC and protein electrophoresis profile are reported in Table 2.

Parameter	Min	Max	Mean (95% CI)	$\pm$ DS
RBC ( $\times 10^6/\mu\text{L}$ )	5.50	10.50	7.98 (7.38-8.57)	1.27
Hb (g/dl)	6.8	12.7	10.1 (9.5-10.8)	1.4
Hct (%)	20.1	39.6	29.7 (27.4-32)	4.9
MCV (f)	28.2	47.2	37.3 (35.5-39.2)	3.9
MCH (pg)	9.1	18	12.8 (12-13.7)	1.8
MCHC (g/dl)	31.9	49.2	34.4 (32.7-36.2)	3.8
RDW (%)	15.2	27.6	19.6 (18.2-20.9)	2.9
PLT ( $\times 10^3/\text{ml}$ )	43	567	326 (247-405)	168
WBC ( $\times 10^3/\text{ml}$ )	5.9	20.3	11.6 (9.7-13.5)	3.9
Neutrophil (%)	19	83	59.8 (52.5-67.1)	15.6
Lymphocytes (%)	10	73	31 (24.2-37.7)	14.3
Monocytes (%)	0	7	2.9 (2.1-3.7)	1.7
Basophils (%)	0	3	0.7 (0.3-1.1)	0.8
Eosinophils (%)	1	15	5.4 (3.5-7.3)	3.9
Total protein (g/dl)	5.6	7.6	6.4 (6.1-6.7)	0.6
Albumin (%)	36.2	52.9	46.1 (43.8-48.3)	4.6
Alpha 1 globulin (%)	1.7	4.4	3.3 (3-3.7)	0.6
Alpha 2 globulin (%)	15	23.8	19.5 (18.1-20.9)	2.8
Beta 1 globulin (%)	2.1	7.6	5.7 (5-6.3)	1.3
Beta 2 globulin (%)	2.5	6.3	4.1 (3.5-4.7)	1.2
Gamma globulin (%)	13.9	39.3	21.1 (17.9-24.3)	6.6
Albumin/globulin ratio	0.5	1.1	0.8 (0.7-0.9)	0.1

**Table 2:** Summary statistics relating to CBC, total protein and protein electrophoretic profile in 20 stray cats infected with *Bartonella henselae* (infection status defined by IFAT  $\geq 1:64$  and/or PCR positive result)

RBC: red blood cells; Hb: hemoglobin; Hct: hematocrit; PCV: packed cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; PLT: platelets; WBC: white blood cells.

The only categorical variables studied that showed a statistically significant association with *Bartonella* infection status (Table 3), were hyperbetaglobulinemia ( $P=0.02$ ) and originating from zone 2 of Milan city ( $P=0.03$ ).

Characteristic	Categories	Total population	Infected(n=20)	Non-infected(n=70)	P value
Age	Young (<6 months)	19 (21.1%)	1 (5.3%)	18 (94.7%)	0.84
	Adult (>6 months)	71 (78.9%)	19 (26.8%)	52 (73.2%)	
Gender	Female	54 (60%)	13 (24.1%)	41 (75.9%)	0.79
	Male	36 (40%)	7 (19.4%)	29 (80.6%)	
BCS	Poor (<3/9)	13 (14.5%)	2 (15.4%)	11 (84.6%)	0.65
	Good (>4/9)	77 (85.5%)	18 (23.4%)	59 (76.6%)	
Colony of					
Origin	Zone 1	11 (12.2%)	2 (18.2%)	9 (81.8%)	1.00
	Zone 2	20 (22.2%)	1 (5.0%)	19 (95.0%)	<b>0.03</b>
	Zone 3	4 (4.4%)	1 (25.0%)	3 (75.0%)	1.00
	Zone 4	3 (3.3%)	0 (0.0%)	3 (100.0%)	1.00
	Zone 5	12 (13.3%)	2 (16.7%)	10 (83.3%)	1.00
	Zone 6	1 (1.1%)	0 (0.0%)	1 (100.0%)	1.00
	Zone 7	11(12.2%)	4 (36.4%)	7 (63.6%)	0.25
	Zone 8	5 (5.5%)	1 (20.0%)	4 (80.0%)	1
	Zone 9	17(18.8%)	6 (35.3%)	11 (64.7%)	0.26
	Suburban	6 (6.7)	3 (50.0%)	3 (50.0%)	0.12
Clinical examination	Healthy	21 (23.3%)	4 (19.0%)	17 (81.0%)	1.00
	Ill	69 (76.7%)	16 (23.2%)	53 (76.8%)	
	Stomatitis	35 (38.9%)	8 (22.9%)	27 (77.1%)	0.98
	Ocular discharge	4 (4.4%)	1 (25.0%)	3 (75.0%)	1
	Nasal discharge	3 (3.3%)	1 (33.3%)	2 (66.7%)	0.53
	Pale mucous membranes	5 (5.6%)	0 (0.0%)	5 (100.0%)	0.58
	Lymphadenopathy	65 (72.2%)	15 (23.1%)	50 (76.9%)	1
	CBC results				
	Presence of anemia (Hct <31.7%)	66 (75.0%)	14 (21.2%)	52 (78.8)	0.92
	Decreased Hb (<10.6 g/dl)	60 (68.2%)	13 (21.7%)	47 (78.3%)	0.93
	Decreased RBC (<6.56 x10 <sup>6</sup> /μl)	19 (21.6%)	1 (5.3%)	18 (94.7%)	0.06
	Thrombocytopenia (<175 x10 <sup>3</sup> /ml)	26 (29.5%)	4 (15.4%)	22 ( 84.6%)	0.78
	Leukocytosis (>18.7 x10 <sup>3</sup> /ml)	6 (6.8%)	1 (16.7%)	5 (83.3%)	1
	Leukopenia (<4.0 x10 <sup>3</sup> /ml)	1 (1.1%)	0 (0.0%)	1 (100%)	1
	Neutrophilia (>14 x10 <sup>3</sup> /ml)	5 (5.7%)	1 (20.0%)	4 (80%)	1
	Neutropenia (<2.3 x10 <sup>3</sup> /ml)	2 (2.3%)	1 (50.0%)	1 (50%)	0.39
	Lymphocytosis (>6.1 x10 <sup>3</sup> /ml)	6 (6.8%)	1 (16.7%)	5 (83.3%)	1
	Lymphopenia (<0.8 x10 <sup>3</sup> /ml)	2 (2.3%)	0 (0.0%)	2 (100.0%)	1
	Eosinophilia (>1.5 x10 <sup>3</sup> /ml)	6 (6.8%)	1 (16.7%)	5 (83.3%)	1
Proteinemia and Electrophoresis	Hyperproteinemia (6-8 g/dl)	3 (3.9%)	0 (0.0%)	3 (100.0%)	1
	Hyperalbuminemia (42-56.6%)	2 (2.63%)	0 (0.0%)	2 (100.0%)	1
	Hyperaglobulinemia (1-3%)	49(64.4%)	13 (26.5%)	36 (73.5%)	0.76
	Hyperβglobulinemia (5.8-8%)	10 (13.1%)	5 (50.0%)	5 (50.0%)	<b>0.02</b>
	Hyperγglobulinemia (13-25.2%)	16 (21.1%)	4 (25.0%)	12 (75.0%)	1.00

**Table 3:** Categorization of different variables in *Bartonella henselae* infected and non-infected cats and study for the association in a population of 90 stray cats of northern Italy. (P value in bold are statistically significant as  $\leq 0.05$ )

BCS: body condition score; CBC: complete blood cell count; RBC: red blood cells; Hb: hemoglobin; Hct: hematocrit; PCV: packed cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; PLT: platelets; WBC: white blood cells.

## Comparison between IFAT and PCR results

A total of 82 samples were analyzed by both IFAT and PCR, of these 14 (17.1%) were seropositive (IFAT titer  $\geq 1:64$ ) and 10 (12.2%) PCR-positive for amplification of *Bartonella* DNA. Five cats out of 82 (6.1%) were both PCR-positive and seropositive for *B. henselae*. Comparison between IFAT and PCR results is reported in Table 4.

Status of cat	PCR results		Total
	Positive	Negative	
IFAT sero-positive	5	9	14
IFAT seronegative	5	63	68
Total	10	72	82

**Table 4:** Comparison between bacteremia and seropositivity for *Bartonella* spp. infection results in 82 cats in northern Italy.

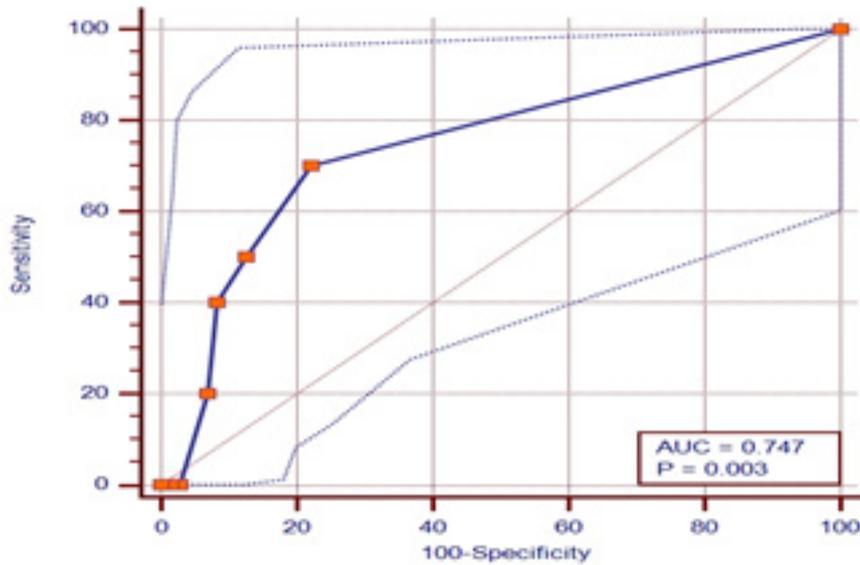
All PCR-positive cats had antibody titers  $\leq 1:256$  and none had the highest antibody titer of 1:1024. Antibody titers were not significantly higher (Mann-Whitney test,  $P=0.06$ ) in PCR-positive cats (highest IFAT titer, 1:256) than in PCR-negative cats (highest IFAT titer, 1:1024). A statistically significant association was found between IFAT titers  $< 1:32$  ( $P=0.004$ ) and 1:128 ( $P=0.037$ ) and PCR results (Table 1). Overall IFAT Se was 50.0% (95%CI 18.7-81.2), Sp 87.5% (95%CI 77.5-94.1), PLR 4.0 (95%CI 1.6-9.5), NLR 0.5 (95%CI 0.3-1.0), PPV 35.7% (95%CI 12.7-64.8) and NPV 92.6% (95%CI 83.6-97.5). The ROC analysis showed that the area under the curve was 0.747 (95% CI, 0.638 to 0.836, Standard error =0.0836, Z statistic =2.950) and was significantly higher ( $P=0.0032$ ) than 0.5 (the value of area indicating no diagnostic utility) (Figure- 1). With the PCR assay considered the gold standard in identifying bacteremic cats (PCR-positive samples), the diagnostic sensitivity and specificity, PPV and NPV of the IFAT at different antibody titers is reported in Table 5. The ROC analysis identified that sensitivity of 100% was achieved at IFAT antibody titer of  $< 1:32$ .

## Discussion

Results of epidemiologic studies have indicated that the worldwide prevalence of *Bartonella* spp. bacteremia in cats ranges from 15% to 55%. In our study 11/90 cats (12.2%) were considered bacteremic, since they tested positive with the PCR assay specific for the *Bartonella* genus. This prevalence is lower than that suggested by worldwide data but higher than previous data on bacteremia in stray cats of Milan city in which prevalence was 5.4% [13]. IFAT was positive for *B. henselae* antibodies in 14/82 (17.1%) cats, but only 5 of these seropositive cats (35.7%) were considered bacteremic, since they were positive with the PCR assay specific for the *Bartonella* genus. Half of bacteremic cats were seronegative and all cats showed low anti-*Bartonella* antibody levels (all PCR-positive cats had IFAT antibody titers  $\leq 1:256$ ). In addition the highest PCR-positive prevalence (30.0%) was significantly associated with IFAT titers  $< 1:32$ .

These results suggest that, as previously demonstrated [16], serological testing, and in particular IFAT, appear to be of limited value for predicting bacteremia. In fact the IFAT had a low PPV (35.7%), and thus is of limited utility in distinguishing between bacteremic and non-bacteremic cats. However, the inability to detect *B. henselae* antibodies appears to be highly predictive of the absence of bacteremia (NPV = 92.7%), and therefore, serologically negative cats may be more suitable for inclusion in a feline blood donor program, bearing in mind that bacteremic cats could be serologically negative. These results are in agreement with previous studies, which also found that serology (IFAT or ELISA) is more useful for exclusion than for confirmation of the infection because of the low PPV (39–46%) compared with the good NPV (87–97%) [13,16-18]. Therefore cats with positive *Bartonella* spp. test results should not be selected as blood donors. Cats with negative *Bartonella* spp. test results are less likely to be harboring the organism and so may be safer donors than cats with positive *Bartonella* spp. test results [9].

ROC analysis found that ideal IFAT cut-off with higher NPV and sensitivity was at titer  $< 1:32$ , in practice this is cats with no anti Ig-*Bartonella henselae* antibodies. No association was found between infection and clinical or hematological alterations and this is in accordance with previous studies in natural and experimentally infected cats [4,5,19,20], and in accordance with the fact that the natural infection is usually asymptomatic in cats, or characterized by mild clinical signs. These data support the hypothesis that cats, even if clinically healthy, can represent a reservoir for *Bartonella*-associated infection. Only one study found associations between antibodies to *Bartonella* spp. and lymphocytosis [14]. Hyperglobulinemia, primarily due to polyclonal gammopathy, was significantly associated with seropositivity to *Bartonella* species in one study [21] and this was partially in accordance with the significant hyperbetaglobulinemia that we found in our study in *Bartonella* spp. infected cats ( $P=0.02$ ).



**Figure 1.** ROC curve for IFAT detection of anti-*Bartonella henselae* IgG antibodies. The Y-axis shows the false positive rate (specificity), and the X-axis shows the true positive rate (sensitivity). A test with the perfect discrimination has a ROC curve that passes through the upper left corner. The area under the curve (AUC) is = 0.747 ( $P= 0.0032$ ). The ROC analysis shows that the cutoff point for the test with the best sensitivity is = 0 (Se: 100.0%; Sp: 0.0%).

Criterion	Sensitivity	95% CI	Specificity	95% CI	PLR	95% CI	PLR	95% CI	PPV	95% CI	NPV	95% CI
$\geq 0$	100.00	69.2 - 100.0	0.00	0.0 - 5.0	1.00	1.0 - 1.0			16.0	8.8 - 25.8		
>0	70.00	34.8 - 93.3	77.78	66.4 - 86.7	3.15	1.7 - 5.7	0.39	0.1 - 1.0	37.5	19.0 - 59.2	93.2	83.3 - 98.1
>1:32	50.00	18.7 - 81.3	87.50	77.6 - 94.1	4.00	1.7 - 9.6	0.57	0.3 - 1.1	43.2	18.8 - 70.4	90.2	80.4 - 96.1
>1:64	40.00	12.2 - 73.8	91.67	82.7 - 96.9	4.80	1.6 - 14.1	0.65	0.4 - 1.1	47.8	18.3 - 78.4	88.9	79.2 - 95.1
>1:128	20.00	2.5 - 55.6	93.06	84.5 - 97.7	2.88	0.6 - 12.9	0.86	0.6 - 1.2	35.4	6.8 - 75.3	85.9	75.9 - 92.9
>1:256	0.00	0.0 - 30.8	97.22	90.3 - 99.7	0.00		1.03	1.0 - 1.1	0.0	0.0 - 85.5	83.6	73.7 - 90.9
>1:512	0.00	0.0 - 30.8	98.61	92.5 - 100.0	0.00		1.01	1.0 - 1.0	0.0	0.0 - 97.9	83.8	74.0 - 91.1
>1:1024	0.00	0.0 - 30.8	100.00	95.0 - 100.0			1.00	1.0 - 1.0			84.0	74.2 - 91.2

**Table 5.** Sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), positive predictive value (PPV), negative predictive value (NPV) of IFAT test compared to PCR results in identifying bacteremic cats for *Bartonella* spp.

Increased globulin concentrations can be explained by the prolonged bacteremia that can be associated with infection by *Bartonella* spp. However in *Bartonella* infections both co-infections and serial reinfection have been documented in cats, both of which could contribute to development of polyclonal gammopathy in a cross sectional study such as this. Causality cannot be determined by analysis of cross-sectional data and it was not possible to test these cats a second time in this study. There were a number of limitations to this study. The primary limitation is the small sample size, which limits the statistical significance of results.

The secondary limitation is the population studied. Free-roaming stray cats should never be considered as potential donors, as they are high-risk cats for blood-borne pathogens, as demonstrated by previous studies in stray cats in northern Italy [12,21-23]. Stray cats have a higher prevalence of *Bartonella* spp. infection than pet cats, mainly due to close contact between infected animals in large groups of cats [24]. Although stray cats represent a sentinel population for a variety of infections, because they receive no prophylaxis and are continually exposed to disease vectors, they provide limited data due to the absence of important anamnestic and clinical information. However stray cats undergoing sterilization in TNR program are usually young cats, and the prevalence of *Bartonella henselae* bacteremia in young cats (< 1 year old) is usually higher than it is in adult cats [16,17]. Other than young age, recognized risk factors for *Bartonella* bacteremia in cats are infestation with fleas, outdoor lifestyle and a multicat environment [9,14,16,17]. Stray cats population are therefore high risk population for *Bartonella* spp. bacteremia and this could facilitate the finding of infected cats whose blood samples are useful in evaluation and comparison of diagnostic methods. In addition with flea infestations of peridomestic animals, stray colony cats contribute to the maintenance of these organisms in close proximity to household environments, and therefore give important information on the risk of *Bartonella* spp. infection for pet cats that share their environments.

Another limitation is that *Bartonella* was investigated only at the genus level, as amplicons derived from blood were not sequenced to distinguish between *B. henselae* and *B. clarridgeiae* PCR products and therefore information about the species infecting the study population was lacking. Cats have been recognized as a reservoir of *Bartonella clarridgeiae* [7]. However, it is likely that *B. clarridgeiae* is present at very low frequencies in the cat population in Italy [11,13,19].

Finally, other organisms such as *Chlamydia* spp. and *Coxiella burnetii*, have been associated with serological cross-reactions in people [26,27] and the same could happen in cats. It is known that antibodies against *B. henselae* generally cross react with *B. clarridgeiae* and other *Bartonella* spp. How these cross-reactions falsely influence the results of serological analysis derived from cats is not known.

## Conclusion

The highest sensitivity for identification of *Bartonella* spp. non-bacteremic cats for inclusion in a feline blood donor program is achieved by using an IFAT cut-off <1:32. However serologically negative cats may still be bacteremic for *Bartonella* spp. Therefore feline blood donors should be screened using both IFAT and PCR (as suggested as the minimum standard by the Consensus Statements of the American College of Veterinary Internal Medicine for the screening of feline blood donors for blood-borne pathogens [1]). Protein electrophoresis should be performed in all cats to be evaluated as blood donors.

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