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First Molecular Detection and characterization of *Borrelia burgdorferi* sensu lato (Lyme disease spirochete) in Egyptian rodents

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SUMMARY

This study was intended to molecularly detect and characterize *Borrelia burgdorferi* sensu lato in Egyptian rodents as an initial step in reservoir competence studies. A total of 30 rodents (26 *Rattus norvegicus* and 4 *Rattus rattus*) were caught at different areas in Giza Governorate, Egypt. EDTA-whole blood was collected and tested for the presence of spirochetal DNA using *ospA* targeting-PCR, and specific amplicons were then subjected to bidirectional sequencing. Positive amplification of 307-bp fragment was detected in 5 (19.2%) *R. norvegicus* (brown or Norway rat), while no borrelial DNA was evident in *R. rattus* (black rat). DNA sequence of the detected strain, designated as *Borrelia burgdorferi* Ghafar, was deposited in the GenBank under accession no. FJ968724. Sequence and phylogenetic analysis showed that Ghafar strain belongs to *B. burgdorferi* sensu stricto (99.6% - 100% similarity) and clearly distinguishable from other *B. burgdorferi* sensu lato genospecies. These results provide the first molecular report addressing existence of *B. burgdorferi* sensu stricto in *Rattus norvegicus* in the country, suggesting that this murine host may act as a reservoir for Lyme disease agent.

Keywords: *Borrelia burgdorferi* sensu lato, Rodents, *Rattus norvegicus*, *Rattus rattus*, PCR, Molecular characterization, Egypt.

INTRODUCTION

Lyme disease is the most prevalent emerging tick-borne zoonosis in North America and Europe (Piacentino and Schwartz, 2002). It is a multisystem infection with dermatologic, neurologic, and rheumatologic manifestations (Steere, 2001). The causative agents of Lyme borreliosis

belong to spirochetes of the *Borrelia burgdorferi* sensu lato complex. This genocomplex encompasses 12 species; 3 heterogenous species have been clearly established as pathogenic to humans: *B. burgdorferi* sensu stricto (North America and Western Europe), *B. afzelii* (Western Europe, Central Europe, and Russia), and *B. garinii* (Europe, Russia, and Northern Asia) (Wilske et al., 1996; Mathiesen et al., 1997; Saint Girons et al., 1998; Wang et al., 1999). It is evident that each species varies ecologically and possesses different pathogenicity with subsequent variable clinical outcomes (Wienecke et al., 1994; Seinost et al., 1999). Previous field and laboratory studies in northern hemisphere demonstrated that Ixodes ticks (*I. ricinus* in Europe, and *I. scapularis* and *I. pacificus* in the United States) and rodents are clearly the critical components in the epidemiology of Lyme borreliosis (Fish, 1995; Barbour, 1998; Walker, 1998). Both transovarial transmission of spirochetes in tick vector and transplacental transmission in rodent reservoir are either rare or do not exist, so that transmission by ticks is essential for maintaining the spirochete in nature (Piesman et al., 1986; Mather et al., 1991). Murine reservoir hosts are proved to be competent (Donahue et al., 1987) and may remain infectious for tick vector lifelong (Schwan et al., 1991). This may be attributed to the facts that rodents are abundant and remain in constant residence in tick-infested sites. To design and implement global efficient control strategies for Lyme disease we have to understand its worldwide natural history and epidemiology. To achieve this goal we have to identify the competent vectors and reservoirs utilized by the disease agents in each particular geographic area and we have to identify the local genospecies of *B. burgdorferi* sensu lato circulating in such area. In Egypt, to date and to the best of our knowledge, only few serological reports of Lyme disease have been published (Haberberger et al., 1989; Hammouda et al., 1995; Helmy, 2000; Helmy et al., 2006) and only one molecular survey has detected the presence of *B. burgdorferi* sensu lato in tick hosts (Adham et al., 2010). However, the competent reservoirs and the complete epidemiological picture of the disease yet to be elucidated. Therefore, this study was intended to molecularly detect and characterize *B. burgdorferi* sensu lato in Egyptian rodents as an initial step in reservoir competence studies. PCR using *ospA*-targeting specific primers and sequencing of the amplified product were used to execute these objectives.

MATERIALS AND METHODS

Rodents and blood sample collection:

Rodents were trapped from different parts in Giza Governorate, Egypt. Captured rodents were brought to the laboratory of biochemistry division at NODCAR,

anaesthetized and morphologically identified to the species level (Barnett, 1963; Nowak and Paradiso, 1983; Avalos and Callahan, 2001). EDTA-whole blood samples were collected and stored at –20°C until DNA extraction. To avoid contamination, aseptic procedures during sampling and handling of specimens were undertaken.

DNA extraction:

Using QIAamp DNA Blood Mini Kit (QIAGEN Inc., CA, USA), total DNA was extracted from 200µl blood samples according to the manufacturer's protocols. DNA concentration and purity were assessed spectrophotometrically and 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:

To prevent contamination, standard PCR routines were applied. Separate laboratory areas were dedicated for DNA extraction, the preparation of reaction mixtures, amplification, and detection of PCR products. Fresh gloves were used with each manipulation; in addition, aerosol-resistant filter pipette tips were used throughout the experiment. Negative controls (PCR-grade water) and positive controls (genomic DNA extract of *B. burgdorferi* sensu lato strain) were included in each experiment to control contaminations and false-negative amplification results. All PCR reagents and DNA polymerase were obtained from the Jena Bioscience (Jena Bioscience, GmbH, Germany) and used as recommended by the supplier. A previously published SL primer set (Table 1) designed to amplify *OspA*-specific target sequences of all three pathogenic genospecies of *B. burgdorferi* sensu lato (*B. burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borrelia garinii*) was used in the PCR (Demaerschalck et al., 1995). Amplification was performed as previously described, with slight modifications. 10µl of each extracted DNA template were amplified in a 50-µl reaction mixture containing 1X buffer, 0.2 mM each deoxynucleoside triphosphate, 5 µM tetramethylammonium chloride (TMA), 2 U of DNA polymerase , and 20 pmoles each primer. The reaction mixture was subjected to 35 cycles of amplification by using an automated thermal cycler (Techne TC512, USA). Each cycle involved heating to 93°C for 1 minute (DNA denaturation), cooling to 60°C for 1 minute (primer annealing), and again heating to 72°C for 1 minutes (primer extension). The amplification was concluded with an extension reaction at 72°C for 5 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels in TAE buffer and were visualized under ultraviolet (UV) transilluminator with ethidium bromide. A positive result was considered a clear band at 307-bp.

Table 1: PCR primers used for detection *B. burgdorferi* sensu lato genospecies.

Primer set	Oligonucleotide sequence	Position & orientation on DNA sequence	Length (bp)	Target species
SL	5'-AAT AGG TCT AAT AAT AGC CTT AAT AGC-3'	21 → 47	27	*B. burgdorferi* s. s., *B. garinii* & *B. afzelii*
	5'-CTA GTG TTT TGC CAT CTT CTT TGA AAA-3'	302 ← 328	27	

Sequencing of PCR products:

Double-stranded PCR products were purified from excised gel bands by using the commercial Agarose Gel Extraction Kit (Jena Bioscience GmbH, Germany) and subjected for bidirectional sequencing using Jena Bioscience facilities. Cycle sequencing reactions were performed using an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 3130 DNA Sequencer, according to the manufacturer's instructions. The *ospA* gene sequence of the *B. burgdorferi* detected in this study is available in the GenBank database under accession no. FJ968724.

Sequence analysis:

DNA sequences were cleaned and aligned by using the BioEdit software (Hall, 1999; http://www.mbio.ncsu.edu/Bioedit/bioedit.html). BioEdit was also used to align the Egyptian sequence with closely related sequences of *ospA* gene retrieved from GenBank. The aligned sequences were exported from BioEdit to PAUP software version 4.0b10 (Swofford, 2003) to phylogenetically analyze the data. Two sequences of *B. lusitaniae* were used as an outgroup taxon for constructing the Neighbor-joining (NJ) tree, which was based on 255 bp of 26 aligned *ospA* DNA sequences. Stability of NJ tree branches was assessed by 1000 bootstrap replications using PAUP.

RESULTS

Collected rodents and PCR:

A total of 30 rodents comprising two species [26 (86.7%) *R. norvegicus* and 4 (13.3%) *R. rattus*] were collected. Five (19.2%) *R. norvegicus* were positive for spirochetal organisms (Figure 1) while any of *R. rattus* showed evidence of borrelia DNA. The overall prevalence rate of infection among captured rodents was 16.7%.

Sequence analysis:

Alignment of the partial *ospA* gene sequences showed that the borrelial *ospA* gene from *R. norvegicus* belongs to the *B. burgdorferi* sensu stricto. The NJ tree revealed that the Egyptian strain clustered in the sensu stricto clad where its branch received 100% bootstrap value (Figure 2). The most distant sequences from the Egyptian isolate were belonging to *B. japonica*. Percent identities as well as geographical and biological origins of some representative strains are compared in Table 2.

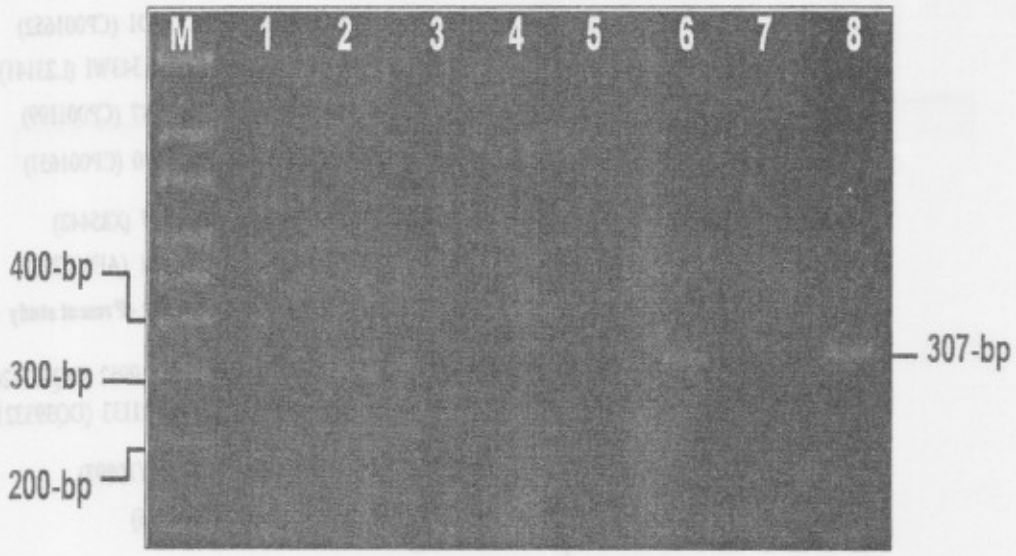

Figure (1): Agarose gel electrophoresis of PCR products using *B. burgdorferi* sensu lato-specific SL primer set. Positive result is indicated by generation of 307-bp fragment. Lane M, molecular size standard marker, 100-bp DNA ladder; lanes 1-6, rodents samples; lane 7, negative control; lane 8, positive control. Only lanes # 2 & 6 are positive.

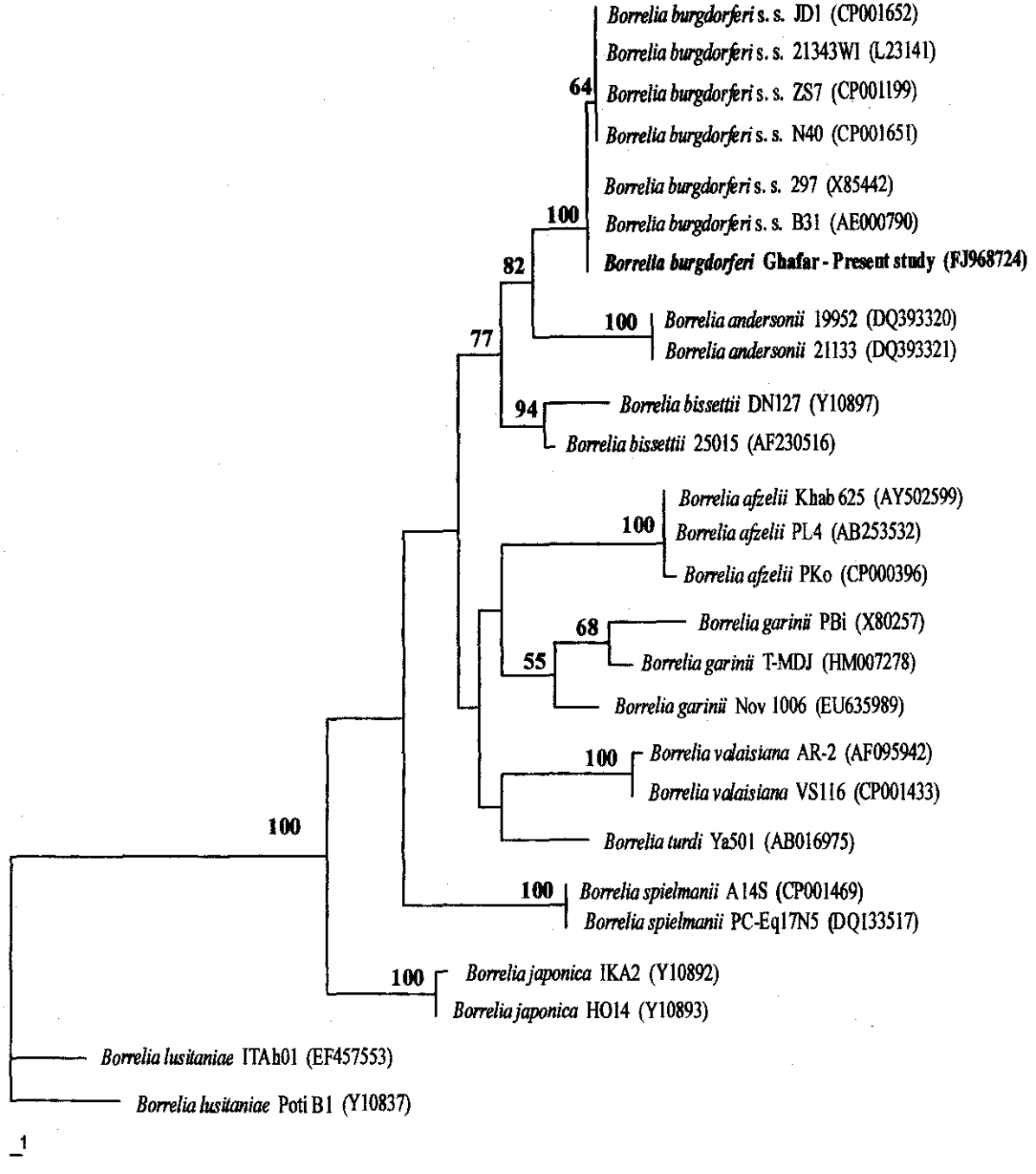

Figure (2): Neighbor-Joining tree based on aligned 302 base pair of OspA gene from different species of *Borrelia*. Two strains of *Borrelia lusitaniae* are used as an outgroup. Bootstrap values of 1000 replications are indicated above the tree branch. The bar scale under the NJ tree represents one nucleotide difference.

Table (2): Percent identity of *B. burgdorferi ospA* region of the Egyptian strain and some selected closely related sequences from GenBank.

Strain	GenBank accession no.	Country	Biological origin	Identity %
B. burgdorferi s. s. 297	X85442	USA	Human, CSF	100
B. burgdorferi s. s. B31	AE000790	USA	*I. scapularis*	100
B. burgdorferi s. s. N40	CP001651	USA	*I. scapularis*	99.6
B. burgdorferi s. s. JD1	CP001652	USA	*I. scapularis*	99.6
B. burgdorferi s. s. ZS7	CP001199	Germany	*I. ricinus*	99.6
B. burgdorferi s. s. 21343WI	L23141	USA	*Peromyscus leucopus*	99.6
B. bissettii DN127	Y10897	USA	*I. pacificus*	93.1
B. bissettii 25015	AF230516	USA	*I. scapularis*	95.1
B. garinii PBi	X80257	Germany	Human, CSF	91.1
B. valaisiana VS116	CP001433	Switzerland	*I. ricinus*	90.7
B. turdi Ya501	AB016975	Japan	*I. turdus*	91.9
B. japonica HO14	Y10893	Japan	*I. ovatus*	88.2

DISCUSSION

The present study aimed to detect and molecularly identify *B. burgdorferi* sensu lato in the Egyptian rodents as a crucial initial step in vectorial competence studies. Selecting rodents as a candidate target for our survey is based on the consideration that rodents are known to be competent reservoir hosts for *B. burgdorferi* genospecies (Donahue et al., 1987); in addition, this murine hosts are abundant in Egypt and some of them are commensals and live in close proximity with people thus posing a risk for human beings.

Choosing Giza Governorate as a substrate area for our sample collection is ascribed to the facts that: (1) *A. phagocytophilum* (the causative agent of human anaplasmosis) and *B. microti* (the causative agent of human babesiosis) have been detected in this locality before (Ghafar, 2003; Ghafar, et al., 2010) and it is established that these organisms have the same natural cycle of transmission as *B. burgdorferi*. (2) in Giza, serological and molecular evidences of *Borrelia* infection have been reported in humans, farm animals and ticks (Helmy, 2000; Helmy et al., 2006; Adham et al., 2010).

Laboratory tests, including microscopic examination, bacterial culture and serological methods, are of limited value to support the diagnosis of Lyme disease since they lack both sensitivity and specificity (Barbour, 1984; Craft et al., 1984). The advent of molecular techniques including PCR and sequencing has been proved to be more accurate in detection and assessing the

diversity of Lyme disease agent (Rosa and Schwan, 1989; Hofmeister et al., 1992). We have utilized *ospA* gene in our PCR experiments. Targeting this gene is based on the fact that this gene is plasmid encoded and is present in multiple copies within each bacterium; therefore, the sensitivity of the assay is greatly increased (Bergstrom et al., 1989; Moter et al., 1994; Xu and Johnson, 1995).

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). Five (19.2%) *R. norvegicus* were PCR positive for spirochetes while any of *R. rattus* showed evidence of *Borrelia* DNA. Occurrence of *B. burgdorferi* in *R. norvegicus* was not a surprise as this murine host was previously reported to be a competent reservoir for the agent of Lyme disease (Smith et al., 1993; Matuschka et al., 1996). It is noteworthy to mention that these rodent samples were previously tested for the occurrence of *B. microti* and it was found that the infection rate of this piroplasm in *R. norvegicus* is significantly higher than that in *R. rattus* (Ghafar et al., 2010). This could be explained by susceptibility of *R. norvegicus* to infection with these tick-borne zoonoses or host preference with certain tick vector. Interestingly, 2 out of the 5 rodents that yielded positive for *B. burgdorferi* were also coinfected with *B. microti*. The relatively higher overall prevalence rate of infection (16.7%) may be biased due to small sample size and the possibility that these positively tested rodents may colonize the same area and exposed to the same infected tick community.

It is well established that genetically variant Lyme disease agents have variable degrees of pathogenicity and clinical outcomes with subsequent different diagnostic and vaccine strategies (van Dam et al., 1993). Therefore, sequencing-based molecular characterization of the circulating genospecies in Egyptian rodents was necessitated.

Our sequence comparisons suggest that the amplicons derived from *R. norvegicus* in this study is really a true *B. burgdorferi* species. Phylogenetic analysis revealed that the current organism clustered with sensu stricto genospecies (Figure 2). Ghafar strain showed 100% identity with strains known to be human pathogen in the US (X85442) and 99.6% - 100% identity with strains detected in the established tick vectors [*I. scapularis* in the US (AE000790, CP001651, CP001652) and *I. ricinus* in Europe (CP001199)] (Table 2). The detected strain could be either endogenous or imported from Europe or the US. Importation of the organism into the country could be either through introduction of infected animals or dispersion of infected ticks via migrating birds, where avian hosts

were proved to be competent for *Borrelia* species (Richter et al., 2000).

Given the previous information, we cannot conclude that *B. burgdorferi* Ghafar strain can cause human infections. Therefore, comparative genomic and antigenic studies with strains causing clinical Lyme borreliosis in the country should be performed. It is noteworthy to mention that in Egypt, to date and to the best of our knowledge, there is only one serological survey for Lyme borreliosis in children with suggestive clinical symptoms (Hammouda et al., 1995); however, the molecular identity of the agent has not been revealed yet.

This study is considered the first molecular characterization of Lyme disease agent in the Egyptian rodents. Detection of *B. burgdorferi* sensu stricto in *R. norvegicus* does not mean that this rodent species is a reservoir for this spirochete; however, this work is a crucial initial step in reservoir competence studies. Identifying both competent reservoir and vector in Egypt will help understanding the global epidemiology of the disease as well as designing and execution of efficient prevention and control measures.

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اول كشف وصفى جزيئى للبوريليا بورجدورفرى سنسو لاتو (ملتوية مرض الليم) فى القوارض المصرية

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الهدف من الدراسة هوالكشف والوصف الجزيئى للبوريليا بورجدورفرى سنسو لاتو فى القوارض المصرية كخطوة مبدئية فى دراسات المستودع المؤهل. تم إصطياد عدد ٣٠ من القوارض (٢٦ من نوع الراتس نورفيجيكس و٤ من نوع الراتس راتس) من أماكن مختلفة من محافظة الجيزة بجمهورية مصر العربية. وقد تم جمع عينات دم غير متخثر من القوارض وإختبارة لوجود الحامض النووى الـ DNA والخاص بملتوية مرض الليم وذلك باستعمال تقنية تفاعل إنزيم البلمرة المتسلسل والذي يستهدف جين الـ ospA ويتبع ذلك معرفة تتابع النيوكليوتيدات فى نواتج تفاعل إنزيم البلمرة المتسلسل.

كانت نسبة الإصابة ١٩.٢% في قوارض الراتس نورفيجيكس و صفر% في قوارض الراتس راتس. تم تحديد تتابع النيوكليوتيدات فى الحامض النووى للسلالة المكتشفة والتى سميت بوريليا بورجدورفرى جعفر ووضع هذا التتابع فى بنك الجينات تحت رقم FJ968724. وبتحليل كل من التتابع وشجرة التطور وجد أن السلالة المكتشفة تتبع البوريليا بورجدورفرى سنسو ستركتو (نسبة تماثل ٩٩.٦% - ١٠٠%). وتعتبر هذه الدراسة أول كشف جزيئي عن البوريليا بورجدورفرى سنسو ستركتو في الراتس نورفيجيكس مقترحا أن يكون هذا النوع من القوارض مستودع مؤهل لمرض الليم فى مصر.