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# Molecular survey of *Babesia microti*, a causative agent of human babesiosis, in rodents in Egypt

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## SUMMARY

In Egypt, to date and to the best of our knowledge, two cases of human babesiosis have been reported; at the same time, very few surveys for detection of *B. microti* in rodents have been published. These studies were solely relied on microscopic examination of stained blood smears; hence, the molecular confirmative evidence for the presence of *B. microti* is not revealed. In addition, the epidemiology of human babesiosis, such as competent vectors and reservoirs is still unclear. The present study is a molecular survey of *B. microti* in wild Egyptian rodents. A total of 30 rodents (26 *Rattus norvegicus* and 4 *Rattus rattus*) were collected from different areas in Giza Governorate and tested for *Babesia*-specific DNA. Nested PCR targeting 18S rRNA gene marker was used to detect the agent in rodents blood. Positive amplification of 154-bp fragment was detected in 8 (30.8%) *R. norvegicus* and 1 (25%) *R. rattus* with a total prevalence rate of 30%. Unidentified *Babesia* was detected in 3 rodents (2 *R. norvegicus* and 1 *R. rattus*) when tested using standard PCR with genus-specific primers. These 3 rodents yielded negative for *B. microti*. Significant correlation ($P<0.05$) between positivity to *B. microti* and splenomegaly was found. The difference between the positive rates for *B. microti* in *R. norvegicus* and in *R. rattus* was also revealed to be significant ($P<0.05$). This study is considered to be the first molecular survey of *B. microti* in Egyptian rodents. These findings confirm the presence of one of the causative agents of human babesiosis in the country with a relative high prevalence among the suspected reservoir

host that warrants more risk for human infections.

**Keywords:** Human babesiosis, *Babesia microti*, Rodents, *Rattus norvegicus*, *Rattus rattus* Molecular survey, Nested PCR

## INTRODUCTION

Babesiosis is an emerging tick-borne zoonotic disease caused by intraerythrocytic piroplasms of genus *Babesia* (Homer et al., 2000; Kjemtrup and Conrad, 2000; Gelfand and Vannier, 2005). Several *Babesia* species are incriminated to cause disease in humans. Of these, *B. microti*, a parasite of rodents, appears to be responsible for human cases in the United States (Parry et al., 1977; Dammin et al., 1981; Persing et al., 1992; Homer et al., 2000; Kjemtrup and Conrad, 2000). On the other hand, *B. divergens* is the predominant species causing human infections in Europe (Gorenflot et al., 1998). Other unnamed species (WA1, CA1, MO1, and EU1) have been documented to cause human babesiosis (Quick et al., 1993; Persing et al., 1995; Herwaldt et al., 1996; Herwaldt et al., 1997; Telford and Spielman, 1998; Herwaldt et al., 2003; Duh et al., 2005). Despite the fact that most infections are subclinical, severe disease is seen in immunocompromized and splenectomized individuals (Rosner et al., 1984; Krause, 2002). In the US, where *B. microti* is the sole agent of human babesiosis, the roles of white footed mouse (*Peromyscus leucopus*) as rodent reservoir and deer tick (*Ixodes scapularis*) as tick vector for human babesiosis, have been well established (Etkind et al., 1980; Piesman and Spielman, 1980; Spielman et al., 1981; Piesman and Spielman, 1982; Piesman et al., 1986; Telford et al., 1993; Mather et al., 1996; Varde et al., 1998, Stafford et al., 1999). Although this piroplasm has been detected in various rodent species in Europe (Shortt and Blachie, 1965; Walter, 1984; Duh et al., 2003) and Asia (van Peen et al., 1977), the complete scenario of epidemiology in these areas is not clear. In Egypt, to date and to the best of our knowledge, only 2 human cases of babesiosis have been reported (Michael et al., 1987; El-Bahnasawy and Morsy, 2008); in addition, very few surveys for detection of *B. microti* in rodents have been published (el Bahrawy et al., 1993; el-Kady et al., 1998). It is noteworthy to mention that these detections in human and murine hosts have solely relied on microscopic examination of stained blood smears. The epidemiology of *B. microti* in Egyptian nature, such as the molecular nature of the present species, competent reservoir and vector, the prevalence of infection, are remained to be elucidated. The objective of the present study was to conduct a molecular survey for detection of *B. microti* in rodents as an initial step for

competence studies; which in turn help in elucidation of the epidemiology of human babesiosis in Egypt.

## MATERIAL AND METHODS

### Rodents and blood samples collection

During 2005, rodents were live-trapped from different parts in Giza Governorate, Egypt. Captured rodents were brought to the laboratory, anaesthetized and identified to the species level (Barnett, 1963; Nowak and Paradiso, 1983; Avalos and Callahan, 2001). Rodents were then bled and EDTA-whole blood samples were collected and stored at -20°C until DNA was extracted. Rodents were then sacrificed, and their spleens were investigated for size. Aseptic procedures during sampling and handling of specimens were implemented to avoid contamination of specimens.

### DNA extraction

Total DNA was extracted from 200 µl of thawed blood samples using QIAamp DNA Blood Mini Kit (QIAGEN Inc., CA, USA) according to the manufacturer's protocols. Purified DNA was eluted from the columns in 200 µl of distilled water and then DNA concentration and purity were assessed spectrophotometrically. Purified DNA was then stored at -20°C till used in PCR. A negative control for the extraction (distilled water) was included with every 10 samples.

### PCR and electrophoresis

Standard PCR routines were used to prevent contamination. DNA extraction, the preparation of reaction mixtures, amplification, and detection of PCR products were all performed in different areas. Fresh gloves were used with each manipulation; in addition, aerosol-resistant filter pipette tips were used throughout the experiment. All PCR reagents and enzyme were obtained from the Jena Bioscience (Jena Bioscience, GmbH, Germany) and used as recommended by the supplier. For screening purposes, oligonucleotide primers, Babesia-F and Babesia-R (Table 1), that amplify the conserved sequence of the 18S rRNA gene of the genus *Babesia* were used in a standard PCR reaction (Inokuma et al., 2003). In this reaction, 5 µl of each extracted DNA template were amplified in a 50-µl mixture containing 20 pmoles of each primer. The reaction involved initial denaturation (95°C for 5 min), followed by 34 cycles (denaturation-95°C for 30 sec, annealing-55°C for 30 sec, extension-72°C for 90 sec) and then final extension at 72°C for 5 min. For specific detection of *B. microti*, nested PCR was performed using outer primers Bab1 and Bab4, and inner primers Bab2 and Bab3 (Table 1). These primer sets also target a fragment of 18S rRNA gene that is specific for the organism. The oligonucleotides amplify 238-bp and

154-bp fragments, respectively (Persing et al., 1992). In nested reaction, extracted template DNA was amplified in 50-µl reaction mixture containing 20 pmoles of each primer. Thermocycler program included initial denaturation (1 min. at 94°C) followed by 35 rounds of temperature cycling (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min). The amplification was concluded with additional final extension at 72°C for 5 min. All PCR reactions were executed in an automated thermocycler (Techne TC512, USA). A panel of "NO DNA" negative controls (PCR-grade water) and positive controls was included in each experiment to control contaminations and false-negative amplification results. For standard PCR, positive *Babesia* DNA sample was used. Unfortunately, *B. microti* positive sample was unavailable for nested PCR; therefore, in the beginning of the experiment we used the available positive sample as internal control, then we used the first obtained *B. microti* positive sample to control downstream PCR amplifications and repeated reactions. PCR products were analyzed by electrophoresis on 1.25% agarose gels and visualized under ultraviolet (UV) transilluminator. A positive result was considered a clear band of expected fragment (Table 1). All positive and negative results were confirmed by at least one repeat. Discordant results (i.e., one negative, one positive) were discarded.

**Table1:** Oligonucleotide sequences of primers used in this study.

| Target organism | Target gene | Primer designation | Length (nt) | Oligonucleotide sequence (5' to 3') | Length of expected band (bp) |
|---|---|---|---|---|---|
| Genus *Babesia* | 18S rRNA | Babesia-F | 20 | GTG AAA CTG CGA ATG GCT CA | ~650 |
| | | Babesia-R | 21 | CCA TGC TGA AGT ATT CAA GAC | |
| *B. microti* (outer) | 18S rRNA | Bab1 | 23 | CTT AGT ATA AGC TTT TAT ACA GC | 238 |
| | | Bab4 | 25 | ATA GGT CAG AAA CTT GAA TGA TAC A | |
| *B. microti* (inner) | 18S rRNA | Bab2 | 25 | GTT ATA GTT TAT TTG ATG TTC GTT T | 154 |
| | | Bab3 | 20 | AAG CCA TGC GAT TCG CTA AT | |

## RESULTS

### Collected rodents

A total of 30 rodents comprising two species [26 (86.7%) *Rattus norvegicus* and 4 (13.3%) *Rattus rattus*] were collected. Postmortem investigation of spleen size revealed that only 6 (5 *R. norvegicus* and 1 *R. rattus*) out of captured rodents had splenomegaly

### Polymerase chain reaction

Results of PCR using both genus *Babesia* and *B. microti*-specific primers in relation to splenomegalia are summarized in Table 2 &

3. Positive cases were recorded by the presence of 650-bp and 154-bp bands, respectively (Figures 1 & 2). A significant correlation between splenomegaly and the presence of specific band corresponds to *B. microti* was also recorded ($P<0.05$). The difference between the positive rates for *B. microti* in *R. norvegicus* and in *R. rattus* was also proved to be significant ($P<0.05$).

**Table 2:** Prevalence of *Babesia* species (using genus primers) in rodents captured in Giza Governorate, Egypt in correlation to splenomegalia.

| Rodent species | No. of PCR positive / No. of rodents tested (%) | | Total |
|---|---|---|---|
| | With splenomegaly | Without splenomegaly | |
| *Rattus norvegicus* | 5/5 (100) | 5[§]/21 (23.8) | 10/26 (38.5) |
| *Rattus rattus* | 1[†]/1 (100) | 0/3[♦] (0) | 1/4 (25) |
| Total | 6/6 (100) | 5/24 (20.8) | 11/30 (36.7) |

§ 2 out of these 5 rodents yielded negative for *B. microti*.
† This animal also gave negative result for *B. microti*.
♦ 1 of these animals tested positive for *B. microti*.

**Table 3:** Prevalence of *B. microti* (using species-specific primers) in rodents captured in Giza Governorate, Egypt in correlation to splenomegalia.

| Rodent species | No. of nested PCR positive / No. of rodents tested (%) | | Total |
|---|---|---|---|
| | With splenomegaly | Without splenomegaly | |
| *Rattus norvegicus* | 5/5 (100) | 3/21 (14.3) | 8/26 (30.8) |
| *Rattus rattus* | 0/1 (0) | 1/3 (33.3) | 1/4 (25) |
| Total | 5/6 (83.3) | 4/24 (16.7) | 9/30 (30) |

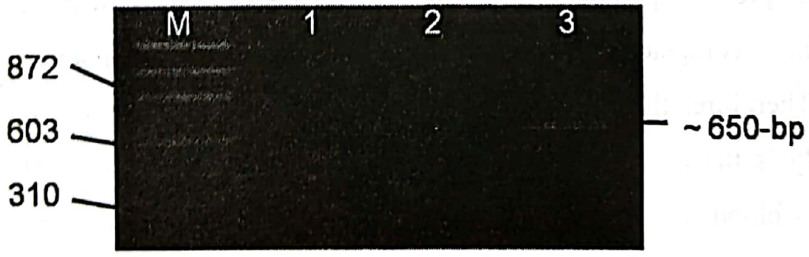


**Figure 1:** Agarose gel electrophoresis of the standard PCR using genus *Babesia*-specific primers (Babesia-F and Babesia-R). Generation of a fragment of ~650 bp (lane 3) indicate positive result. Lane M, molecular size standard marker, ΣX174 DNA- *Hae* III Digest (bp).

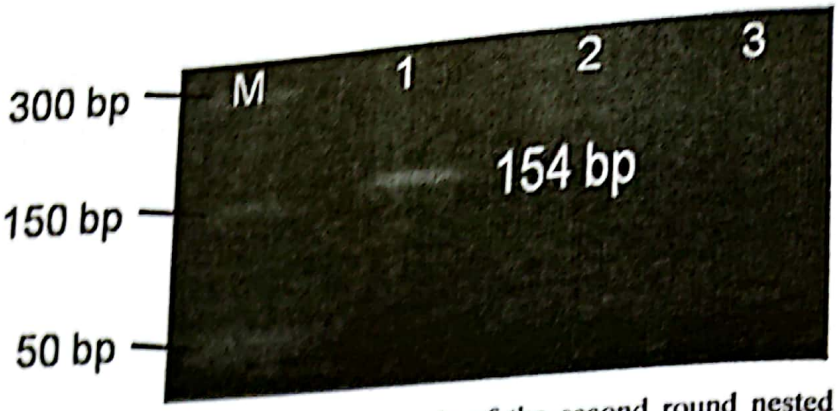


**Figure 2:** Agarose gel electrophoresis of the second round nested PCR using *B. microti*-specific primers (Bab2 and Bab3). Positive result is indicated by generation of 154 bp fragment (lane 1). Lane M, molecular size standard marker, FastRunner DNA ladder.

## DISCUSSION

Microscopic detection of *B. microti* in Egyptian rodents has been reported (el Bahrawy et al., 1993; el-Kady et al., 1998); however, the molecular confirmative evidence of the present organism has not been revealed yet. To design and implement efficient control strategies for any vector-borne zoonosis, first we have to molecularly identify the present pathogen and then identify the competent vectors and reservoirs. Therefore, the main task of the present study is the molecular detection of *B. microti* in blood of rodents using nested PCR. In addition, this survey looks for the occurrence of other *Babesia* species circulating in rodents community.

Selecting rodents as a candidate target for our survey is based on the following considerations: (1) rodents were known to be competent reservoir hosts for *B. microti* in the US (Fay and Rausch, 1969; Healy et al., 1976; Watkins et al., 1991; Burkot et al., 2000) and proved to harbor the organism in Europe (Shortt and Blachie, 1965; Walter, 1984; Duh et al., 2003) and Asia (van Peen et al., 1977). (2) babesia has been detected in Egyptian Black rat (*R. rattus*) and Norway rat (*R. norvegicus*) by microscopic examination (El Bahrawy et al., 1993). (3) rodents are known to be competent reservoir hosts for *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, the organisms that share vector and reservoir with *B. microti* (Spielman, 1976; Pancholi et al., 1995; Hofmeister et al., 1998) and have been detected in Egypt (Ghafar, 2003). (4) rodents are abundant in Egypt and some species are commensals and live in close proximity with people posing a risk for human beings.

Choosing Giza governorate as a prime substrate for our study is based on the fact

that *A. phagocytophilum* has been detected in ticks in this area before (Ghafar, 2003).

For template DNA isolation we used EDTA-whole blood samples, which has been proved to have a superior sensitivity over extraction from spleen tissues (Welc-Faleciak et al., 2007). Nested PCR was employed as the detection technology. This method has a higher sensitivity and specificity than other diagnostic procedures and demonstrated superior usefulness in *Babesia* prevalence surveys (Krause et al., 1996a; Zamoto et al., 2004; Sinski et al., 2006). For PCR, the 18s rRNA gene was targeted for amplification; which is the most widely used marker in epidemiological surveys for *Babesia*-genus organisms. This is based on the facts that this gene is present in all eukaryotic organisms, has highly conserved sequences for all *Babesia* species and highly variables regions allowing designing of highly specific primers (Skotarczak, 2008). Primers Bab1 and Bab4 were first used by Persing et al., (1992) in diagnosis of human babesiosis. Few years later, Krause et al., (1996b) used the same primer pairs for detection of human infections with *B. microti* and concluded that these oligonucleotides are sensitive and specific as the use of Giemsa-stained blood smears and lab animal inoculation. .

Out of 30 rodents captured, 26 (86.7%) were identified as *R. norvegicus* and 4 (13.3%) were *R. rattus*. The abundance of the first species recorded in this study agrees with other previous reports (Younis et al., 1995; Soliman et al., 2001)

For screening purposes and to see if there is *Babesia* species other than *B. microti* circulating in Egyptian rodents, *Babesia* genus-specific primers in a standard PCR was used. This procedure revealed that 11 (36.7%) out of 30 rodents harbored *Babesia* DNA, indicating a relatively high prevalence of the disease. Table 2 shows that all rodents with splenomegaly tested positive for babesia. This result is consistent with other obtained by Welc-Faleciak et al., (2007). Table 3 summarizes the results of nested PCR detection of *B. microti* in relation to splenomegalia. A total of 9 animals showed amplicons of specific 154-bp. All these animals were proved to be positive for *Babesia* genus. Appearance of ~ 650 bp band in electrophoresis of PCR amplicons of 2 rodents (2 *R. norvegicus* and 1 *R. rattus*), which were negative for *B. microti* indicates the presence of another *Babesia* species circulating in rodent community. Sequence analysis of these amplicons is required to gain information about the molecular identity of the present agent.

The fact that 9 out of the 11 positive cases for *babesia*-genus were proved to be positive for *B. microti* indicates that the most prevalent *Babesia* species circulating in rodents is *B. microti*. One animal of the 3

*R. rattus* that did not show splenomegaly and proved to be negative for *Babesia*-genus PCR, revealed the presence of a 154-bp band specific for *B. microti*. The plausible explanation that could account for this result is that animal was recently infected and showed low parasitemia. Consequently, low copy number of the genetic marker was present in extracted nucleic acid that could not be detected in standard PCR but still detectable by the more sensitive nested procedures.

Significant correlation ($P<0.05$) was found between infection with *B. microti* and splenomegalia. This result was not a surprise as it is well known that splenomegaly is a common symptoms of babesiosis in both animal and human hosts. *R. norvegicus* has been seen to be more sensitive to infection with *B. microti* than *R. rattus* ($P<0.05$). It is noteworthy to mention that, detection of *B. microti* in rodents does not mean that rodents are competent reservoirs for this agent; therefore, reservoir competence studies should be conducted.

This study is the first molecular report addressing existence of *B. microti* in rodents population in Egypt. It confirms that one of the agents causing human babesiosis is present in the country and at a relatively high prevalence in their suspected reservoir host, thus posing a risk for human infections. In addition, this report paves the road ahead for competence studies.



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# المسح الجزيئى للبابيزيا ميكروتى، المسبب لمرض البابيزيوزس الآدمى، فى القوارض فى مصر

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حتى الآن في جمهورية مصر العربية قد تم تسجيل حالتين من مرض البابيزيوزس الأدمى، وفى نفس الوقت هناك عدد قليل من البحوث المنشورة والتي كشفت عن وجود البابيزيا ميكروتى في القوارض. وهذه الدراسات قد اعتمدت فقط على الفحص الميكروسكوبي لشرائح الدم المصبوغة، وعلية فان الدليل الجزيئي المؤكد لوجود هذه المسببات المرضية لم يثبت بعد، كما أن وبائية مرض البابيزيوزس الأدمى من حيث المستودع المؤهل والناقل المؤهل ما زالت غير واضحة.

تعتبر هذه الدراسة مسح جزيئي للكشف عن وجود البابيزيا ميكروتى في القوارض المصرية حيث أنة قد تم اصطياد عدد ٣٠ من القوارض (٢٦ من نوع الراتس نورفيجيكس و ٤ من نوع الراتس راتس) من أماكن مختلفة من محافظة الجيزة بجمهورية مصر العربية وتم الكشف عن البابيزيا ميكروتى في دم هذه القوارض باستعمال تقنية تفاعل إنزيم البلمرة المتسلسل المتداخل والذي يستهدف جين الـ 18S rRNA.

كانت نسبة الإصابة ٣٠.٨% في قوارض الراتس نورفيجيكس و ٢٥% في قوارض الراتس راتس بينما كانت نسبة الإصابة الكلية ٣٠%. كما تم الكشف عن وجود فصائل بابيزيا غير محددة في ٣ حيوانات (٢ راتس نورفيجيكس و ١ راتس راتس) وذلك عند اختبار القوارض بواسطة تقنية تفاعل إنزيم البلمرة المتسلسل العادي والذي يستعمل بادئات خاصة لرتبة البابيزيا. وهذه الحيوانات الثلاثة قد أعطت نتيجة سلبية في اختبار البابيزيا ميكروتى. كانت هناك علاقة ايجابية بين الإصابة بمرض البابيزيا ميكروتى وعرض تضخم الطحال، كما وجد أيضا أن القوارض من النوع الراتس نورفيجيكس أكثر حساسية للإصابة بالمرض من النوع الراتس راتس.

وتعتبر هذه الدراسة أول مسح جزيئي للكشف عن البابيزيا ميكروتى في القوارض المصرية والذي يؤكد وجود مسبب من مسببات مرض البابيزيوزس الأدمى في مصر وبنسبة عالية في مستودعات المرض المشتبه فيها مما يمثل خطورة للإصابات الآدمية.