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Isolation and molecular identification of Brucella *spp. in bovine herds kept at householders in the Delta region of Egypt by MALDI-TOF and AMOS-PCR*

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Summary
Brucellosis is a widespread disease in Egypt which cause huge economic losses in the dairy industry. The present study aims at isolating and identifying *Brucella* (*B.*) spp. circulating in bovine and buffalo dairy herds kept at farmers houses in four districts of the Delta region of Egypt. One hundred and five tissue specimens were collected from seropositive cattle and buffaloes. The samples included 10 vaginal swabs, 3 placentas, 3 uteri and 86 supra-mammary lymph nodes from dams, as well as 3 stomach contents from aborted fetuses. Matrix-assisted laser desorption ionization (MALDI) and the conventional biotyping techniques were used for preliminary identification of isolates into the genus level. AMOS-PCR was applied to differentiate *Brucella* isolates into species level. Nineteen *Brucella* strains have been identified, four *B. abortus* strains were recovered from cattle and 15 *B. melitensis* strains were isolated from both cattle (n = 8) and buffaloes (n = 7). The predominant occurrence of *B. melitensis* in bovines raises the fact that *B. melitensis* clone can cross species barriers and can establish a permanent reservoir in cattle and buffaloes. Presence of culture-positive animals at householders represent a high-risk factor for human infection. This knowledge is of significant importance in the control of brucellosis in bovines.

Brucellosis is an infectious zoonotic disease worldwide caused by *Brucella* (*B.*) species. The disease might have serious public and animal health impact as infections are often associated to chronic debilitation in humans and reproduction failure in animals (Godfroid *et al.* 2011). In the sexually mature female animals, the disease provokes metritis, abortion, stillbirth, retention of the placenta and drop in milk production, in males the disease can cause orchitis and epididymitis. Infertility may be seen in both sexes (Ducrotoy *et al.* 2014). Up-to-date, the genus *Brucella* comprises of 12 accepted phenotypically recognized species; *B. abortus, B. suis, B. melitensis*, *B. neotomae, B. ovis, B. canis, B. inopinata, B. microti, B. pinnepedails, B. ceti, B. papponi,* and *B. vulpex*. Only *B. melitensis, B. suis* except bv 2, *B. abortus* and to some extent *B. canis* are well-known pathogens for humans (Chiliveru *et al.* 2015), while the zoonotic potential of the others are not proved yet. Regarding their capability of causing disease in humans, *B. melitensis* is considered the most virulence species followed by *B. suis*, and *B. abortus* which, amongst the three species, is considered the mildest one (Galinska and Zagorski 2013).

In Egypt, *Brucella* was reported for the first time in 1939 (Refai 2002). In the 1980ies, attention was directed to the animal diseases when an open-door policy was applied and Friesian cows were imported to establish governmental farms. In that period, the incidence of brucellosis in some farms reached 38% (Refai 2002). Recent National serological investigations gave indirect proof of the presence of *Brucella* in cattle, buffalo, sheep, and goat herds nationwide (Wareth *et al.* 2014a). In Egypt only few laboratories are capable of isolating *Brucella*. Despite the implementation of the control program,

the disease is still endemic among livestock and humans. Buffaloes and cattle are a valuable and economic component of Egyptian rural household and number of buffaloes is higher than in any other neighboring country in the Near East region. Control programs have been instituted by the general organization of veterinary services (GOVS) to prevent the spread of brucellosis in the country particularly in large dairy herds (Wareth *et al.* 2014a). More than 70% of total livestock populations are owned by smallholders. Few cattle and buffaloes are kept in the household to produce milk, meat, and dairy products for home consumption or to sell often unpasteurized products in the local markets. The current study was aimed to identify the *Brucella* species circulating in small buffalo and cattle dairy herds in different localities of Delta region in Egypt using Matrix-assisted laser desorption ionization (MALDI), conventional and AMOS PCRs.

A total of 105 seropositive animals (90 cattle, 15 buffaloes) were used in the study. All animals were serologically positive for *Brucella* by Rose Bengal Test (RBT) and ELISA within the surveillance and eradication program instituted by the GOVS, Egypt. The animals originated from Qalyubia, Gharbia, Dakahliya, and Menofia. From each animal one sample was collected at abattoirs. Different tissue specimens have been taken: 86 lymph nodes, 3 uteri, 3 placenta and 10 vaginal swabs from dams, as well as 3 stomach contents from aborted fetuses. The specimens were collected aseptically and were kept in an icebox and transferred directly to the laboratory for isolation. The organ surface was burned and the internal parts were smeared by the swab. The swabs were plated on *Brucella* agar for primary isolation and subsequent identification of *Brucella* species was done according to colony morphology, Gram stain, motility, CO_2 requirement, H_2S production, phage lysis, and the biochemical tests e.g. catalase, oxidase and urease tests (Alton *et al.* 1988). A single colony was selected from the *Brucella* selective agar media and sub-cultured again on blood agar media to obtain uncontaminated colonies. After 48 hours of incubation, one colony was picked and submitted to Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF) for genus *Brucella* identification as described previously (Murugaiyan *et al.* 2014). The score values between 2.0 and 2.29 considered 'secure genus identification and probable species identification'.

Genomic DNA was extracted with the High Pure template preparation kit (DNA HP kit, Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions. Two pairs of primers amplifying different regions of the *Brucella* genome were used, the primer B4 and B5 that amplify a 223 bp fragment of the 31-kDa outer membrane protein. PCR assay conditions were performed in 25 µl of a reaction mixture containing final concentrations of 10 pmol/µl for each primer with 0.2 µl of 5 U/µl of Taq DNA polymerase (Promega), and 10 mM/1µl of deoxynucleotide triphosphates (dNTP) (Eppendorf). One µl DNA template was added to each reaction mixture. The PCR mixtures were overlaid with 2.5 µl of PCR-Puffer (Genaxxon) and complete with HPLC until 25 µL. The amplification was performed at a denaturation temperature of 93 °C for 5 min. This was followed by 35 cycles at 90 °C for 60 sec, 60 °C for 60 sec, 72 °C for 60 sec and 1 final extension at 72 °C for 5 minutes. Positive control (*B. abortus* 544 DNA) and negative control (HPLC water) were included in each reaction. After amplification, all reaction mixtures were analyzed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and photographed. Visible bands of appropriate sizes of (223 bp) for *Brucella* spp. were considered positive reactions.

To confirm results of MALDI-TOF and secure identification of *Brucella* species, the AMOS-PCR (*B. abortus, B. melitensis, B. ovis* and *B. suis*-PCR) was done according to Bricker and Halling and Matope and colleagues (Bricker and Halling 1994, Matope *et al.* 2009) with few modifications. Briefly, the PCR was performed in a final volume of 25 µl consisting of a reaction mixture containing 10 × PCR buffer (Genaxxon) without Mgcl. 0.2 µM each of *B. abortus, B. melitensis, B. ovis, B. suis*, and IS711-specific primer added to the reaction (Table I). 10 mM of dNTPs (Eppendorf), 0.2 µl of 5 U/µl of Taq DNA polymerase (Eppendorf) were added and complete with HPLC water until 25 µL. 1 µl DNA template was added to the 24 µl reaction mixture. The cycling conditions utilized were initial denaturation of 95 °C for 5 min; followed by 30 cycles of amplification using the following parameters: at 95 °C for 60 sec, annealing for 120 sec at 58 °C, extension for 120 sec at 72 °C, and a final elongation step of 72 °C for 5 min. The PCR products were separated by electrophoresis

Table I. *Primers used in conventional and AMOS-PCR for identification of* Brucella *in samples collected from seropositive cattle and buffaloes in the Delta region of Egypt.*

Conventional PCR primer	Nucleotide sequence 5'-3 '	Amplicon size
B4 primer	TGG CTC GGT TGC CAA TAT CAA	223 bp
B5 primer	CGC GCT TGC CTT TCA GGT CTG	
AMOS-PCR		-
B. abortus	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC	498 bp
B. melitensis	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	731 bp
B. ovis	CGG-GTT-CTG-GCA-CCA-TCG-TCG	976 bp
B. suis	GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG	285 bp
IS711	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT	-

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Table II. *Number of collected samples and number of* Brucella *isolates obtained from seropositive cattle and buffaloes in the Delta region of Egypt.*

Source of samples	Cattle			Buffaloes		
	No. of samples	No. of isolates	%	No. of samples	No. of isolates	%
Lymph node	74	8	10.8%	12	5	41.6%
Uterus	3	1	33.3%	-	-	0
Placenta	3	2	66.6%	-	-	0
Stomach content	3	1	33.3%	-	-	0
Vaginal swabs	7	-	0	3	2	66.4%
Total	90	12	13.3%	15	7	46.6%

using 1.5% agarose gel (w/v). Visible bands were considered positive reactions at appropriate sizes of 498 bp for *B. abortus*, 731 bp for *B. melitensis*, 976 bp for *B. ovis* and 285 bp for *B. suis*.

As shown in Table II, the bacterial examination of the collected samples from seropositive cattle and buffaloes revealed 19 *Brucella* strains, 12/90 strains were recovered from cattle and 7/15 were recovered from buffaloes. All recovered isolates showed the characteristic colonial morphology of *Brucella* on *Brucella* agar, *Brucella* selective media, and Blood agar. The colonies were convex, round, 1-2 mm. in diameter, with smooth margins, round edges, translucent and of golden color (pale honey-colored). All isolates were Gram-negative, non-motile, catalase and oxidase-positive. They required CO_2 for growth and produced H_2S. Conventional PCR and MALDI-TOF confirmed the classical bacteriology results on the 19 strains as *Brucella* with clear bands and score values > 2.3, respectively. The score > 2 is considered positive for genus *Brucella*, while the score values between 2.3 and 3.0 indicate 'highly probable species identification'. MADI-TOF identified four strains as *B. abortus* and 15 strains as *B. melitensis*. AMOS-PCR differentiated four *B. abortus* strains at appropriate band size of 498 bp, while a total of 15 strains were identified as *B. melitensis* at appropriate band size of 731 bp. All *B. abortus* strains identified in the current study were recovered from cattle samples, while *B. melitensis* strains were isolated from cattle (n = 8) and buffaloes (n = 7).

Brucellosis is endemic and prevalent in the Middle East, in the Mediterranean basin, in some countries of Africa and Asia, and in some areas of Latin America. However, still the epidemiological situation is not clear in some of those regions. The present work was planned to isolate *Brucella* spp. from cattle and buffaloes in different governorates of the Delta region in Egypt. Bacteriological examination of 105 collected samples produced 19 isolates, twelve strains were isolated from cattle and seven from buffaloes. Failure of isolation from the other cases may be due to a low number of viable *Brucella* organisms in specimens and/or due to massive contamination with other bacteria (Wareth *et al.* 2014b). *B. abortus* was isolated from cattle, while *B. melitensis* was isolated from both cattle and buffaloes. Previously it was assumed that brucellosis in cattle was mainly caused by *B. abortus*, less frequently by *B. melitensis* and rarely by *B. suis* (Radostits *et al.* 2000). In recent years, *B. melitensis* has been described as cause of great outbreaks in cattle and now is becoming a worldwide emerging problem and difficult to be controlled. This is likely due to the ineffective vaccination programs and lack of knowledge regarding the causative agents in different hosts (Alvarez *et al.* 2011). The major limitation of the control program in Egypt is the difficulty to detect all infected animals, especially carriers hosts i.e. dogs, cats, rats (Wareth *et al.* 2017). Seronegative/culture-positive animals within the herds also play a significant role in re-emerging and dissemination of the infection (El-Diasty *et al.* 2018). In Egypt, *B. melitensis* bv3 and *B. abortus* bv1 are the predominant source of brucellosis. *B. melitensis* have been isolated from sheep, goat, cattle, buffaloes, rats and Nile catfish (Wareth *et al.* 2014a), while *B. abortus* has been isolated from cattle, buffaloes and once from dog and cat (Wareth *et al.* 2017). Isolation of *B. melitensis* from cattle and buffaloes, the non-original hosts, can be attributed to raise sheep and goats together with cattle and buffaloes. The current results are in accordance with previous studies that highlighted the fact that *B. melitensis* clone can cross species barriers and establish a reservoir in cattle and buffaloes (Abd-El Halim *et al.* 2017, Hosein *et al.* 2018, Wareth *et al.* 2014a). Different molecular typing methods such as PCR techniques can differentiate *Brucella* at the species and the biovars level (Al Dahouk *et al.* 2005, Bricker and Halling 1994). These methods require little expertise, easy to perform and provide valuable alternatives to biochemical typing (Fretin *et al.* 2008). Application of MALDI-TOF MS was used successfully for microbial identification into the subspecies level, demonstrating that this technology is also a potentially effective tool for detection of microorganisms (Croxatto *et al.* 2012). Combination of AMOS-PCR and MALDI-TOF were able to identify *Brucella* spp. from a single colony in a short time and are promising techniques for fast and accurate identification. The results obtained in the current study should be considered during control and eradication program of brucellosis. All strains were recovered from small dairy farms which should be included in the control programs.

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