Seroprevalence and Real-time PCR Detection of Brucellosis in Abattoirs Animals as a Potential Route of Infection in Egypt

Amira M. Zakaria, Salwa F. Ahmed, and Mohamed S. Motawae

Abstract—Brucellosis is among the most common zoonosis worldwide, posing serious economic concerns. In Egypt, it is endemic among animals and humans. This work intends to evaluate conventional serological and molecular approaches as tools for studying the prevalence of brucellosis within animals in abattoirs in two large Egyptian provinces. Two hundred and thirty (n=230) blood and serum samples were collected from 2-3 years old male calves in two abattoirs. Rose Bengal test (RBT) and modified in-house ELISA were used to determine the seroprevalence of brucellosis in animals at abattoirs, while quantitative TaqMan real-time PCR (RT-PCR) was used to characterize Brucella species. The overall prevalence of brucellosis in both provinces was 53.9%, 75.2%, and 79.1% as determined by ELISA, RBT, and RT-PCR assays, respectively. Brucella DNA from serum and blood samples was successfully amplified. A total of n=182 samples (79.1%) were identified by real-time PCR amplification for the IS711 gene to contain DNA of Brucella genus, n=118 (64.8%) were reported to contain B. abortus, n=85 (46.7%) were reported to contain B. melitensis, and n=44 (24.17%) comprised both species of bacteria. This study endorses the rose Bengal test as a sensitive and cost effective serological test for detection of brucellosis and real-time PCR as a distinguishing tool to detect causative agents. Our findings indicate a significantly high prevalence of anti-Brucella antibodies and DNA in blood and serum samples, which poses a crucial threat to public health in Egypt.

Index Terms— Brucellosis, Abattoirs, Molecular typing, Seroprevalence, Real-time PCR, Egypt

I. INTRODUCTION

Brucellosis is among the most important zoonotic diseases that induce abortion in cattle. It is prevalent worldwide and impacts both wild and domestic animals (Maleknejad, 2007). The significance of this disease is increasing due to several reasons, including its fast spread, difficulty to control and prevent, and time consuming and costly treatment (Koneman, 1997). It also has critical consequences on the trading of livestock and animal products, inhibiting social and economic development of breeders (Chand and Sharma, 2004).

Brucellosis is a zoonotic disease caused by the bacterial genus Brucella (Garrity, 2001), which can be transmitted from animals to humans via contaminated food products, direct exposure to infected animals, or inhalation of aerosols. The disease is common and historic, known by several names, such as Mediterranean fever, Malta fever, gastric remittent fever, and undulant fever (Johnson, 1992).

Humans are inadvertent hosts. However, brucellosis remains a significant global public health issue and is the most frequent zoonotic disease. In endemic regions, human brucellosis poses crucial impacts on public health (Erdenebaatar, 2004). Brucella melitensis is regarded as the most popular species causing human brucellosis, with complications in immunizing free-ranging goats and sheep (Wareth, 2014). In countries where elimination in animals is not achievable (through vaccination and/or exclusion of diseased animals), human infection prevention relies on raising awareness, spreading knowledge, taking food-safety and laboratory safety precautions, and increasing occupational hygiene.

Brucellosis is considered a notifiable disease worldwide (Hegazy, 2009). Existing information on brucellosis and other zoonotic diseases in Egypt is relatively limited. Thus, the aim of this work is to fill gaps in knowledge on brucellosis in this part of Egypt. This work was done in coordination with veterinarians and workers at the focal abattoir serving the whole governorate, including neighboring villages.

II. MATERIALS AND METHODS

A. Study Area (location and study duration)

A cross-section study was conducted in two Egyptian provinces (Cairo and Ismailia) over a period of 15 months, from March 2015 to May 2017. One principle abattoir from each province was targeted as a focal point. The first one (AB1) was in Ismailia at Tal-Alkabeer region, while the second (AB2) was in Greater Cairo at Al-Moneeb region. These two slaughterhouses were selected for being the main ones serving a considerably large population and for including previously recorded brucellosis cases among workers and veterinarians (data under publication).

B. Ethical statement

The protocol for collection of animal materials was approved by district veterinary and agricultural authorities. The sampling process was carried out during daily slaughtering on the slaughtering panel so that no IRB was needed.

C. Sampling

Labeled sterile vacutainers were used to collect blood from two hundred and thirty (n=230) male calves 2-3 years
old, on the slaughtering panel. Up to one hundred and fifty-three (n=153) blood samples were collected from Ismailia and seventy-seven (n=77) from Greater Cairo. Each blood sample was divided into two portions: the first portion (5 ml) was collected in citrate-anticoagulant vacutainers for DNA extraction and PCR, while the second portion (5 ml) was collected in anticoagulant-free bottles kept in slanted position prior to being transported to the laboratory for serum separation (O’Leary, 2006).

III. SEROLOGICAL ASSAYS

A. Rose Bengal Test (RBT)

Rose Bengal test was done to serologically estimate the prevalence of brucellosis in Egyptian abattoirs. All serum samples were tested for agglutination against the Brucella antigen using PriocHECK® Brucella Rose Bengal Test Kit (Pronics, Switzerland), via a qualitative method according to the enclosed instructions. Equal volumes up to 30 µl from each serum sample and from the Brucella antigen were mixed together and manually agitated for 2 minutes. Agglutination was considered a positive result, indicating the presence of specific anti-Brucella antibodies in the animal serum. No agglutination was considered a negative result, indicating absence of specific anti-Brucella antibodies (Altwegg and Bohl, 1985). To increase test sensitivity, all positive sera were diluted by isotonic saline into 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 dilutions and examined semi quantitatively (Baum et al., 1995).

B. In-house modified ELISA for bovine brucellosis diagnosis

A modified in-house ELISA assay was performed for rapid diagnosis of bovine brucellosis as described by (Fadeel et al., 2006 and 2011). 96-well ELISA plates (ICN Biomedicals, OH) were coated with the B. abortus antigen (BD). Serum samples were diluted using the appropriated conjugating buffer to 1:160 and 1:320 dilutions. 100 µl from samples, blank, and positive and negative controls were added to the coated plates in two wells (duplicate), covered with a lid, and incubated for one hour at 37°C in a humid chamber. Contents were then discarded, washed three times, and then loaded with the conjugate (Anti-Bovine IgG (H+L)–Peroxidase antibody produced in goat KPL). Plates were then incubated for 30 min at 37°C in a humid chamber. Then contents were discarded and the plates washed three times. 100 µl of the prepared substrate (Sigma-Fast OPD tablets) solution were added to each well. Plates were then incubated in the dark for 30 min at room temperature (approx. 25°C-27°C). 50 µl of stopping solution (1 M H2SO4) were added to each well. The developed color intensity (optical density OD) was recorded at 492 nm. An OD cutoff point of the assay was set to be 0.38. Any sample showing OD ≥ 0.38 was considered initially reactive and at serum titer > 1:320 reported positive for anti-Brucella antibodies. Samples showing OD > 0.38 at 1:160 and < 0.38 at 1:320 were considered equivocal. Samples showing OD < 0.38 at 1:160 were considered non-reactive and reported as negative for anti-Brucella antibodies.

C. Genomic DNA Extraction

Total DNA was extracted from blood and serum samples using a genomic DNA mini kit (QiAGen, QIAamp mini prep, USA) according to the manufacturer’s protocol. The extracted DNA was stored at -20°C for later use. DNA concentration and purity of stock solutions were measured by a NanoDrop ND-1000 spectrophotometer at 260 and 280 nm.

D. TaqMan Real-time PCR Assay

The real-time PCR assays were carried out for differentiation of Brucella species, as described by Hinic et al., 2008, using 3 primer pairs and 3 dual labeled (FAM and BHQ) probes, purchased from Biosearch Technologies.

### Table I: Primes and probes used in Real-Time PCR assays

<table>
<thead>
<tr>
<th>PCR</th>
<th>Target Seq</th>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
<th>Species Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IS711</td>
<td>Brucella genus</td>
<td>GCTGAAAGTGCACACCAGT</td>
<td>GGCTACACCAGTGGAAT</td>
<td>FAM-AAGCAACACCGGTGTATG-</td>
</tr>
<tr>
<td>2</td>
<td>BMEJ00466</td>
<td>B. melitensis</td>
<td>TCGCATGCCAGTTTCAA</td>
<td>CCAGCTTTGCCCCATTC</td>
<td>FAM-CTCGGATGTCGCCACAA-BHQ-</td>
</tr>
<tr>
<td>3</td>
<td>BruAb2_0168</td>
<td>B. abortus</td>
<td>GCACACACTTTCACCAACAA</td>
<td>CCCGTTCTGACACCAGACT</td>
<td>FAM-TGGAAACGACCTTTGCAGGAGATCBHQ-</td>
</tr>
</tbody>
</table>

### Table II: Number and percentage of positive results per test used for the detection of brucellosis in male calves in the two slaughterhouses (abattoirs).

<table>
<thead>
<tr>
<th>Province</th>
<th>Sample No</th>
<th>ELISA</th>
<th>RBT</th>
<th>Brucella Genus</th>
<th>B. Abort</th>
<th>B. melitises</th>
<th>Both spp</th>
<th>Unidentified Brucella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ismailia</td>
<td>153</td>
<td>75 (49)</td>
<td>106 (69.3)</td>
<td>109 (71.2)</td>
<td>64 (41.8)</td>
<td>32 (20.9)</td>
<td>13 (8.4)</td>
<td>26 (16.99)</td>
</tr>
<tr>
<td>Cairo</td>
<td>77</td>
<td>50 (64.9)</td>
<td>67 (87)</td>
<td>74 (96.1)</td>
<td>54 (70.1)</td>
<td>53 (68.8)</td>
<td>31 (40.25)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>Total prevalence</td>
<td>230</td>
<td>124 (53.9)</td>
<td>173 (75.2)</td>
<td>183 (79.5)</td>
<td>118 (64.8)</td>
<td>85 (46.7)</td>
<td>44 (24.17)</td>
<td>28 (15.38)</td>
</tr>
</tbody>
</table>

### Table III: Distribution of true positive samples as confirmed by the three tests in the study area with percentage

<table>
<thead>
<tr>
<th>Province</th>
<th>Sample No</th>
<th>Sample positive for all assays (ELISA, RBT, RT-PCR), number(%)</th>
<th>Ismailia</th>
<th>50</th>
<th>74</th>
<th>118 (64.8)</th>
<th>85 (46.7)</th>
<th>44 (24.17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cairo</td>
<td>77</td>
<td></td>
<td>40 (26)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total prevalence</td>
<td>230</td>
<td>79 (40)</td>
<td></td>
<td></td>
<td></td>
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DOI: [http://dx.doi.org/10.24018/ejmed.2019.1.5.105](http://dx.doi.org/10.24018/ejmed.2019.1.5.105)
USA (Table 1). The typical 25 µL reaction contained the following: 12.5 µL TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 300 nM of each forward and reverse primer, 200 nM of the labeled probe, and 2.5 ng of sample DNA. TaqMan real-time PCR reactions were developed in StepOnePlus equipment (Applied Biosystems). The reaction mixture was initially incubated at 95°C for 5 min. Amplification was performed with 45 denaturation cycles at 95°C for 20s and annealing and extension at 60°C for 1 min. Samples exhibiting sigmoid amplification peaks with below 40 cycle thresholds (CTs) were considered positive.

IV. RESULTS

A. Prevalence of Brucella in the two provinces

A total of n=230 blood and serum samples were obtained from two focal abattoirs in Egypt. The prevalence of brucellosis in the targeted locations varied according to the test used for detection (Table 2). The overall prevalence of brucellosis in the two provinces was n=124 (53.9%), n=173 (75.2%), and n=182 (79.1%), as determined by ELISA, RBT, and RT-PCR assays, respectively (Figure 1). In Ismailia, n=153 samples were collected, and the prevalence was reported to be n=75 (49%), n=106 (69.3%), and n=109 (71.2%), as assayed by ELISA, RBT, and RT-PCR, respectively. In Cairo, the prevalence was reported to be n=49 (63.6%), n=67 (87%), and n=74 (96.1%), as estimated by ELISA, RBT, and RT-PCR, respectively.

Samples showing positive results using the three selected assays were defined as true positive (Table 3). In Ismailia, up to n=40 (26%) out of n=153 were reported as true positive, while in Cairo true positives were n=39 (50.6%) out of n=77 samples. A total of n=79 (40%) out of the total samples collected from the two provinces were positive for the three tests and defined as true positive.

B. Identification of Brucella using real-time PCR

A total of n=182 samples (79.1%) were identified by real-time PCR assay for the IS711 gene to contain DNA of the Brucella genus. n=118 (64.8%) were reported to contain B. abortus, and n=85 (46.7%) were reported to contain B. melitensis. n=44 (24.17%) contained both species. Since only oligonucleotides for two species were applied to detect only abortus and melitensis spp., (species in up to 28 samples positive for Brucella genus could not be identified.

V. DISCUSSION

Brucellosis in Egypt is endemic among animals and humans (Aidaros, 2005, McDermott and Arimi S., 2002). The diagnosis of brucellosis is difficult as rapid and reliable identification of the causative Brucella species is challenging with any diagnostic approach currently available relevant to serology (Amin, 2012). Massive funds to monitor and eliminate brucellosis were invested in Egypt during the past thirty years, with only limited achievement. Unsatisfactory success of the Egyptian surveillance plan was a consequence of inappropriate detection, in addition to prevalence of the disease countrywide (Wareth et al., 2014).

Our findings pointed out high prevalence of Brucella (40%) among the assayed animals. The relatively high prevalence of brucellosis in the two provinces can be explained by the fact that most abattoirs animals were imported from Sudan, where brucellosis is substantially prevalent (Gwida et al., 2011).

Molecular detection of brucellosis using PCR has frequently been employed as a supplementary choice. Genus-specific PCR assays are affordable diagnostic tests for screening, as they are able to identify the causative agent with minimal concentrations of DNA. Our results are in concurrence with earlier findings (Zerva et al., 2001) that B. abortus and B. melitensis DNA from bovine serum and blood samples can be successfully amplified.

Serological investigation of brucellosis is presumptive proof of disease, while laboratory confirmation requires isolation of bacteria or identification of Brucella DNA using PCR. Thus, the diagnostic window of Brucella serology should be complemented by bacteriological or molecular...
diagnosis (Abdel-Razik et al., 2008). The results confirmed the ability of real-time PCR assay to detect Brucella DNA even in seronegative animals, which recommends the use of PCR as a tool for routine diagnosis. This was previously reported by Amin et al., 2001.

In the current study, PCR assays detected Brucella DNA in ELISA-negative samples. Our data promote real-time PCR assays for risk analysis examination during trading and transport of livestock between importing and exporting countries. The inability of PCR to detect Brucella in ELISA-positive samples was described elsewhere. The reason for this is the fact that antibody titers remain high for a long period of time after infection, independent of bacterial circulation or the presence of DNA. However, false positive ELISA results were caused by cross-reactions with the LPS of other bacteria. Further supplementary studies are recommended to exclude the presence of other cross-reactive bacteria, such as Yersinia and Salmonella.

In developing countries like Egypt, conventional assays of serum samples are employed for screening for the presence of brucellosis. They play an essential role in surveillance strategies of the disease (Hassanain and Ahmed, 2012). In light of a previous research about brucellosis in Egypt, this work is the first to report B. abortus and B. melitensis DNA in sera samples of male calves, since previous publications were mainly based on blood specimens.

Brucella cultivation is a hazardous process, and the technique is limited to few laboratories in Egypt. Thus, Brucella was not isolated in this study. Detection of DNA of both species, B. abortus and B. melitensis, in the same animal (24%) in this study shows that one host can be infected by two different species of bacteria at the same time. The potential host scope of Brucella additionally relies on reproducing conditions (Hegazy et al., 2011). It was previously documented that the pathogen’s ability to overcome species barriers through close contact with different animal species and cohabitation has increased (El-Sherbini et al., 2007).

VI. CONCLUSION

Endemic regions suffer lack of efficiency and pose an unfavorable impact on human health. Control of brucellosis in animals and humans relies on the reliability of strategies utilized for detection and diagnosis of the causative agent. Lacking preventive measures and uncontrolled transport between Egyptian governorates to and from animal markets play a crucial role in occurrence of brucellosis. Detection of brucellosis is challenging as it depends on herds serology and the isolation of bacteria. The serological tests are restrictive in terms of affectability and specificity, as they are not and cannot be standardized nor suitable to differentiate between affected and immunized animals. The present results ought to be considered while evaluating the epidemiological pattern in a certain region and during the application of control measures. Further research is needed, including isolation of the organism and biotyping, to achieve an epidemiological proof of transmission of Brucella between animals.

CONFLICT OF INTEREST

The authors declare that they approve this version and that there is no conflict of interest.

FUNDING INFORMATION

The authors acknowledge the American Society for Microbiology (ASM) for partially funding this work through the small grant for biosafety/biosecurity research program in Egypt (2016).

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