Identification, Genotyping and Antimicrobial Susceptibility Testing of *Brucella* spp. Isolated from Livestock in Egypt

Aman Ullah Khan ¹,²,³, Waleed S. Shell ⁴, Falk Melzer ¹, Ashraf E. Sayour ⁵, Eman Shawkat Ramadan ⁶, Mandy C. Elschner ¹, Amira A. Moawad ¹,²,⁷, Uwe Roesler ², Heinrich Neubauer ¹ and Hosny El-Adawy ¹,8,

¹ Institute of Bacterial Infections and Zoonoses, Friedrich-Loeffler-Institut, 07743 Jena, Germany; AmanUllah.Khan@fli.de (A.U.K.); falk.melzer@fli.de (F.M.); Mandy.Elschner@fli.de (M.C.E.); amira.moawad@fli.de (A.A.M.); heinrich.neubauer@fli.de (H.N.)
² Institute for Animal Hygiene and Environmental Health, Free University of Berlin, 14163 Berlin, Germany; Uwe.Roesler@fu-berlin.de
³ Department of Pathobiology, College of Veterinary and Animal Sciences, 35200 Jhang, Pakistan
⁴ Central Laboratory for Evaluation of Veterinary Biologics, Agricultural Research Center, 11517 Abbassa-Cairo, Egypt; tarikwaleedshell@hotmail.com
⁵ Department of Brucellosis, Animal Health Research Institute, Agricultural Research Center, 12618 Dokki-Giza, Egypt; shoofa@dr.com
⁶ Animal Reproduction Research Institute, Agricultural Research Center, 12556 Al Ahram-Giza, Egypt; emanramadan1311971@gmail.com
⁷ Provincial Laboratory, Institute of Animal Health Research, 35516 Mansoura, Egypt
⁸ Faculty of Veterinary Medicine, Kafr Elsheikh University, 33516 Kafr El-Sheikh, Egypt

* Correspondence: hosny.eladawy@fli.de

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**Abstract:** Brucellosis is a highly contagious zoonosis worldwide with economic and public health impacts. The aim of the present study was to identify *Brucella* (B.) spp. isolated from animal populations located in different districts of Egypt and to determine their antimicrobial resistance. In total, 34 suspected *Brucella* isolates were recovered from lymph nodes, milk, and fetal abomasal contents of infected cattle, buffaloes, sheep, and goats from nine districts in Egypt. The isolates were identified by microbiological methods and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Differentiation and genotyping were confirmed using multiplex PCR for *B. abortus*, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* (AMOS) and Bruce-ladder PCR. Antimicrobial susceptibility testing against clinically used antimicrobial agents (chloramphenicol, ciprofloxacin, erythromycin, gentamicin, imipenem, rifampicin, streptomycin, and tetracycline) was performed using E-Test. The antimicrobial resistance-associated genes and mutations in *Brucella* isolates were confirmed using molecular tools. In total, 29 *Brucella* isolates (eight *B. abortus* biovar 1 and 21 *B. melitensis* biovar 3) were identified and typed. The resistance of *B. melitensis* to ciprofloxacin, erythromycin, imipenem, rifampicin, and streptomycin were 76.2%, 19.0%, 76.2%, 66.7%, and 4.8%, respectively. Whereas, 25.0%, 87.5%, 25.0%, and 37.5% of *B. abortus* were resistant to ciprofloxacin, erythromycin, imipenem, and rifampicin, respectively. Mutations in the *rpoB* gene associated with rifampicin resistance were identified in all phenotypically resistant isolates. Mutations in *gyrA* and *gyrB* genes associated with ciprofloxacin resistance were identified in four phenotypically resistant isolates of *B. melitensis*. This is the first study highlighting the antimicrobial resistance in *Brucella* isolated from different animal species in Egypt. Mutations detected in genes associated with antimicrobial resistance unravel the molecular mechanisms of resistance in *Brucella* isolates from Egypt. The mutations in the *rpoB* gene in phenotypically resistant *B. abortus* isolates in this study were reported for the first time in Egypt.
Keywords: Brucella; Egypt; antimicrobial resistance; resistance-associated genes; mutation

1. Introduction

Brucellosis is considered as a common bacterial zoonotic disease of high prevalence in countries of the Middle East and the Mediterranean region, as well as some parts of Central and South America, Africa, and Asia [1,2]. Brucellosis is caused by bacteria of various species of the genus Brucella (B.) that are genetically highly related [3,4]. Brucella is a Gram negative, facultative intracellular pathogen classically causing infections in sheep and goats (B. melitensis), rams (B. ovis), bovines (B. abortus), canines (B. canis), pigs (B. suis), and rodents (B. neotomae) [5,6]. Brucellosis also affects terrestrial wildlife (B. microti) and marine mammals (B. ceti and B. pinnipedialis) [7]. However, the cross infection of animal species with brucellae has also been reported [8]. Brucellosis in livestock is causing high economic losses to livestock industry due to poor health, debility and loss of quality livestock products [9]. In humans, brucellosis causes severe acute febrile illness that becomes chronic if left untreated [10]. In developing countries, brucellosis is common but neglected disease, which has been endemic in Egypt for thousands of years and is present with a high prevalence in animals today [11–14]. Prevalence ranges from 2.47% to 26.66% in various livestock populations and this has a great socio-economic impact [15]. In Egypt, B. abortus, B. suis and B. melitensis strains were isolated from livestock having high levels of phylogenetic variability within each species [12]. The incidence of human brucellosis is 0.28–95 per 100,000 inhabitants per year in Egypt [16,17]. Humans get infected via the ingestion of contaminated raw milk, unpasteurized dairy products, handling of infected animals, animal discharges or dealing with Brucella cultures [18,19].

The diagnosis of brucellosis is still challenging and usually relies on serological tests [20], which are applied in vitro (milk or blood). Exceptionally, in vivo (allergic tests) are used. The isolation of brucellae and detection of Brucella DNA by PCR are the methods that allow definitive diagnosis [21]. Although confirmation of the disease is achieved by bacterial culture and identification, Brucella is difficult to grow and bacterial culturing is time consuming. Additionally, this method poses a risk to laboratory personnel and requires specific biosafety measures [22]. Hence, culture and biochemical typing remain the “gold standard” for the diagnosis of Brucella infection [23], including biochemical tests like CO₂ requirement, H₂S production, and dye sensitivity. Urease, oxidase, and catalase tests are also used for the typing of Brucella spp. [24]. A comparatively new method like matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged for microbiological identification [25]. It is an economical, easy, rapid and accurate method based on the automated analysis of the mass distribution of bacterial proteins [26]. A recently published study indicates that MALDI-TOF MS can accurately identify 99.5% and 97% of Brucella strains at the genus and species level, respectively that minimizing laboratory hazards. However, there are limitations in terms of sub-species level identification [27]. Brucella identification and species differentiation can be accomplished using genus-specific Brucella PCR (B4/B5), AMOS-PCR, and Bruce-ladder PCR [28–32].

The intracellular location of brucellae in reticuloendothelial cells and their predilection sites (e.g., bone) limit the penetration of most antibiotics. Antimicrobial regimes with quinolones, doxycycline, rifampicin, streptomycin, and aminoglycoside alone or in combination are used to treat brucellosis [33]. Regular treatment failure and numerous reports of relapses of brucellosis following therapy exist ranging from 5% to 15% in uncomplicated cases [34]. Recently, the antimicrobial resistance in Brucella is emerging in brucellosis endemic regions of the world (e.g., Egypt, Qatar, Iran, Malaysia, and China) [34].

There is no proper legislation in Egypt regulating the use of antimicrobials. Some compounds such as quinolones, tetracycline, beta-lactams, aminoglycosides and imipenem are still overused non-therapeutically in Egypt to treat various human infections [35–37]. This improper use of antimicrobials results in the emergence of multidrug resistant bacteria [38–41]. The use of antimicrobials
in farm animals to promote growth or as prophylaxis also contributes to the development of resistant bacteria and plays a key role in their spread along the food chain [42]. Antimicrobial resistance in zoonotic pathogens is an additional risk because it will limit disease treatment options in public health and veterinary settings [43]. None of the available studies highlights detailed antimicrobial susceptibility patterns of *Brucella* isolates from livestock in Egypt.

The use of antimicrobial susceptibility testing is the solution for appropriate control and treatment of brucellosis [44,45]. Micro-dilution and/or gradient strip (*E*-test) methods are used to establish minimum inhibitory concentration (MIC) for antimicrobials [45,46]. PCR assays and the subsequent sequencing of genes associated with resistance are used to identify the genetic bases of resistance [47–49].

Resistance to commonly used antimicrobials is mediated by mutations of *rpoB* gene (rifampicin), *gyrA, gyrB, parC, parE* genes (quinolones), *erm, mef, msr* (macrolides) or the presence of *tet* genes (tetracyclines), *mecA* (beta-lactams) and *floA* (trimethoprim) [50]. Mutations in the *rpoB* and *gyrA* genes may occur naturally or can be induced in vitro [45,47,51,52].

This study aimed to isolate, identify and biotype *Brucella* strains from livestock in various regions of Egypt. Antimicrobial resistance and its genetic basis are to be investigated in the gained *Brucella* isolates.

2. Materials and Methods

2.1. Isolation and Identification

A total of 34 suspected *Brucella* isolates were recovered from clinical specimens of lymph nodes, milk and fetal stomach contents from infected cattle, buffaloes, sheep and goats located in Giza, Beheria, Asyut, Qalyubia, Beni-Suef, Ismailia, Dakahlia, and Monufia governorates/districts in Egypt (Table 1).

Bacterial isolation and identification were performed in Biological Safety Level-3 (BSL-3) laboratory. Isolates were inoculated on calf blood agar, *Brucella* medium and *Brucella* selective medium plates (Oxoid GmbH, Wesel, Germany) at 37 °C in the absence and presence of 5–10% CO2 for up to 2 weeks. Typically, round, glistening, pinpoint and honey drop-like cultures were picked and stained with Gram and modified Ziehl-Neelsen staining (MZN) methods. Subsequent biochemical tests, motility test, hemolysis on blood agar and agglutination with monospecific sera were performed [24,53]. Isolates were stored at −20 °C for further processing.

Identification by MALDI-TOF MS

Bacterial identification was additionally carried out using MALDI-TOF MS as described previously [27,54]. Briefly, pure cultures of suspected *Brucella* were obtained by incubating inoculated chocolate PolyViteX (PVX) agar plates (bioMérieux, Marcy-l’Étoile, France) for 48 h at 37 °C in the presence of 5% CO2. Samples were reliably inactivated in Biological Safety Level-3 laboratory. Approximately 10 colonies from culture medium were suspended in 50 μL of sterile HPLC water and mixed carefully. Formic acid (*v/v 70%) was added for the inactivation of brucellae and for extraction of proteins. Then, 1 μL of tested sample and *Brucella* reference strains were added onto spots of a steel target plate. After inactivation, the plate was dried at room temperature followed by the addition of 0.5 μL of 100% ethanol to each well. Finally, spots were overlaid with 1 μL of reconstituted alpha-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA, USA).

Spectra were acquired with an Ultraflex instrument (Bruker Daltonics GmbH, Bremen, Germany). Analysis was done with the Biotyper 3.1 software (Bruker Daltonics GmbH, Germany) as per the manufacturer’s instructions to exclude spectra with outlier peaks or anomalies.

Logarithmic score values (0–3.0) were determined by automatically calculating the proportion of matching peaks and peak intensities between the test spectrum and the reference spectra in the database. The identification was considered reliable when the score between 2.3 and 3.0. A logarithmic score of 1.7–2.299 was reported as ‘probable genus identification’, indicating that identification was
reliable only at the genus level. When the logarithmic score was <1.7, the spectrum was reported as ‘not reliable identification’, indicating that sample could not be identified.

2.2. Genomic DNA Extraction and Purification

DNA was extracted from heat inactivated pure Brucella culture (biomass) using the HighPure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. DNA quantity and purity were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

2.3. Molecular Identification and Differentiation

The presence of the Brucella genus-specific bscp31 gene [55] and Brucella-specific insertion sequence 711 (IS711) [29] was investigated for Brucella genus identification. Briefly, PCR was performed using 25 µL reaction mixture containing 18.3 µL HPLC water, 2.5 µL 10x PCR buffer (Genaxxon bioscience GmbH, Ulm, Germany), 1 µl of 10mM dNTP (Thermo Fisher Scientific, USA), 1 µL each forward (5′-TGG CTC GGT TGC CAA TAT CAA-3′) and reverse primer (5′-CGC GCT TGC CTT TCA GGT CTG-3′) (Jena Bioscience, Germany), 0.2 µL of 5U/µL Taq-polymerase (Genaxxon bioscience GmbH, Ulm, Germany) and 1 µL DNA template.

PCR condition was initiated by initial denaturation at 93 °C for 5 min, followed by 35 cycles of denaturation at 90 °C for 60 s, annealing at 60 °C for 60 s and elongation at 72 °C for 60 s and final elongation step at 72 °C for 5 min. PCR products (223 bp) were analyzed on 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

The AMOS-PCR was performed to differentiate Brucella species [29,32] followed by a multiplex Bruce-ladder PCR assay for strain and biovar typing [30,56]. The list of primers and primer sequences for AMOS-PCR and Bruce-ladder PCR were geared from previously published [29] and [30], respectively. Briefly, for AMOS-PCR, PCR was performed using 25 µL reaction mixture containing 9.5 µL of 2x Qiagen Master mix (Qiagen, Germany), 1 µL of 10 pmol primer mix and 2 µL DNA template. Initial denaturation at 95 °C for 5 min, was followed by 30 cycles of denaturation at 95 °C for 60 s, annealing at 58 °C for 2 min and elongation at 72 °C for 2 min and a final elongation step at 72 °C for 5 min. The Bruce-ladder PCR was performed using 12.5 µL reaction mixture containing 4.25 µL HPLC water, 6.25 µl of 2x Qiagen Master mix (Qiagen, Germany), 1 µL of 2 pmol/µL primer mix and 1 µL DNA template. Initial denaturation at 95 °C for 15 min, was followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s, elongation at 72 °C for 3 min and a final elongation step at 72 °C for 10 min.

The PCR products from each PCR were separated by electrophoresis using 1.5% agarose gels (120 V for 60 min for conventional and AMOS-PCR and 130 V for 60 min for Bruce-ladder PCR). Gels were stained with ethidium bromide and photographed using a gene snap camera (Syngene Pvt Ltd., Cambridge, UK).

2.4. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of B. melitensis and B. abortus isolates was performed against eight clinically relevant antimicrobial agents (chloramphenicol, ciprofloxacin, erythromycin, gentamicin, imipenem, rifampicin, streptomycin and tetracycline) using gradient strip method (E-test, bioMerieux, Marcy L’Etoile, France) as described previously [48]. Briefly, a suspension of bacteria adjusted to 0.5 McFarland standard units was inoculated on Mueller-Hinton plates (Oxoid GmbH, Wesel, Germany) supplemented with 5% sheep blood and the gradient strips were applied. The plates were incubated at 37 °C with 5% CO₂ for 48 h before reading. As MIC breakpoints for clinically used antimicrobials are not yet established for brucellae, the guidelines for slow-growing bacteria (Haemophilus influenzae) were used as an alternative [57]. Quality control assays were performed using E. coli (161008BR3642, DSM 1103, ATCC 25922). The susceptibility profiles of Brucella isolates are presented as resistant and susceptible using minimum inhibitory concentrations (MIC), MIC₅₀ and MIC₉₀. The interpretations
were performed using CLSI (The Clinical and Laboratory Standards Institute) [57] and EUCAST (The European Committee on Antimicrobial Susceptibility Testing) [58] using the criteria for slow growing bacteria. For rifampin, the strains were also classified as intermediate (Table 2).

2.5. Molecular Detection of Antimicrobial Resistance-Associated Genes

The PCR assays were performed as described previously [47,49,52,59] to detect the antimicrobial resistance-associated genes, i.e., catB, gyrA and gyrB, rpoB, Aac genes and tet genes for chloramphenicol, ciprofloxacin, rifampicin, streptomycin, gentamicin and tetracycline, respectively (Supplementary Table S1). The primers used for amplification of the rpoB gene were designed by using submitted sequences for the rpoB gene of B. abortus (accession number AY562181) [47]. PCR was performed using 25 µL reaction mixture containing 2x Qiagen Mastermix, 10 pmol each forward and reverse primer (Table 1) and 5 µl DNA template. PCR was carried out by initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing (temperatures for each primer are given in Table 1) for 60 s, elongation at 72 °C for 60 s and a final elongation step at 72 °C for 10 min. Twenty microliters of each reaction mixture were analyzed by gel electrophoresis (1% agarose gel with ethidium bromide).

2.6. PCR Amplicon Sequencing and Data Analysis

Amplified PCR products for gyrA, gyrB and rpoB genes were purified using Qiagen QIAquick Gel extraction kit (Qiagen, Germany) and sent for sequencing (Eurofins Genomics Germany GmbH, Ebersberg, Germany). All consensus sequences were aligned and compared to the reference Brucella genes obtained from NCBI for detection and evaluation of nucleotide diversity and mutations using the software Geneious® R11.1.5 (https://www.geneious.com). The sequences of gyrA (CP034103 and AE017223), gyrB (CP007760 and SDWB01000001) and rpoB (AY562181 and AY540346) genes of B. melitensis and B. abortus were geared from Gene bank and used as reference. Amino acid sequences were determined along with nucleotide sequences to identify missense mutations using BLAST.

3. Results

3.1. Microbiological Identification

Based on microbiological and biochemical characteristics, 21 strains were typed as B. melitensis biovar 3, eight strains were B. abortus biovar 1 and five samples were identified as Achromobacter species (Table 1). The results of MALDI-TOF MS confirmed five isolates as Achromobacter species while the remaining 29 isolates were identified as Brucella species (Table 1).

3.2. Molecular Identification and Differentiation

Brucella DNA of 24 isolates from cattle, three from buffaloes, one from a sheep and one from a goat were amplified with the genus specific assay. AMOS-PCR and Bruce-ladder PCR differentiated these 21 isolates as B. melitensis (17 from cattle, two from buffaloes, 1 from a sheep and 1 from a goat) and 8 isolates as B. abortus (seven from cattle and one from a buffalo). All isolates were confirmed as field strains (Table 1).

3.3. Antimicrobial Susceptibility Profiling

The in vitro MIC values against eight antimicrobial agents of all 29 Brucella isolates were determined by the gradient strip method (E-test). The MIC values along with MIC50 and MIC90 are summarized in Table 2.

In this study, 76.19%, 19.04%, 76.19%, 66.66%, and 4.76% of the B. melitensis isolates were resistant to ciprofloxacin, erythromycin, imipenem, rifampicin/rifampin and streptomycin, respectively. While, 25%, 87.5%, 25%, and 37.5% of B. abortus isolates were phenotypically resistant to ciprofloxacin, erythromycin, imipenem and rifampicin/rifampin, respectively. All 29 Brucella isolates were sensitive
to chloramphenicol, gentamicin, and tetracycline. Four isolates of *B. melitensis* (19.04%) and one *B. abortus* isolate showed multidrug resistance against ciprofloxacin (fluoroquinolones), erythromycin (macrolides), imipenem (carbapenems) and rifampicin (ansamycins).

### 3.4. Detection of Antimicrobial Resistance-Associated Genes and Mutations

Genes associated with antimicrobial resistance (*catB*, *Aac* and *tet* (*tetA*, *tetB*, *tetM* and *tetO*) conferring resistance to chloramphenicol, streptomycin/gentamicin and tetracycline, respectively) were not identified either in resistant or sensitive isolates. The *gyrA*, *gyrB* and *rpoB* genes were amplified in all isolates.

Mutations in *rpoB* gene associated with a rifampicin-resistant *B. melitensis* and *B. abortus* phenotypes were detected at different positions (Table 3).

Mutations in *gyrA* gene associated with phenotypic-ciprofloxacin resistance were detected at positions 167 (ATG to AAG/methionine to arginine), 197 (CCC to CGC/proline to arginine), 202 (CGC to AGC/arginine to serine), 235 (GGT to CGT/glycine to arginine), 941 (GCC to GAC/alanine to aspartic acid), 944 (GTG to GAG/valine to glutamic acid), 944-945 (GTG to GGA/valine to glycine), 946 (GCC to TCC/alanine to serine) and 962 (AAC to ACC/asparagine to threonine) in *B. melitensis* (Table 4).

Three-point mutations were also detected in *gyrB* gene at position 1141 (AAG to GAG/Lysine to Glutamine), 1144 (ATC to CTC/Isoleucine to leucine) and 1421 (TCA to TTA/Serine to Leucine) in phenotypically resistant *B. melitensis* isolates (Table 4).

Repeated mutations were detected at positions 676, 677 (TAC to CTG/tyrosine to leucine) and 1435 (AAG to CAG/lysine to glutamine) in the *rpoB* gene of phenotypic resistant *B. melitensis* isolates while the same was recorded at position 2890 (CGT to GGT/arginine to glycine) in the *rpoB* gene of *B. abortus* isolates. No mutation was detected in *gyrA* and *gyrB* gene of *B. abortus* strains.
| Sample ID | Animal Species | Origin of Sample | Type of Sample | Growth with CO₂ | Slide Agglutination A-M-R-Serum | MALDI-TOF MS | Molecular Identification |
|-----------|----------------|------------------|----------------|----------------|-------------------------------|-------------|-----------------------|
| 18RB17227 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17228 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17229 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella melitensis       | B. melitensis |
| 18RB17230 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. melitensis) | B. melitensis |
| 18RB17231 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | * NA         | Achromobacter spp.       | -ve          |
| 18RB17232 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | NA          | Achromobacter spp.       | -ve          |
| 18RB17233 | Cattle         | Giza             | Lymph node     | +/+/-           | +/-/-/-                      | B. abortus 1  | B. abortus              | B. abortus |
| 18RB17234 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | NA          | Achromobacter spp.       | -ve          |
| 18RB17235 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17236 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. melitensis) | B. melitensis |
| 18RB17237 | Cattle         | Giza             | Lymph node     | + + +           | + + + +                      | NA          | Achromobacter spp.       | -ve          |
| 18RB17238 | Cattle         | Giza             | Lymph node     | + + +           | + - -                          | B. abortus 1  | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17239 | Cattle         | Giza             | Lymph node     | + + +           | + - -                          | NA          | Achromobacter spp.       | -ve          |
| 18RB17240 | Cattle         | Beheira          | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17241 | Cattle         | Beheira          | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17242 | Cattle         | Beheira          | Lymph node     | +/-/-           | +/-/-/-                      | B. abortus 1  | B. abortus              | B. abortus |
| 18RB17243 | Cattle         | Beheira          | Lymph node     | + + +           | + + + +                      | B. abortus 1  | B. abortus              | B. abortus |
| 18RB17244 | Buffalo        | Asyut            | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17245 | Buffalo        | Asyut            | Lymph node     | +/-/-           | +/-/-/-                      | B. abortus 1  | B. abortus              | B. abortus |
| 18RB17246 | Goat           | Beni-Suef        | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17247 | Cattle         | Asyut            | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17248 | Cattle         | Qalyubia         | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17249 | Cattle         | Qalyubia         | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17250 | Sheep          | Beni-Suef        | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17251 | Cattle         | Beni-Suef        | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17252 | Cattle         | Ismailia         | Lymph node     | + + +           | + + + +                      | B. melitensis 3 | Brucella spp. (B. abortus) | B. melitensis |
Table 1. Cont.

| Sample ID  | Animal Species | Origin of Sample | Type of Sample | Growth with CO₂ | Slide Agglutination A-M-R-Serum | MALDI-TOF MS | Molecular Identification |
|------------|----------------|------------------|----------------|-----------------|---------------------------------|-------------|--------------------------|
| 18RB17253  | Cattle         | Ismailia         | Lymph node     | +               | +     | +     | +ve  | +ve  | -ve  | -ve  | B. melitensis 3         | Brucella spp. (B. abortus) | B. melitensis |
| 18RB17254  | Cattle         | Ismailia         | Lymph node     | +               | +     | +     | +ve  | +ve  | -ve  | -ve  | B. melitensis 3         | Brucella spp. | B. melitensis |
| 18RB17255  | Cattle         | Beheira          | Fetal stomach content | +/-  | +/-  | +/-  | +ve  | -ve  | -ve  | B. abortus 1 | B. abortus |
| 18RB17256  | Cattle         | Dakahlia         | Lymph node     | +/-  | +/-  | +/-  | +ve  | -ve  | -ve  | B. abortus 1 | B. abortus |
| 18RB17257  | Cattle         | Monufia          | Lymph node     | +/-  | +/-  | +/-  | +ve  | -ve  | -ve  | B. abortus 1 | B. abortus |
| 18RB17258  | Cattle         | Monufia          | Milk           | +     | +     | +    | +ve  | +ve  | -ve  | B. melitensis 3 | Brucella spp. (B. abortus) |
| 18RB17259  | Cattle         | Qalyubia         | Lymph node     | +/-  | +/-  | +/-  | +ve  | -ve  | -ve  | B. abortus 1 | B. abortus |
| 18RB17260  | Buffalo        | Qalyubia         | Lymph node     | +     | +     | +    | +ve  | +ve  | -ve  | B. melitensis 3 | Brucella spp. (B. microti) |

*NA-not applicable, a Positive, b Negative, c Brucella medium, d Brucella selective medium, e Brucella blood agar.

Table 2. Antimicrobial resistance profiles of 21 B. melitensis and 8 B. abortus isolated from livestock species in Egypt against 8 clinically used antibiotics using E-test. Breakpoint and Minimal Inhibitory Concentration (MIC₅₀, MIC₉₀) for B. melitensis and B. abortus used in this study according to CLSI and EUCAST recorded for H. influenzae [57,58] were provided.

| Antibiotic       | Class           | Sensitive (mg/L) | Intermediate (mg/L) | Resistant (mg/L) | R (%) | MIC₅₀ (mg/L) | MIC₉₀ (mg/L) | R (%) | MIC₅₀ (mg/L) | MIC₉₀ (mg/L) |
|------------------|-----------------|-----------------|--------------------|-----------------|-------|--------------|--------------|-------|--------------|--------------|
| Chloramphenicol  | Phenicols       | ≤2              | 4                  | ≥8              | 0.0   | 1            | 2            | 0.0   | 0.25         | 0.5          |
| Ciprofloxacin    | Fluoroquinolones| ≤0.06           | –                  | >0.06           | 76.19 | 0.12         | 0.25         | 25.0  | 0.02         | 0.06         |
| Erythromycin     | Macrolides      | –               | –                  | ≥16             | 19.04 | 4            | 8            | 87.5  | 1            | 32           |
| Gentamicin       | Aminoglycosides | –               | –                  | ≤4              | 0.0   | 11           | 11           | 0.0   | 0.12         | 0.5          |
| Imipenem         | Carbapenems     | ≤2              | –                  | ≥2              | 76.19 | 8            | 8            | 25.0  | 1            | 4            |
| Rifampicin       | Ansamycins      | ≤1              | 2                  | ≥4              | 66.66 | 4            | 8            | 37.5  | 2            | 4            |
| Streptomycin     | Aminoglycosides | –               | –                  | ≥16             | 4.76  | 1            | 2            | 0.0   | 0.25         | 0.5          |
| Tetracycline     | Tetracyclines   | ≤2              | 4                  | ≥8              | 0.0   | 0.06         | 0.12         | 0.0   | 0.03         | 0.12         |

- Not determined
Table 3. Detection of mutations in rpoB gene associated with rifampicin resistance in *B. melitensis* and *B. abortus*.

| ID     | Brucella spp. | RIF Resistance | Mutation Sites | Mutation | Amino Acid Change | NCBI (Accession No.) |
|--------|---------------|----------------|----------------|----------|-------------------|---------------------|
| 18RB17227 | *B. melitensis* | 4              | 676, 677       | TAC to CTC | Tyrosine to leucine | MN544028, MN544042, MN544056, MN544070, MN544084 |
|         |               |                | 1816           | GAT to GAA | Aspartic acid to glutamic acid |                     |
|         |               |                | 1818           | GTC to GCC | Valine to alanine |                     |
|         |               |                | 1820, 1822     | GTT to ATA | Valine to isoleucine |                     |
|         |               |                | 1824, 1825     | TAC to TTT | Tyrosine to phenylalanine |                     |
|         |               |                | 1826, 1828     | CTG to GTC | Leucine to valine |                     |
|         |               |                | 1829, 1831     | TCG to GAC | Serine to aspartic acid |                     |
|         |               |                | 1835, 1837     | ATG to GGC | Methionine to glycine |                     |
|         |               |                | 1838           | GAA to AAA | Glutamic acid to lysine |                     |
|         |               |                | 1842, 1843     | GAA to GGT | Glutamic acid to glycine |                     |
| 18RB17228 | *B. melitensis* | 4              | 676, 677       | TAC to CTC | Tyrosine to leucine | MN544029, MN544043, MN544057, MN544071, MN544085 |
|         |               |                | 3901, 3902     | TAC to ACC | Tyrosine to threonine |                     |
|         |               |                | 1011           | AAC to AGC | Asparagine to serine |                     |
| 18RB17229 | *B. melitensis* | 4              | 676, 677       | TAC to CTC | Tyrosine to leucine | MN544030, MN544044, MN544058, MN544072, MN544086 |
|         |               |                | 1456, 1458     | AAC to AGC | Asparagine to serine |                     |
|         |               |                | 1787           | GAA to AAG | Glutamic acid to lysine |                     |
|         |               |                | 2491           | AAG to ACG | Lysine to threonine |                     |
|         |               |                | 1435           | ACC to CCC | Threonine to proline |                     |
| 18RB17230 | *B. melitensis* | 8              | 676, 677       | TAC to CTC | Tyrosine to leucine | MN544031, MN544045, MN544059, MN544073, MN544087 |
|         |               |                | 1798, 1799     | AAG to CAG | Lysine to glutamine |                     |
|         |               |                | 1801, 1802     | AAG to GAG | Lysine to glycine |                     |
|         |               |                | 1804, 1806     | GGT to GTT | Valine to leucine |                     |
|         |               |                | 1807           | ATC to TCC | Threonine to serine |                     |
|         |               |                | 2209, 2210     | ATC to TCC | Isoleucine to serine |                     |
| 18RB17235 | *B. melitensis* | >8             | 676, 677       | TAC to CTC | Tyrosine to leucine | MN544032, MN544046, MN544060, MN544074, MN544087 |
|         |               |                | 1469           | GTC to GGC | Valine to glycine |                     |
Table 3. Cont.

| ID     | Brucella spp. | RIF Resistance | Mutation Sites | Mutation | Amino Acid Change                  | NCBI (Accession No.)       |
|--------|---------------|----------------|----------------|----------|------------------------------------|----------------------------|
| 18RB17236 | B. melitensis | 8              | 676, 677       | TAC to CTC | Tyrosine to leucine               | MN544033, MN544047, MN544061, MN544075, MN544089 |
| 18RB17238 | B. melitensis | 16             | 677, 1780, 1786, 1788, 2869, 2871 | TAC to TTC, TAT to GAT, AAG to CAA, CGT to GGG | Tyrosine to phenylalanine, Tyrosine to aspartic acid, Lysine to glutamine, Arginine to glycine | MN544034, MN544048, MN544062, MN544076, MN544090 |
| 18RB17240 | B. melitensis | 16             | 2494, 2496     | TCG to CTC | Serine to leucine                 | MN544035, MN544049, MN544063, MN544077, MN544091 |
| 18RB17241 | B. melitensis | 6(8)           | 1435, 2870, 2871 | AAG to CAG, CGT to CCG | Lysine to glutamine, Arginine to proline | MN544036, MN544050, MN544064, MN544078, MN544092 |
| 18RB17246 | B. melitensis | 4              | 676, 678, 1436, 1437, 2870, 3896, 3901 | TAC to CTT, AAG to ACA, CGT to CCT, TAC to AAC, ACG to CCG | Tyrosine to leucine, Lysine to threonine, Arginine to proline, Tyrosine to asparagine, Threonine to proline | MN544037, MN544051, MN544065, MN544079, MN544093 |
Table 3. Cont.

| ID       | *Brucella* spp. | RIF Resistance | Mutation Sites | Mutation | Amino Acid Change | NCBI (Accession No.) |
|----------|-----------------|----------------|---------------|----------|-------------------|----------------------|
| 18RB17249 | *B. melitensis* | 4              | 1435, 1437    | AAG to GTA | Lysine to valine  | MN544038, MN544052, MN544066, MN544080, MN544094 |
|          |                 |                | 2170          | GGC to CGC | Glycine to arginine|                      |
|          |                 |                | 2203, 2205    | ATC to TTT | Isoleucine to phenylalanine |                      |
|          |                 |                | 2869          | CGT to GGT | Arginine to glycine |                      |
|          |                 |                | 3152, 3153    | GTG to GGT | Valine to glycine |                      |
|          |                 |                | 3154, 3156    | CAG to GCA | Glutamine to alanine |                      |
|          |                 |                | 3157          | CGC to AGC | Arginine to serine |                      |
| 18RB17253 | *B. melitensis* | 4              | 1435          | AAG to CAG | Lysine to glutamine | MN544039, MN544053, MN544067, MN544081, MN544095 |
|          |                 |                | 1745          | GCC to GGC | Alanine to glycine |                      |
| 18RB17258 | *B. melitensis* | 6              | 676, 677      | TAC to CTC | Tyrosine to leucine | MN544040, MN544054, MN544068, MN544082, MN544096 |
|          |                 |                | 2501, 2502    | CAC to CCA | Histidine to proline |                      |
| 18RB17260 | *B. melitensis* | 4              | 1435          | AAG to CAG | Lysine to glutamine | MN544041, MN544055, MN544069, MN544083, MN544097 |
|          |                 |                | 3670, 3672    | CAG to TAT | Glutamine to tyrosine |                      |
| 18RB17233 | *B. abortus*    | 4              | 703, 704      | ACT to CTT | Threonine to leucine | MN544013, MN544016, MN544019, MN544022, MN544025 |
|          |                 |                | 709, 710      | ACC to CAC | Threonine to histidine |                      |
|          |                 |                | 1457, 1458    | AAG to ACA | Lysine to threonine |                      |
|          |                 |                | 1460          | GAA to GGA | Glutamic acid to glycine |                      |
|          |                 |                | 2512          | ACC to CCC | Threonine to proline |                      |
|          |                 |                | 2515, 2517    | TCG to CTC | Serine to leucine |                      |
|          |                 |                | 2890, 2892    | CGT to GGG | Arginine to glycine |                      |
|          |                 |                | 3123          | GAC to GAG | Aspartic acid to glutamic acid |                      |
|          |                 |                | 3124, 3125    | GAC to ATC | Aspartic acid to isoleucine |                      |
Table 3. Cont.

| ID          | Brucella spp. | RIF Resistance | Mutation Sites | Mutation       | Amino Acid Change       | NCBI (Accession No.) |
|-------------|---------------|----------------|----------------|-----------------|------------------------|----------------------|
| 18RB17242   | B. abortus    | >4             | 698, 699       | TAC to TTT     | Tyrosine to phenylalanine | MN544014,           |
|             |               |                | 1457, 1458     | AAG to ACA     | Tyrosine to threonine   | MN544017,           |
|             |               |                | 1460           | GAA to GGA     | Glutamic acid to glycine| MN544020,           |
|             |               |                | 1789           | ATC to GTC     | Isoleucine to valine    | MN544023,           |
|             |               |                | 1801           | TAT to GAT     | Tyrosine to aspartic acid| MN544026            |
|             |               |                | 2887           | GAG to AAG     | Glutamic acid to lysine  |                     |
|             |               |                | 2890           | CGT to GGT     | Arginine to glycine     |                     |
| 18RB17245   | B. abortus    | 4              | 709            | ACC to CCC     | Threonine to proline    | MN544015,           |
|             |               |                | 2890           | CGT to GGT     | Arginine to glycine     | MN544018,           |
|             |               |                |                |                |                        | MN544021,           |
|             |               |                |                |                |                        | MN544024,           |
|             |               |                |                |                |                        | MN544027            |

Table 4. Detection of mutations in gyrA and gyrB genes associated with ciprofloxacin resistance in B. melitensis.

| ID          | Brucella spp. | CIP Resistance | Gene | Mutation Sites | Mutation       | Amino Acid Change       | NCBI (Accession No.) |
|-------------|---------------|----------------|------|----------------|-----------------|------------------------|----------------------|
| 18RB17230   | B. melitensis | 0.5            | gyrA | 167            | ATG to AGG      | Methionine to arginine| MN536677            |
|             |               |                |      | 197            | CCC to CGC      | Proline to arginine   |                     |
|             |               |                |      | 202            | CGC to AGC      | Arginine to serine    |                     |
|             |               |                |      | 235            | GGT to CGT      | Glycine to arginine   |                     |
| 18RB17235   | B. melitensis | 0.25           | gyrA | 944, 945       | GTG to GGA      | Valine to glycine     | MN536678            |
|             |               |                |      | 946            | GCC to TCC      | Alanine to serine     |                     |
| 18RB17238   | B. melitensis | 0.25           |      | 941            | GCC to GAC      | Alanine to aspartic acid| MN536679            |
|             |               |                |      | 944            | GTG to GAG      | Valine to glutamic acid|                     |
| 18RB17254   | B. melitensis | 0.12           |      | 962            | AAC to ACC      | Asparagin to threonine| MN536680            |
| 18RB17230   | B. melitensis | 0.5            |      | 1144           | ATC to CTC      | Isoleucine to leucine | MN536681            |
| 18RB17244   | B. melitensis | 0.25           |      | 1141           | AAG to GAG      | Lysine to glutamine   | MN536682            |
| 18RB17252   | B. melitensis | 0.12           |      | 1421           | TCA to TTA      | Serine to Leucine     | MN536683            |
| 18RB17254   | B. melitensis | 0.12           |      | 1421           | TCA to TTA      | Serine to Leucine     | MN536684            |
4. Discussion

Brucellosis is a zoonotic disease of public health importance and is still endemic in many countries including Egypt [17,20]. In this study, the phenotypic and molecular characterization of Brucella isolates from cattle, buffaloes, sheep and goats obtained from different geographical locations of Egypt was performed. Additionally, the molecular basis of antimicrobial resistance in Brucella isolates from Egypt is reported for the first time. These results contribute to a better understanding of geographic transmission and spread of brucellae in livestock in Egypt and pave a way for specific treatment and control of the disease in animals and as well as in humans.

For the accurate diagnosis of brucellosis, isolation of bacteria or molecular proof along with suggestive clinical signs is needed. Brucellae were isolated in this study from milk, lymph nodes and fetal stomach contents as recommended in previous reports [24,60].

Twenty-one B. melitensis bv3 and 8 B. abortus bv1 were isolated from cattle, buffaloes, sheep and goats from Giza, Beheria, Asyut, Qalyubia, Beni-Suef, Ismailia, Dakahlia and Monufia governorates. Previous reports were described previously that Brucella was prevailing in the country [12]. The isolation of B. melitensis from cattle and buffaloes in this study may be attributed to mixed farming of large and small ruminants as mentioned previously [13].

Still brucellosis is a challenge to treat in humans, particularly after delayed diagnosis of the infection. The WHO (World Health Organization) recommended treatment include high oral doses of rifampicin, doxycycline or tetracycline and trimethoprim-sulfamethoxazole. Although streptomycin and tetracycline are considered as powerful therapeutic agents against brucellosis, their higher toxicity limits their use [52,61]. Quinolones are promising alternatives to treat human brucellosis as they have good bioavailability and affinity for bone and soft tissues [51].

Only one study from Brazil reported reduced antimicrobial sensitivity in brucellae isolated from cattle [62]. However, the emergence of brucellae isolated from humans phenotypically resistant to ciprofloxacin, gentamycin, streptomycin, rifampicin and trimethoprim-sulfamethoxazole was reported in Egypt, Iran, Qatar, China, Norway and Malaysia [46,48,63–65]. Phenotypically rifampicin resistant B. melitensis isolates were also reported from Norway in imported cases from the Middle East, Asia or Africa [45]. Probable rifampicin resistance was noted in 19% of a large collection of B. melitensis isolates from humans in Egypt between 1999 to 2007 [65]. However, none of those isolates were investigated further to confirm the basis of resistance or reduced susceptibility.

In this study, a notable phenotypic resistance against ciprofloxacin (76.19%) was detected in B. melitensis strains isolated from animals. In contrast, none of the mentioned studies reported ciprofloxacin resistance in clinical isolates of humans and animals before. However, antimicrobial resistance against quinolones has been reported in in vitro studies of B. melitensis from Greece and France [49,52].

An alarming high number of rifampicin resistant (66.66%) B. melitensis isolates was found in this study. Previous reports from Egypt (19%), [65], Norway (24%) [45] and Kazakhstan (26.4%) [66] described comparatively low resistance. Hence, these findings are in agreement with previously published reports from Egypt that clearly showed an increase in antimicrobial resistance in various other human pathogens [37]. Reduced rifampicin susceptibilities in B. melitensis strains were also reported from Iran, Malaysia, China, and Kazakhstan [46,48,63,64,66].

The most striking finding of the present study was the emergence of phenotypic antimicrobial resistance against erythromycin (19.04%), imipenem (76.19%) and streptomycin (4.76%) in B. melitensis isolates. However, the increased use of these antimicrobials in Egypt in veterinary and human practices may be the cause of the emerging of this resistance [37].

The phenotypic antimicrobial resistance against ciprofloxacin (25%), erythromycin (87.5%), imipenem (25%) and rifampicin (37.5%) of B. abortus isolated in this study was not proved previously. Multidrug resistant strains of B. abortus isolated from cattle in this study were reported previously in Brazil [62]. Four isolates of B. melitensis and one isolate of B. abortus showed multidrug resistance against ciprofloxacin, erythromycin, imipenem and rifampicin. These findings are in agreement with
the results of Barbosa Pauletti et al. who find corresponding resistance among \textit{B. abortus} isolates from cattle in Brazil [62]. All \textit{B. melitensis} and \textit{B. abortus} isolates in this study were sensitive to chloramphenicol, gentamicin and tetracycline. These findings are comparable to previously published reports in Egypt, China, Qatar and Kazakhstan [46,48,65,66].

The target for rifampicin action in \textit{Brucella} as well as in other bacteria is the beta-subunit of the DNA dependent RNA polymerase (RNAP) encoded by \textit{rpoB} gene [47,51]. In this study, mutations were identified in \textit{rpoB} gene associated with phenotypic rifampicin resistant \textit{Brucella} strains isolated from clinical specimens of animals in Egypt. Mutations were detected in all phenotypically resistant brucelae. Multiple and variable mutations were noted in each isolate along with few commonly shared mutations among many isolates. Frequent mutations at positions 676, 677-TAC to CTC (tyrosine to leucine, 38%) and 1435-AAG to CAG (lysine to glutamine, 23.8%) in the \textit{rpoB} gene of phenotypically resistant \textit{B. melitensis} were detected. These mutations are different from previously reported mutations (in vitro mutations) associated with rifampicin resistance in \textit{Brucella} [47].

Johansen et al. reported mutations in phenotypic rifampicin resistant or intermittently resistant \textit{B. melitensis} isolates [45], which in agreement with the findings of this study with additional mutations were detected as well as in intermediate rifampicin resistant \textit{B. melitensis}.

To the best of our knowledge, this study is the first report that proved mutations in the \textit{rpoB} gene of rifampicin resistant \textit{B. abortus} strains. Frequent mutations were detected at position 2890-CGT to GGT (arginine to glycine, 37.5%).

Fluoroquinolone/quinolone resistance in \textit{Brucella} is multifactorial by nature in addition to obvious mutations of the \textit{gyrA}, \textit{gyrB}, \textit{parC} and \textit{parE} genes [51,52]. In this study, the mutations in \textit{gyrA} and \textit{gyrB} genes in phenotypically resistant \textit{B. melitensis} and \textit{B. abortus} to ciprofloxacin were investigated. The mutations in \textit{gyrA} did not correspond with fluoroquinolone resistance mutations described by Turkmani et al. [49], although they investigated mutations in vitro selected fluoroquinolone resistant \textit{Brucella} mutants. The mutations in the \textit{gyrB} gene detected at positions 1141-AAG to GAG (lysine to glutamine), 1144-ATC to CTC (isoleucine to leucine) and 1421-TCA to TTA (serine to leucine) of \textit{B. abortus} were investigated. The mutations in \textit{gyrA} or \textit{gyrB} genes. However, the role of \textit{parC}, \textit{parE} and efflux systems cannot be ruled out for fluoroquinolone resistance [51] as we did not investigate the changes in \textit{parC} and \textit{parE} genes.

Genes responsible for resistance against chloramphenicol (\textit{catB}), gentamicin (\textit{Aac}) and tetracycline (\textit{tetA}, \textit{tetB}, \textit{tetM} and \textit{tetO}) were not detected in all investigated \textit{Brucella} isolated in this study, which in accordance with the phenotypic antimicrobial susceptibility results of isolated \textit{Brucella} isolates. It is also worth mentioning that all resistant \textit{Brucella} strains were isolated from animals and they showed resistance to antimicrobials clinically used in humans practice, suggesting that the source of these \textit{Brucella} strains may be of human origin. These findings point to the fact that inter-species and intra-host species \textit{Brucella} transmission is common, but spillback may occur also when chronic human brucellosis is mistreated and resistant strains are shedded [67]. A likely scenario would be the animal keeper interface.

The emergence of antimicrobial resistance (AMR) in bacteria is a public health issue globally and already compromises the treatment options regarding effectiveness of antimicrobials and control of several bacterial infections especially caused by gram-negative bacteria [68]. Wide spreading AMR in these bacteria is likely to persist and even worsen in future due to the uncontrolled use of antimicrobials. Rifampicin and ciprofloxacin are effective against intracellular bacteria like \textit{Brucella} [33]. Higher phenotypic resistance in \textit{Brucella} against these antimicrobials is likely to limits the treatment effectiveness, owing to the increased number of infections. Emergence of multidrug resistance \textit{Brucella} in livestock species in this study may pose serious threat to humans as these bacteria often transferred from animals to humans through food chain [69]. Being a zoonotic pathogen and given the emergence of increased antimicrobial resistance in \textit{Brucella} species, the situation with respect to hospital care may worsen and limits the treatment options in public health settings.
5. Conclusions

Brucellosis is a contagious and often communicable worldwide zoonosis with high morbidity and low mortality. There has been a tremendous increase in inter-host-species infection in the recent decades, especially in developing countries where farm animal species are kept on the same premises without biosecurity precautions. The disease is endemic in Egypt and B. melitensis and B. abortus have been reported as the main causative agents of brucellosis in humans and animals. High phenotypic resistance against ciprofloxacin, erythromycin, and imipenem were detected in Brucella spp. isolated from different districts and animals species reflecting a broad geographical distribution. The molecular identification of mutations in antimicrobial resistance associated genes highlight the mechanism of resistance in Brucella spp. There is a need for further insights into the epidemiology and spread of antimicrobial resistant Brucella in Egypt. The WHO regimes have to be reevaluated and awareness among physicians about AMR needs to be raised.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/12/603/s1, Table S1: List of primers and primer sequences used for detection of antimicrobial associated resistance mechanism.

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