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Detection of *Bartonella henselae* in feline erythrocytes in Egypt by using Giemsa staining, transmission electron microscopy, and polymerase chain reaction

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Abstract

Bartonella species (*Bartonella* spp.) have gained recognition as a significant human pathogen, implicated in a wide range of diseases. Among these, *Bartonella henselae* infection has been extensively studied for its primary occurrence in cats and its role in the development of cat-scratch disease in humans. While light microscopy and transmission electron microscopy (TEM) have traditionally played crucial roles in identifying causative agents of infectious diseases, including *Bartonella* spp., the accuracy of these methods in identifying *Bartonella* spp. remains undefined. Therefore, this study aims to bridge this gap by employing both light microscopy and TEM to detect *Bartonella* in feline blood samples and to confirm *B. henselae* with polymerase chain reaction (PCR). Examination of blood smears stained with Giemsa and toluidine blue semithin sections by using light microscopy revealed the presence of intraerythrocytic corpuscles, suggesting *Bartonella* infection in six out of 33 examined cat blood samples. TEM findings corroborated these observations, showcasing the engulfment of bacteria by the erythrocyte membrane, along with the presence of some *Bartonella* spp., adhering to the erythrocyte wall. PCR-based molecular detection confirmed the presence of *B. henselae* in these six samples. It is concluded that light microscopy and TEM are considered valuable in the screening of cats' blood for the potential presence of *Bartonella*. However, further molecular techniques are essential for precise identification and confirmation of specific *Bartonella* spp.

Research Highlights

- Giemsa-stained blood smear and semithin section showed potential intraerythrocytic *Bartonella* spp. corpuscles.
- TEM demonstrated the engulfment of *Bartonella* spp. by the erythrocyte membrane, along with the presence of some *Bartonella* spp. adhering to the erythrocyte wall.
- Molecular analysis of blood samples from cats by PCR unveiled that six out of 33 (18.18%) samples tested positive for *B. henselae* infection.

KEYWORDS

Bartonella, cat-scratch disease, feline erythrocyte, Giemsa stain, TEM

1 | INTRODUCTION

Bartonella species (*Bartonella* spp.) are regarded as emerging vector-borne zoonotic pathogens associated with a broad spectrum of diseases with global distribution (Krügel et al., 2022; Lamas et al., 2008). *Bartonella* are small, gram-negative bacteria capable of infecting domestic and wild mammals, as well as humans (Dehio & Sander, 1999). *Bartonella* infections of humans and animals are often characterized by an intraerythrocytic bacteremia (Regier et al., 2016). Within the animal kingdom, cats, dogs, and rodents serve as the primary reservoirs for *Bartonella* spp., and vectors, or cat scratches, represent the notable means of infection (Carithers, 1985; Jacomo et al., 2002).

Among *Bartonella* spp., numerous investigations have shown that *Bartonella henselae* infection mainly occurs in cats, with the bacteria infecting feline erythrocytes (Jacomo et al., 2002; Kordick et al., 1997). Although it has been known that the infected cats are usually asymptomatic, the experience of chronic recurring bacteremia has been reported (Breitschwerdt & Kordick, 1995). Furthermore, it is well documented in humans that *B. henselae* is responsible for the development of cat-scratch disease (CSD), which is characterized by chronic enlargement of lymph nodes, inflammation of the endocardium, brain disorder, convulsions, hepatosplenomegaly, and/or kidney disorder (Boulouis et al., 2005; Lamas et al., 2008). This infection can also induce bacillary angiomatosis (BA) which has been primarily seen in immunity disorder human patients (Boulouis et al., 2005). Although domestic animal and human *Bartonella* infections may resolve spontaneously and without evident symptoms (Jacomo et al., 2002), there are instances where neglecting the condition can pose significant risks.

In addition to *B. henselae*, other *Bartonella* spp. have been known to be human pathogens such as *Bartonella bacilliformis*, the agent of Carrion's disease, *Bartonella quintana*, the agent of trench fever and BA, and *Bartonella elizabethae*, which can cause endocarditis (Daly et al., 1993; Welch et al., 1992). In addition, *Bartonella vinsonii* has been found in a case of canine endocarditis and has been reported to cause human endocarditis (Breitschwerdt et al., 1995; Schwartzman, 1996). The other *Bartonella* spp. including *Bartonella clarridgeiae*, *Bartonella doshiae*, *Bartonella grahamii*, *Bartonella peromysci*, *Bartonella talpae*, and *Bartonella taylorii* have been isolated from the blood of various animals, and have not been known to induce disease in the infected mammal species (Birtles et al., 1995; Lawson & Collins, 1996). These *Bartonella* spp. can be transmitted by a wide variety of arthropods including ticks (*Ixodes pacificus*), fleas (*Ctenocephalides felis*), lice (*Pediculus humanus corporis*), and sandflies (*Lutzomyia verrucarum* and *Lutzomyia peruensis*) (Chang et al., 2001; Cotté et al., 2008; Jacomo et al., 2002).

In Egypt, very few studies have investigated the *Bartonella* spp. infection in cats and humans. Among these studies, a prevalence of antibodies to *Bartonella* spp. was detected using a modified agglutination test in the sera from 105 out of 178 (59.6%) feral cats from Abou-Rawash, Giza (Al-Kappany et al., 2011). Moreover, *B. henselae* has been recognized by using polymerase chain reaction (PCR) in 8% (6/75) of blood samples from cats (Sayed et al., 2022). Furthermore, a recent study has reported three out of 100 (3%) cats with strict indoor lifestyles in Cairo city tested PCR-positive for *Bartonella* spp., and two of these positive samples were identified positive for *B. henselae* (Zarea et al., 2023). In humans, *B. henselae* seroprevalence of 46% has been reported in Egypt using immunofluorescent assay (Sayed et al., 2022).

Several laboratory diagnostic procedures have been developed to identify *Bartonella* including bacterial isolation, serological tests, and molecular techniques (Agan & Dolan, 2002; Jensen et al., 2000; Okaro et al., 2017). Because of the fastidious nature of this bacteria, *Bartonella* isolation on culture media is usually unsuccessful, laborious, and requires an incubation period of 4–8 weeks (Okaro et al., 2017). Moreover, some studies have examined blood smears (Billeter et al., 2008) and transmission electron microscopy (TEM) for detection of *B. bacilliformis* (Benson et al., 1986) and *B. henselae* (Diddi et al., 2013; Kordick & Breitschwerdt, 1995; Pitassi et al., 2007). Moreover, electron microscopy has been a pivotal technique in identifying the causative agents of infectious diseases (Golding et al., 2016; Goldsmith & Miller, 2009; Hazelton & Gelderblom, 2003). Nonetheless, the question of how accurate are light microscopy and TEM in identifying *Bartonella* infection is still undefined. Thus, this study was designed to use rapid methods to identify *Bartonella* spp. in feline blood samples, by using both light microscopy and TEM and to correlate the findings with the molecular detection of *B. henselae* by using PCR. The focus on the molecular detection of *B. henselae* among *Bartonella* species is due to its role in causing severe vasoproliferative tumors, such as BA and bacillary peliosis, particularly in immunocompromised individuals.

2 | MATERIALS AND METHODS

2.1 | Samples collection

A total of 33 healthy cats, aged 3 months to 4 years, were investigated at a veterinary pet clinic in Assiut Governorate, Egypt, between March 2016 and March 2018. These cats included 18 females and 15 males. A blood sample (1 mL) was obtained from the cephalic vein of the examined cats. Briefly, the cat was positioned near the edge of the examination table, with its head facing toward the person taking the blood sample. One hand gently restrained the cat's head by supporting it underneath, while the other hand was placed behind the cat's elbow, extending the limb slightly forward. The fur over the cephalic vein on the upper forelimb was clipped, and the site was cleaned aseptically. To raise the vein, the thumb was positioned on the dorsal side of the forelimb at the elbow, with a slight lateral rotation to bring the vein into the optimal position for blood sampling. Following sampling, blood was placed in tubes with the 3.4% sodium citrate as an anticoagulant.

2.2 | Bacterioscopy with Giemsa staining

The Giemsa staining procedures were carried out as previously mentioned (Bentzel et al., 2008). Briefly, a single drop of whole blood was

applied onto a glass slide and left to air-dry for 30 min. Following this, the slides were fixed using 96% ethyl alcohol for 2 min, followed by drying and staining with Giemsa for 8-min. After a brief rinse with distilled water, the slides were left to air-dry at room temperature. Following that, the slides were examined under light microscopy (Olympus microscope with a digital Toupcam L3CMOS10000KPA camera), and digital images were captured and quantified for the percentage of the infected erythrocytes.

2.3 | Semithin sections and transmission electron microscopy

Blood samples (500 µL) were centrifuged at 1200 rotations per minute for 5 min at room temperature. After discarding the supernatants, the remaining pellets were prepared for TEM examination by holding in a fixative composed of 3% paraformaldehyde-glutaraldehyde and 0.1-M phosphate buffer (pH 7.3) at 4°C for 24 h (Karnovsky, 1965). The fixed samples were washed in 0.1-M phosphate buffer (pH 7.3) and were fixed in 1% osmium tetroxide in 0.1-M phosphate buffer (pH 7.3). Following that, the samples were dehydrated in a graded series of ethanol (50%, 70%, 90%, and 100%), fixed in Epon, and polymerized at 60°C for 24 h, and then at 75°C for 48 h. Semithin sections (1 mm thick) were cut with Richert Ultracuts (Leica, Germany), and stained with toluidine blue (Bancroft & Gamble, 2008), and were examined under light microscopy (Olympus microscope with a digital Toupcam L3CMOS10000KPA camera). Ultrathin sections (70-nm thick) were cut with Ultrotom VRV (LKB Bromma, Germany), stained with uranyl acetate and lead citrate (Reynolds, 1963), and were examined by JEOL100CX II transmission electron microscope at the Electron Microscopy Unit of Assiut University, Egypt.

2.4 | Molecular detection of *B. henselae*

Molecular identification of *B. henselae* was performed in the colors for research laboratory (Member of Clinilab group, Cairo, Egypt).

2.4.1 | Extraction of DNA

The deoxyribonucleic acid (DNA) was extracted from feline blood samples (33) by using the QIAamp DNA Mini Kit (Cat. No. 51403). The extraction procedure strictly followed the manufacturer's instructions.

2.4.2 | Amplification via PCR

For molecular detection of *B. henselae*, the extracted DNA was amplified using a primer targeting the citrate synthase gene (*gltA*). The primers were used: BartogltA forward: 5′-TTCCGYCTTATGGGTTTTGG-3′ and Bartohenselae: 5′-CATTTCTGTTGGAAATCCTAG-3′ (Fard et al., 2016). PCR reaction mixture prepared including 13 µL of Taq Master Mix (Taq PCR Master Mix Kit, Cat. No. 201445), 1 µL of both forward and reverse primers (0.40 p/mol each), 100 pg/µL to 100 ng/µL DNA template, and deionized water was added to a final volume of 25 µL. The amplification process included a 5 min initial denaturation stage at 95°C, 35 cycles of 45 s each at 95°C, 45 s at 56°C, and 45 s at 72°C, in addition to a 7 min extension step at 72°C. Five microliters of each of the amplified PCR products were examined in 1.6% (w/v) agarose gels in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer, stained with ethidium bromide, transilluminated under ultraviolet light, and photographed. AMPLIRUN® *B. henselae* DNA control (Vircell, Cat. No. MBC005) was used as positive control and distilled water was used as a negative control. The amplicon size is 246 bp.

3 | RESULTS

3.1 | Light microscopical examination

Examination of Giemsa-stained blood smears using light microscopy suggested the occurrence of *Bartonella* spp. inside the erythrocyte of six out of 33 examined cat blood samples (Figure 1). The percentage of the infected erythrocytes in these samples was 34.38% ± 6.19%.

3.2 | Semithin sections and TEM examination

Examination of semithin sections of blood samples that were positive for Giemsa stain revealed the presence of varied-sized pale-stained corpuscles within erythrocytes, suggesting potential *Bartonella* spp. infection. Moreover, small dark-stained structures were observed attached to the outer surfaces of the erythrocytes (Figure 2).

Additionally, TEM findings aligned with those from light microscopy, proposing the presence of *Bartonella* spp. within the

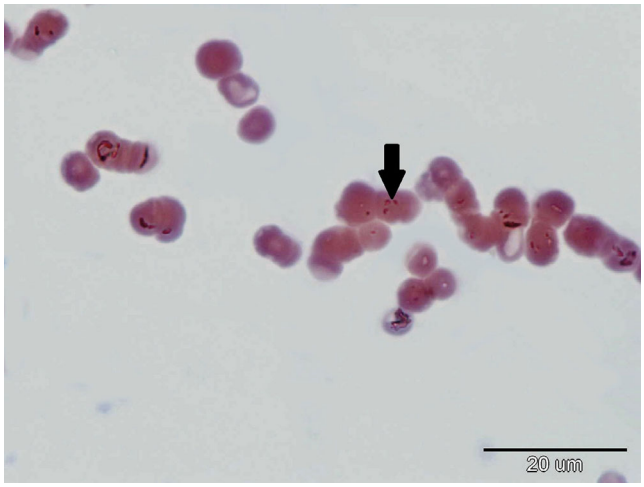


FIGURE 1 Giemsa-stained blood smear showing intraerythrocytic corpuscles compatible with *Bartonella* spp., (black arrow). Scale bar = 20 µm.

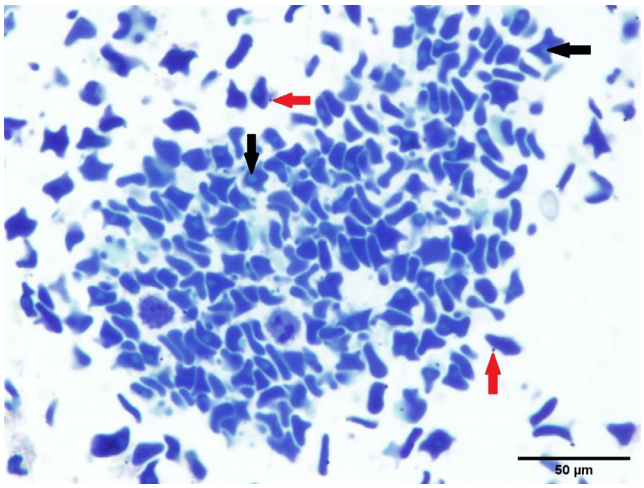


FIGURE 2 Semithin section stained with toluidine blue reveals the presence of varied-sized pale-stained *Bartonella* spp., corpuscles within erythrocytes (black arrows), with small dark-stained structures attached to the outer surfaces of the erythrocytes (red arrows). Scale bar = 50 µm.

erythrocytes, where the bacteria appeared to be engulfed by the erythrocyte membrane (Figure 3a,b). Notably, adherence of some *Bartonella* spp. to the erythrocyte wall was also observed (Figure 4).

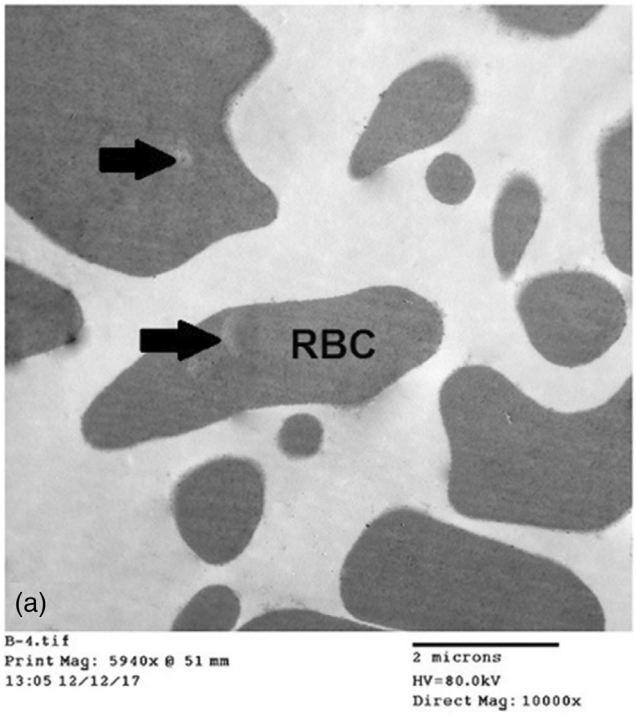
3.3 | Molecular detection of *B. henselae* by PCR

Molecular detection of *B. henselae* by PCR was performed on the collected 33 blood samples, and the outcomes revealed the identification of *B. henselae* in the six samples that were positive for Giemsa stain, with a rate of 18.18% (6/33 samples) (Figure 5).

4 | DISCUSSION

Cats are regarded as the principal host of *B. henselae* (Kordick et al., 1995). Infection between cats depends principally on the cat flea (*C. felis*) (Chomel et al., 1996). Although infected cats usually remain asymptomatic, bacteremia may persist for several months to several years (Abbott et al., 1997). Following experimental infection, cats suffer from fever, transient anemia, neurological disorder, and endocarditis (Guptill et al., 1997; Kordick et al., 1999; Kordick & Breitschwerdt, 1997).

Examination of Giemsa-stained blood films uncovered the recognition of *Bartonella* spp. inside the blood cells of six examined cats (Figure 1). Giemsa-stained blood films have been used as a diagnostic method for the detection of the intraerythrocytic presence of various pathogens including *B. henselae*. (Abbas et al., 2018; Hong et al., 2019; Le et al., 2008; Peng et al., 2021; Sanchez Clemente et al., 2016; Staggemeier et al., 2010; Torres et al., 2018; Zhou et al., 2014). In addition, invasion of feline erythrocytes with *B. henselae* was previously detected by scanning confocal microscopy (Mehock



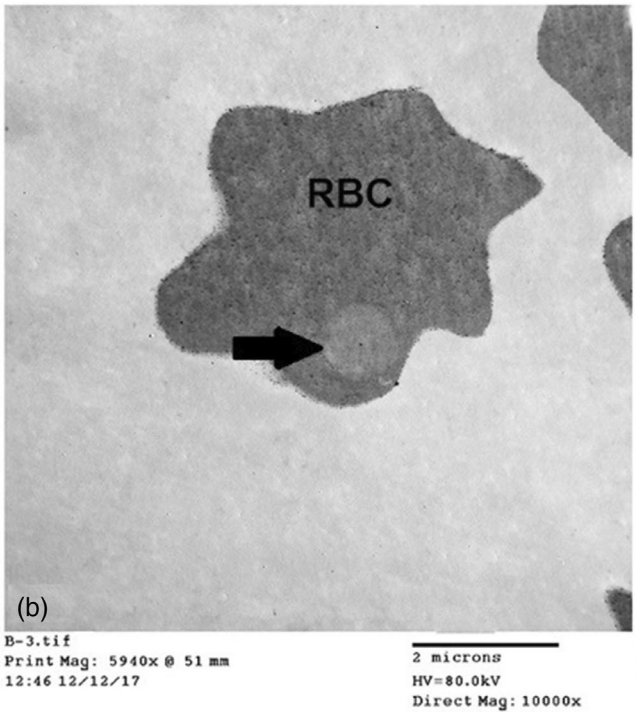


FIGURE 3 (A, B) Transmission electron micrographs showing invasion of cat red blood cells (RBC) with *Bartonella* spp., (black arrows). Uranyl acetate and lead citrate stain, scale bar = 2 µm.

et al., 1998). This potential intraerythrocytic localization of *Bartonella* spp. was confirmed by semithin sections that revealed the presence of varied-sized pale-stained corpuscles within erythrocytes (Figure 2). Interestingly, the transmission electron micrograph unveiled the engulfment of bacteria by the erythrocyte membrane (Figure 3),

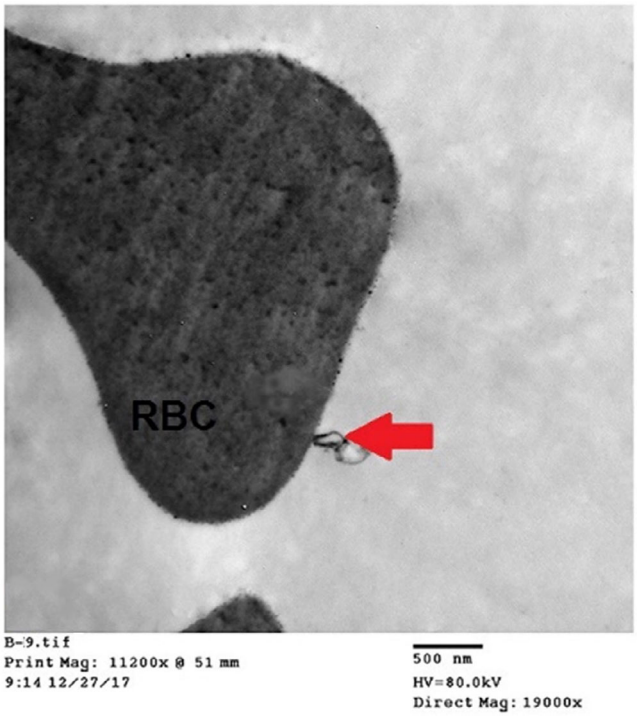


FIGURE 4 Transmission electron micrograph show adherence of *Bartonella* spp., (red arrow) to the wall of feline red blood cells (RBC). Uranyl acetate and lead citrate stain, scale bar = 500 nm.

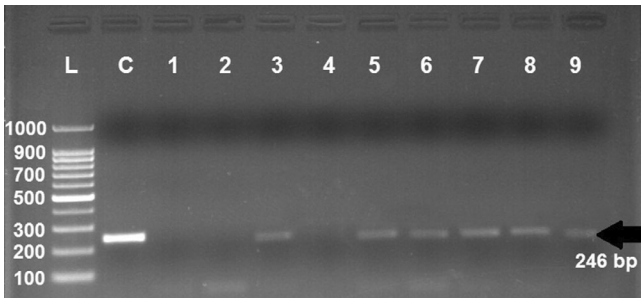


FIGURE 5 Amplification of *gltA* gene of *B. henselae* in blood samples of cats by using PCR primer BartogltA and Bartohenselae. Lane L: DNA Ladder (1 Kb); Lane C: positive control; Lane 3, 5, 6, 7, 8, and 9 *B. henselae* positive samples; Lane 1, 2, and 4 *B. henselae* negative samples.

accompanied by the observation of some bacteria adhering to the erythrocyte wall (Figure 4).

In agreement with our results, blood smears of cats from South Brazil were examined by Giemsa stain and revealed the identification of *Bartonella* spp. inside the red blood cells (Staggemeier et al., 2010). Moreover, several TEM studies reported the intraerythrocytic presence of *B. henselae* in cats (Diddi et al., 2013; Pitassi et al., 2007).

It is noteworthy to mention that Giemsa stain and TEM examination are not sufficient to diagnose *Bartonella* infection in cat blood. However, they are considered valuable screening methods that need further confirmation. Molecular detection techniques, such as PCR, prove valuable in confirming or excluding the presence of these agents. In this study, amplification of the *gltA* gene revealed *Bartonella* infection in six cats (18.18%) out of the total 33 examined cats. The results of both the Giemsa stain and TEM examination were concurrent with the results of molecular diagnosis.

Previous investigations have unveiled considerable variations in the prevalence rates of *B. henselae* when using PCR. Earlier studies have reported lower prevalence rates of *B. henselae* including, Portugal (6.7%) (Childs, 1995), Germany (13%) (Sander et al., 1997), Switzerland (8.3%) (Glaus et al., 1997), Japan (9.1%) (Maruyama et al., 1998), United Kingdom (9.4%) (Birtles et al., 2002), Czech Republic (8%) (Melter et al., 2003), Sweden (2.2%) (Olsson Engvall et al., 2003), Turkey (8.2%) (Celebi et al., 2009), South of Brazil (10.63%) (Staggemeier et al., 2010), and Egypt (2%) (Zarea et al., 2023). On the contrary, higher *B. henselae* prevalence rates have been detected in some studies conducted in the Philippines (61%) (Chomel et al., 1999), Brazil (RJ, 90%) (Souza, 2009), and South Korea (33.3%) (Kim et al., 2009). The variations in the reported prevalence among cat populations across various territories can be linked to changes in climate (Boulouis et al., 2005). Another contributing factor could be the level of flea infestation, which tends to be higher in warm and humid areas compared to colder regions (Kreppel et al., 2016).

5 | CONCLUSION

The use of light microscopy and TEM is indeed valuable in the initial screening of cats' blood for the presence of *Bartonella* spp. These methods allow for the visual identification of morphological characteristics indicative of *Bartonella* infection. However, given the limitations of microscopy, such as potential misidentification due to similar-looking organisms and the need for highly trained personnel, further molecular confirmation is needed. A large-scale epidemiological study is recommended to investigate *B. henselae* in cats and humans to explore the genetic characterization of the *Bartonella* strains circulating in Egypt.

AUTHOR CONTRIBUTIONS

Reem M. Alsaadawy: Methodology; investigation; validation; formal analysis; writing—original draft; conceptualization; data curation. **Amal S. M. Sayed:** Conceptualization; supervision; methodology; validation; writing—review and editing; project administration. **Magda M. Ali:** Conceptualization; supervision; validation; writing—review and editing; methodology. **Sary Kh. Abd-Elghaffar:** Writing—review and editing; methodology.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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