Aus dem
Friedrich-Loeffler-Institut,
Bundesforschungsinstitut für Tiergesundheit,
Institut für Bakterielle Infektionen und Zoonosen, Jena

eingereicht über das
Institut für Tier- und Umwelthygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Molecular Epidemiology, Genotyping and Antimicrobial Susceptibility Studies on *Brucella* spp. Isolated from Livestock

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Aman Ullah Khan
Tierarzt aus Lahore, Pakistan

Berlin 2020
Journal-Nr.: 4243

mbvberlin | mensch und buch verlag

Aus dem
Friedrich-Loeffler-Institut,
Bundesforschungsinstitut für Tiergesundheit,
Institut für Bakterielle Infektionen und Zoonosen, Jena

eingereicht über das
Institut für Tier- und Umwelthygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Molecular Epidemiology, Genotyping and Antimicrobial Susceptibility Studies on *Brucella* spp. Isolated from Livestock

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Aman Ullah Khan
Tierarzt aus Lahore, Pakistan

Berlin 2020
Journal-Nr.: 4243

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Uwe Rösler
Zweiter Gutachter:	Prof. Dr. Heinrich Neubauer
Dritter Gutachter:	Univ.-Prof. Dr. Stefan Schwarz

Deskriptoren (nach CAB-Thesaurus):
Cattle, Buffaloes, Sheep, Goats, Camels, Pigs, *Brucella*, *Brucella abortus*, *Brucella melitensis*, Brucellosis, Serum, Antibodies, MALDI-TOF, Antimicrobial succeptibility testing, Multilocus sequence typing, Mulitlocus VNTR analysis, Core-genome single neucleotide polymorphism analysis, Virulence, Epidemiology, Microbiology, Egypt

Tag der Promotion: 06.10.2020

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <https://dnb.de> abrufbar.

ISBN: 978-3-96729-072-1
Zugl.: Berlin, Freie Univ., Diss., 2020
Dissertation, Freie Universität Berlin
D188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

To my parents

Sabir Khan

&

Mussarat Bibi

Table of contents

Table of contents

Table of contents

List of abbreviations

AMR	:Antimicrobial resistance
AMOS-PCR	:*Abortus*, *melitensis*, *ovis* and *suis* polymerase chain reaction
AUC	:Area under the curve
B.	:*Brucella*
BAPAT	:Buffered Acidified Plate Antigen Test
BC	:Before Christ
BCV	:Brucellae containing vesicle
BMT	:Bulk milk tank
BTWC	:Biological and Toxin Weapons Convention
CAMP	:Cationic antimicrobial peptide
CARD	:Comprehensive Antibiotic Resistance Database
CDC	:Centers for Disease Control and Prevention
c-ELISA	:Competitive enzyme linked immunosorbent assay
CFT	:Complement fixation test
cgMLST	:core-genome Multilocus Sequence Typing
CLSI	:Clinical and Laboratory Standards Institute
DNA	:Deoxyribose nucleic acid
DOR	:Diagnostic odd ratio
EGP	:Egyptian pound
ELISA	:Enzyme linked immunosorbent assay
erm	:erythromycin ribosome methylase genes
ER	:Endoplasmic reticulum
EUCAST	:European Committee on Antimicrobial Susceptibility Testing
FAO	:Food and Agricultural Organization
FAT	:Florescent Antigen-antibody Assay
FPA	:Fluorescent Polarization Assay
FMD	:Foot and Mouth Disease
GDP	:Gross Domestic Product
gyrA	:DNA gyrase subunit A
gyrB	:DNA gyrase subunit B
HPLC	:High Performance Liquid Chromatography
IFA	:Immunofluorescent Assay
i-ELISA	:Indirect ELISA
IL	:Interleukins
LAMP	:Loop-Mediated Isothermal Amplification
LFA	:Lateral Flow Assay
LPS	:Lipopolysaccharide
MALDI-TOF MS	:Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
mecA	:Methicillin resistance gene
MET	:Mercaptoethanol Test
mef	:Macrolide efflux genes
MIC	:Minimum Inhibitory Concentration
msr	:erythromycin resistance ATP-binding protein
MLST	:Multilocus Sequence Typing
MLVA	:Multiple Locus Variable Number of Tandem Repeats Analysis
MRT	:Milk Ring Test
MSAT	:Modified Standard Tube Agglutination Test
MprF	:Multiple peptide resistance factor
n.a.	:Not applicable
n.d.	:Not defined
NCBI	:National Centers for Biotechnology Information
NGS	:Next Generation Sequencing

List of abbreviations

OIE	:Office International des Epizooties
OMPs	:Outer Membrane Proteins
PAMPs	:Pathogen-Associated Molecular Pattern
PCR	:Polymerase Chain Reaction
R	:Rough
RBPT	:Rose Bengal plate agglutination test
ROC	:Receiver operating characteristics
RT-PCR	:Real Time Polymerase Chain Reaction
rpoB	:β subunit of bacterial RNA polymerase
S	:Smooth
SAT	:Serum Agglutination Test
SNP	:Single Nucleotide Polymorphism
Spp.	:Species
TAT	:Tube Agglutination Test
tet	:Tetracycline resistance protein
T4SS	:Type IV Secretion System
TLR	:Toll-like receptor
TNF	:Tumor Necrotic Factor
UN	:United Nations
µm	:Micrometer
µl	:Microliter
VNTR	:Variable Number of Tandem Repeats
WGS	:Whole genome sequencing
WHO	:World Health Organization

Introduction

According to the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the “Office International des Epizooties” (OIE), brucellosis is considered as the second most important zoonosis worldwide after rabies [1]. The disease is well controlled in some countries but still endemic in many others with the highest records in the Middle East and Central Asian regions including Egypt [2-4].

Brucellosis is caused by bacteria of the genus *Brucella* (*B.*) which are gram-negative bacterium, aerobic, non-spore-forming, facultative intracellular coccobacilli or short rods (0.6 to 1.5μm) in length and (0.5 to 0.7μm) in width [5]. For many years, six ‘classical’ species were identified largely based on distinct host specificity, pathogenicity, and phenotypic characteristics [6]. *Brucella abortus* (biovars 1-7) is causing infection in cattle and buffaloes, *B. melitensis* (biovars 1-3) in sheep and goats, *B. suis* (biovars 1-5) in pigs, hares, reindeer and rodents, *B. canis* in dogs, *B. ovis* in sheep and *B. neotomae* in rodents [6]. In the recent past, newly described species are causing infections in dolphins and porpoises (*B. ceti*), seals (*B. pinnipedialis*) and in voles and foxes (*B. microti*) [7-9]. Another new species (*B. inopinata)* was isolated from a human breast implant [10]. Recently, *B. papionis* was described from an isolate from baboons (*Papio* spp.) [11]. More recently, another new species (*B. vulpis*) was isolated from mandibular lymph nodes of red foxes (*Vulpes vulpes*) [12]. *Brucella* spp. infect not only their preferred hosts but also other domestic and wild animal species, which in turn can act as reservoirs of the disease for other animal species and humans [13-15]. Brucellosis in livestock is causing high economic losses to the livestock industry due to poor health, debility, and loss of quality livestock products [16]. In humans, it is severe acute, febrile illness that often becomes chronic if left untreated [17]. *Brucella melitensis*, *B. abortus*, *B. suis,* and *B. canis* are pathogenic to humans. Infection can be transmitted to humans through direct contact with infected animals, birth products, or animal discharges, and through consumption of contaminated milk, milk products, or meat [18]. There have been few case reports which highlighted human to human transmission possible through blood transfusion, bone marrow transplant, sexual intercourse, and breastfeeding to infants [18-20].

The diagnosis of brucellosis is still challenging and usually relies on serological tests [21] which are applied *in vitro* (milk or blood). Exceptionally, *in vivo* allergic tests are used. Isolation of brucellae and detection of *Brucella* DNA by PCR are the methods that allow definitive diagnosis [22]. *Brucella* species differentiation can be accomplished using AMOS-PCR and Bruce-Ladder (multiplex PCR) PCR [23,24]. The availability of new and advanced molecular detection and typing methods have contributed to improved laboratory diagnosis.

These molecular methods could serve as important alternatives to culture methods for the confirmation and may also provide valuable epidemiological tools to trace sources of infection [25]. However, with the development in molecular biology, whole genome sequencing (WGS) can provide more details for epidemiological investigations and typing of *Brucella* species. Therefore, molecular methods such as Multilocus Sequence Typing (MLST), Multiple Loci VNTR (Variable Number of Tandem Repeats) Analysis (MLVA), and more recently, whole-genome sequencing (WGS) typing methods are additionally used to discriminate between *Brucella* strains, to provide higher resolution genetic clustering, and to identify outbreaks [26-30].

The clinical management of brucellosis is of particular concern because of high initial treatment failure and relapse rates. Antimicrobial regimes with quinolones, doxycycline, rifampicin, streptomycin, and aminoglycoside alone or in combination are used to treat brucellosis [31]. Numerous reports regularly fail and on relapses of brucellosis, following therapy exists ranging from 5 to 15% in uncomplicated cases [32]. Recently, antimicrobial resistance in *Brucella* is emerging in brucellosis endemic regions of the world (e.g. Egypt, Qatar, Iran, Malaysia, and China) [32]. Antimicrobial resistance in zoonotic pathogens is an additional risk because it will limit disease treatment options in public health and veterinary settings [33]. None of the available studies highlights detailed antimicrobial susceptibility patterns of *Brucella* isolates from animals in Egypt.

Brucellosis is endemic in Egypt for thousands of years and is present with high prevalence in animals today [3]. Prevalences range from 2.47% to 26.66% in various livestock populations having great socioeconomic impact [34]. However, brucellosis is still underdiagnosed in various parts of the country challenging sero-surveillance programs. In Egypt, *B. abortus, B. suis*, and *B. melitensis* strains were isolated from livestock with high levels of phylogenetic variability within each species [35]. The incidence of human brucellosis is ranging from 0.28-95 per 100,000 inhabitants per year in Egypt [36,37].

Brucellosis in humans was reported in a scientific report from Egypt for the first time in 1939. Since then the disease remained endemic at high levels among cattle, buffaloes, sheep, and goats and is still representing a public health hazard. There are only a few seroprevalence reports of camel brucellosis in Egypt, as the disease has not received much attention. Although, brucellosis in pigs has not been reported in Egypt, a surveillance report using Rose Bengal Plate Agglutination Test (RBPT) was performed previously showing 12.6% seropositivity [38]. Moreover, the current epidemiological situation of prevailing *Brucella* species in livestock (especially in camels and pigs) needs classification. The global emergence of antimicrobial resistance in *Brucella* species urges surveillance and research

on antimicrobial resistance development in *Brucella* strains circulating in livestock species in Egypt.

Based on the previously mentioned information and available literature, the current study aimed to:

1. Gain insights into the current situation of brucellosis in farm animal species in Egypt.
2. Provide additional knowledge and update on seroprevalence of brucellosis in pigs reared in close contact with other animals and humans in Egypt.
3. Assessment of available serological assays for diagnosis of camel brucellosis and molecular identification of *Brucella* species in camels reared in Egypt.
4. Identification and differentiation of *Brucella* species isolated from brucellosis outbreaks in livestock species in Egypt.
5. Study the antimicrobial susceptibilities in *Brucella* isolated from animals against antimicrobials used in human medicine.
6. Study the molecular basis of antimicrobial resistance in phenotypically resistant *Brucella* strains.
7. Epidemiology and genotyping of *Brucella* strains isolated from livestock in Egypt based on whole-genome sequencing typing methods.

References

1. Schelling, E.; Diguimbaye, C.; Daoud, S.; Nicolet, J.; Boerlin, P.; Tanner, M.; Zinsstag, J. Brucellosis and q-fever seroprevalences of nomadic pastoralists and their livestock in chad. *Preventive veterinary medicine* **2003**, *61*, 279-293.
2. Pappas, G.; Papadimitriou, P.; Akritidis, N.; Christou, L.; Tsianos, E.V. The new global map of human brucellosis. *The Lancet. Infectious diseases* **2006**, *6*, 91-99.
3. Wareth, G.; Hikal, A.; Refai, M.; Melzer, F.; Roesler, U.; Neubauer, H. Animal brucellosis in egypt. *J Infect Dev Ctries* **2014**, *8*, 1365-1373.
4. Kirk, M.D.; Pires, S.M.; Black, R.E.; Caipo, M.; Crump, J.A.; Devleesschauwer, B.; Dopfer, D.; Fazil, A.; Fischer-Walker, C.L.; Hald, T.*, et al.* World health organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: A data synthesis. *PLoS medicine* **2015**, *12*, e1001921.
5. Liu, D. Brucella. In *Molecular medical microbiology*, Elsevier: 2015; pp 1781-1788.
6. Whatmore, A.M. Current understanding of the genetic diversity of brucella, an expanding genus of zoonotic pathogens. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* **2009**, *9*, 1168-1184.
7. Isidoro-Ayza, M.; Ruiz-Villalobos, N.; Perez, L.; Guzman-Verri, C.; Munoz, P.M.; Alegre, F.; Barberan, M.; Chacon-Diaz, C.; Chaves-Olarte, E.; Gonzalez-Barrientos, R.*, et al.* Brucella ceti infection in dolphins from the western mediterranean sea. *BMC veterinary research* **2014**, *10*, 206.
8. Hernandez-Mora, G.; Palacios-Alfaro, J.D.; Gonzalez-Barrientos, R. Wildlife reservoirs of brucellosis: Brucella in aquatic environments. *Revue scientifique et technique (International Office of Epizootics)* **2013**, *32*, 89-103.
9. Godfroid, J.; Scholz, H.C.; Barbier, T.; Nicolas, C.; Wattiau, P.; Fretin, D.; Whatmore, A.M.; Cloeckaert, A.; Blasco, J.M.; Moriyon, I.*, et al.* Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. *Preventive veterinary medicine* **2011**, *102*, 118-131.
10. Scholz, H.C.; Nockler, K.; Gollner, C.; Bahn, P.; Vergnaud, G.; Tomaso, H.; Al Dahouk, S.; Kampfer, P.; Cloeckaert, A.; Maquart, M.*, et al.* Brucella inopinata sp. Nov., isolated from a

breast implant infection. *International journal of systematic and evolutionary microbiology* **2010**, *60*, 801-808.
11. Whatmore, A.M.; Davison, N.; Cloeckaert, A.; Al Dahouk, S.; Zygmunt, M.S.; Brew, S.D.; Perrett, L.L.; Koylass, M.S.; Vergnaud, G.; Quance, C.*, et al.* Brucella papionis sp. Nov., isolated from baboons (papio spp.). *International journal of systematic and evolutionary microbiology* **2014**, *64*, 4120-4128.
12. Scholz, H.C.; Revilla-Fernandez, S.; Dahouk, S.A.; Hammerl, J.A.; Zygmunt, M.S.; Cloeckaert, A.; Koylass, M.; Whatmore, A.M.; Blom, J.; Vergnaud, G.*, et al.* Brucella vulpis sp. Nov., isolated from mandibular lymph nodes of red foxes (vulpes vulpes). *International journal of systematic and evolutionary microbiology* **2016**, *66*, 2090-2098.
13. Shoukat, S.; Wani, H.; Ali, U.; Para, P.A.; Ara, S.; Ganguly, S. Brucellosis: A current review update on zoonosis. *Journal of Immunology and Immunopathology* **2017**, *19*, 61-69.
14. Diaz Aparicio, E. Epidemiology of brucellosis in domestic animals caused by brucella melitensis, brucella suis and brucella abortus. *Revue scientifique et technique (International Office of Epizootics)* **2013**, *32*, 43-51, 53-60.
15. Godfroid, J.; Garin-Bastuji, B.; Saegerman, C.; Blasco, J.M. Brucellosis in terrestrial wildlife. *Revue scientifique et technique (International Office of Epizootics)* **2013**, *32*, 27-42.
16. Singh, B.B.; Dhand, N.K.; Gill, J.P. Economic losses occurring due to brucellosis in indian livestock populations. *Prev Vet Med.* **2015**, *119*, 211-215.
17. Godfroid, J. Brucellosis in livestock and wildlife: Zoonotic diseases without pandemic potential in need of innovative one health approaches. *Arch Public Health.* **2017**, *75*, 6.
18. Palanduz, A.; Palanduz, S.; Guler, K.; Guler, N. Brucellosis in a mother and her young infant: Probable transmission by breast milk. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* **2000**, *4*, 55-56.
19. Arroyo Carrera, I.; Lopez Rodriguez, M.J.; Sapina, A.M.; Lopez Lafuente, A.; Sacristan, A.R. Probable transmission of brucellosis by breast milk. *Journal of tropical pediatrics* **2006**, *52*, 380-381.
20. Lubani, M.; Sharda, D.; Helin, I. Probable transmission of brucellosis from breast milk to a newborn. *Tropical and geographical medicine* **1988**, *40*, 151-152.
21. El-Diasty, M.; Wareth, G.; Melzer, F.; Mustafa, S.; Sprague, L.D.; Neubauer, H. Isolation of *brucella abortus* and *brucella melitensis* from seronegative cows is a serious impediment in brucellosis control. *Vet Sci.* **2018**, *5*, 4.
22. Godfroid, J.; Nielsen, K.; Saegerman, C. Diagnosis of brucellosis in livestock and wildlife. *Croat Med J.* **2010**, *51*, 296-305.
23. Bricker, B.J.; Halling, S.M. Enhancement of the brucella amos pcr assay for differentiation of brucella abortus vaccine strains s19 and rb51. *Journal of clinical microbiology* **1995**, *33*, 1640-1642.
24. Lopez-Goni, I.; Garcia-Yoldi, D.; Marin, C.M.; de Miguel, M.J.; Barquero-Calvo, E.; Guzman-Verri, C.; Albert, D.; Garin-Bastuji, B. New bruce-ladder multiplex pcr assay for the biovar typing of brucella suis and the discrimination of brucella suis and brucella canis. *Veterinary microbiology* **2011**, *154*, 152-155.
25. Franco, M.P.; Mulder, M.; Gilman, R.H.; Smits, H.L. Human brucellosis. *The Lancet. Infectious diseases* **2007**, *7*, 775-786.
26. Al Dahouk, S.; Fleche, P.L.; Nockler, K.; Jacques, I.; Grayon, M.; Scholz, H.C.; Tomaso, H.; Vergnaud, G.; Neubauer, H. Evaluation of brucella mlva typing for human brucellosis. *Journal of microbiological methods* **2007**, *69*, 137-145.
27. Le Fleche, P.; Jacques, I.; Grayon, M.; Al Dahouk, S.; Bouchon, P.; Denoeud, F.; Nockler, K.; Neubauer, H.; Guilloteau, L.A.; Vergnaud, G. Evaluation and selection of tandem repeat loci for a brucella mlva typing assay. *BMC microbiology* **2006**, *6*, 9.
28. Sacchini, L.; Wahab, T.; Di Giannatale, E.; Zilli, K.; Abass, A.; Garofolo, G.; Janowicz, A. Whole genome sequencing for tracing geographical origin of imported cases of human brucellosis in sweden. *Microorganisms* **2019**, *7*, 398.
29. Georgi, E.; Walter, M.C.; Pfalzgraf, M.T.; Northoff, B.H.; Holdt, L.M.; Scholz, H.C.; Zoeller, L.; Zange, S.; Antwerpen, M.H. Whole genome sequencing of brucella melitensis isolated from 57 patients in germany reveals high diversity in strains from middle east. *PloS one* **2017**, *12*, e0175425.
30. Janowicz, A.; De Massis, F.; Ancora, M.; Camma, C.; Patavino, C.; Battisti, A.; Prior, K.; Harmsen, D.; Scholz, H.; Zilli, K.*, et al.* Core genome multilocus sequence typing and single nucleotide polymorphism analysis in the epidemiology of brucella melitensis infections. *Journal of clinical microbiology* **2018**, *56*.

31. Ariza, J.; Bosilkovski, M.; Cascio, A.; Colmenero, J.D.; Corbel, M.J.; Falagas, M.E.; Memish, Z.A.; Roushan, M.R.; Rubinstein, E.; Sipsas, N.V.*, et al.* Perspectives for the treatment of brucellosis in the 21st century: The ioannina recommendations. *PLoS Med.* **2007**, *4*.
32. del Pozo, J.S.G.; Solera, J. Treatment of human brucellosis-review of evidence from clinical trials. In *Updates on brucellosis*, InTech.: 2015.
33. Agunos, A.; Pierson, F.W.; Lungu, B.; Dunn, P.A.; Tablante, N. Review of nonfoodborne zoonotic and potentially zoonotic poultry diseases. *Avian Dis.* **2016**, *60*, 553-575.
34. Eltholth, M.M.; Hegazy, Y.M.; El-Tras, W.F.; Bruce, M.; Rushton, J. Temporal analysis and costs of ruminant brucellosis control programme in egypt between 1999 and 2011. *Transbound Emerg Dis.* **2017**, *64*, 1191-1199.
35. Menshawy, A.M.; Perez-Sancho, M.; Garcia-Seco, T.; Hosein, H.I.; Garcia, N.; Martinez, I.; Sayour, A.E.; Goyache, J.; Azzam, R.A.; Dominguez, L.*, et al.* Assessment of genetic diversity of zoonotic brucella spp. Recovered from livestock in egypt using multiple locus vntr analysis. *Biomed Res Int* **2014**, *2014*, 353876.
36. Dean, A.S.; Crump, L.; Greter, H.; Schelling, E.; Zinsstag, J. Global burden of human brucellosis: A systematic review of disease frequency. *PLoS Negl Trop Dis.* **2012**, *6*.
37. Pappas, G.; Papadimitriou, P.; Akritidis, N.; Christou, L.; Tsianos, E.V. The new global map of human brucellosis. *Lancet Infect Dis.* **2006**, *6*, 91-99.
38. Ashraf, M.; Hassan, A.; Raafat, M.; Fathia, A. Occupational health hazard of egyptian employees in contact with wastage nourished swine. *Journal of American Science* **2011**, *7*.

C H A P T E R 1.

Review of literature

Update on animal brucellosis in Egypt

1. Introduction

Brucellosis is an ancient disease and regarded as one of the most widespread, globally important zoonoses and is known as infectious or enzootic abortion, epizootic abortion, slinking of calves, ram epididymitis and Bang's disease in animals. The disease in humans was named Mediterranean fever, Malta fever, undulant fever, gastric remittent fever, Crimean fever, gastric fever, contagious abortion and Rock fever [1]. Brucellosis often induces chronic and incapacitating disease with low mortality [2]. Brucellosis has been stamped out in many developed countries (Europe, New Zealand, Japan, and Australia) but is still endemic in Africa, Middle East, Mediterranean countries, Asia, and Latin America [2-4]. New foci of human brucellosis have emerged mainly in Asia and particularly in Central Asia while the situation in certain countries of the near east (e.g., Syria) is rapidly worsening [5]. Brucellosis is endemic in livestock causing high economic losses by decreased milk production, abortion, weight loss, weak offspring, infertility, lameness, and even death due to acute metritis and retained fetal membranes. It also affects humans in terms of poor health and debility [6].

Brucellosis is caused by a bacterium called "*Brucella*" that may affect a range of mammals including humans, cattle, buffaloes, sheep, goats, pigs, rodents, and marine mammals [7]. The pathogen belongs to the CDC category B agents as it has characteristics of an agent for bioterrorism [2,8]. The pathogen is highly contagious and it has been revealed that only 10 to 100 bacteria would be enough to cause disease. CDC declared that it was the first biological weapon developed in the 1950s in the US [9,10].

1.1. The pathogen

The genus *Brucella* (*B.*) belongs to the *Brucellaceae* (family III), with *Mycoplana* and *Ochrobactrum*, of the order *Rhizobiales* in the class *α-Proteobacteria* of the phylum *Proteobacteria* [11]. The genera *Brucella*, *Bartonella*, *Rickettsia,* and *Ehrlichia* in these families cause infections in mammals [12,13]. Only the genus *Brucella* and *Bartonella* share common features of mammalian cell infection in the order *Rhizobiales*. However, major differences exist between *Brucella*, a facultative intracellular pathogen, and *Bartonella*, an obligate intracellular pathogen and the genome of *Brucella* spp. is 50–100% larger than that of *Bartonella* spp. [14]. The genus *Brucella* includes 12 highly genetically related species namely *B. abortus, B. melitensis, B. suis, B. ovis, B. canis, B. neotomae, B. ceti, B. pinnipedialis, B. microti, B. inopinata, B. papionis* and *B. vulpis* [15]. The organisms in genus *Brucella* share >90% homology in the DNA. This is why it has been considered a monospecific genus [16]. The genome of *Brucella* is very stable and contains two circular chromosomes of variable size depending upon *Brucella* spp. except for *B. suis* biovar 3 which contains a single chromosome [17]. There has been no evidence of

extrachromosomal DNA or plasmids in *Brucella* so far. Based on the susceptibility to lytic phages, *Brucella* is divided into six groups, which are important for typing and taxonomy [18]. Each species was named based on antigenic and biochemical characteristics and primarily its host species specificity [12].

Brucellae are tiny bacteria ranging from 0.5-0.7μm (width) to 0.6-1.5μm (length) in size, are intracellular and Gram-negative coccobacilli. Fresh cultures of brucellae appear as pinpoint, punctate, non-hemolytic, and non-pigmented colonies on agar plates. Colonies of smooth (S) *Brucella* strains are 0.5–1 mm in diameter, translucent, circular, and raised but become less convex and more opaque with a dull, dry, and yellowish-white granular appearance after sub-cultivation or prolonged culture (>4 days) [19]. These changes are caused by the dissociation of brucellae from smooth to rough (R) forms [20]. Colony morphology is termed as smooth and rough dependent on the structure of lipopolysaccharides (LPS) [21]. The smooth type has a complete structure of LPS (Lipid A, a core oligosaccharide, and an O-side sugar chain) while rough types lacks the O-side chain [21]. All brucellae except *B. ovis* and *B. canis* are smooth types [13]. Brucellae stain faintly with safranin. They are strict anaerobe. However, some strains require 5-10% carbon dioxide for their primary isolation. Brucellae are always catalase-positive but the production of oxidase, urease, and hydrogen sulfide is variable in different species [22] e.g. *B. papionis* does not produce H_2S [23]. Most of the brucellae especially *B. suis* and *B. ovis* can reduce nitrate to nitrite by producing nitrate reductase [19]. Phage susceptibility pattern, growth on dye (Thionin and Fuschin) containing media, agglutination with monospecific antisera (A, M, R) and production of H_2S discriminates brucellae at the biovar level [19,24].

2. Transmission

The source of brucellosis is usually an undiagnosed infected animal introduced into the herd without prior screening or accidental contact with an infected reservoir [25]. Chronically infected animals may spread the disease horizontally via milk and reproductive tract discharges [26] and vertically to their newborn calves [27]. Recently, unusual reservoir hosts e.g. dogs, cats, camels, equines, and wildlife were assumed to be potential sources of spillover infection to humans and animals and vice versa [6,28]. Scavengers might be reducing brucellosis transmission in natural conditions by removing contaminated birth materials [29]. *Brucella* spp. are highly communicable and can be spread by contact with aborted materials, discharges, semen, feces, hygroma fluids, and fomites of infected animals [30]. Transmission of brucellosis by vectors such as ticks, flies, fleas, and mosquitoes from infected to healthy herds has never been reported [31]. Humans can get the infection through direct contact with infected animals or materials and consumption of contaminated animal products like unpasteurized milk or milk products, liver, spleen, udder, kidney, and testes. Rare infections via breast milk, sexual intercourse, blood transfusion,

tissue transplantation or aerosols have been reported [32,33]. Brucellosis is also an occupational hazard to butchers, laboratory personnel, and farmers, along with veterinary medical practitioners [34]. Wounds may act as a portal of entry for the bacterium when in close contact with contaminated biomaterial [35]. A possible role of birds in the transmission of brucellosis remains unclear [36].

3. Clinical microbiology and pathobiology

Brucella species, in general, are host specific and classically cause infections in sheep and goats (*B. melitensis*), rams (*B. ovis*), bovines (*B. abortus*), canines (*B. canis*), pigs (*B. suis*), and rodents (*B. neotomae*) [37,38], terrestrial wildlife (*B. microti*) and marine mammals (*B. ceti* and *B. pinnipedialis*) [39]. *Brucella* species infect not only their preferred hosts but also other domestic and wild animal species, which in turn can act as reservoirs of the disease for other animal species and humans [40,41]. *Brucella melitensis, B. abortus, B. suis* biovars 1-4*,* and rarely *B. canis* cause infection in humans [42]. Brucellae may survive up to 100 days in fresh cheese, 30 days in ice cream, and up to 6 weeks in cream at 4°C [43]. *B. melitensis*, *B. abortus,* and *B. suis* cause considerable morbidity in countries where brucellosis persists in domestic animals [44].

Pathogenesis of brucellae remains cryptic as these microorganisms are believed to lack classical virulence factors e.g. capsule, fimbriae, flagella, exotoxins, endotoxic lipopolysaccharide (LPS), exoproteases, cytolysins, spore formation, plasmids, epitope shift or drift and even type I, II and III secretion system [45]. Lipopolysaccharides of the cell wall act as major virulence factors of brucellae which is different to the enterobacterial endotoxins of other Gram-negative bacteria such as *Escherichia coli*. Smooth LPS brucellae (*B. abortus, B. melitensis* and *B. suis*) tend to invade host cells more efficiently than rough LPS brucellae (*B. canis* and *B. ovis*) as their O-LPS chain facilitates entry, intracellular survival, and cell apoptosis evasion into the phagocytes [45,46]. Brucellae also get the advantage of a weak immune response by the host. Besides LPS, Outer Membrane Proteins (OMPs), phosphatidylcholine, a Type IV Secretion System (T4SS), BvrR/BvrS regulatory system, pathogen-associated molecular pattern (PAMPs) and cyclic-ß-(1-2) glucan are important factors for brucellae intracellular pathogenesis [47].

Usually, brucellae penetrate the mucosa of oral and nasal cavities after ingestion or through conjunctiva or skin abrasions [48]. After initial localization in lymph nodes, *B. abortus* infects the gravid uterus during bacteremia and multiplies in trophoblast which results in massive accumulation in the placenta [49]. Enrichment of placental tissue with erythritol along with progesterone may stimulate the growth of *B. abortus*. In males, it grows also in testes and causes orchitis and epididymitis [50]. The bacterium replicates preferably in phagocytic cells of the reticuloendothelial system and trophoblast cells of the placenta in pregnant animals

[1,51]. Brucellae can survive reactive oxygen intermediates and nitric oxide (NO) killing in host phagocytes following the prevention of phagolysosomal fusion by activation of bacterial genes thus remodeling the intracellular compartment and subsequently replicate intracellularly [21]. This can/may lead to latent and chronic infection [22]. They are then drained to the regional lymph nodes and are disseminated systemically into various predilection sites depending upon the host e.g. reproductive tracts of male and female animals, bones, joints and the nervous system in humans, the gravid uterus of pregnant ruminants and lymphoid tissues (e.g. spleen). At these locations, the bacteria provoke clinical signs e.g. abortion, placentitis and fever, and are secreted heavily with body secretions after abortion e.g. with the placenta but also with milk [45,52].

The vital element of immunity that marks the survival of the host and hence upkeep the chronic infective state is interferon-gamma (IFN-gamma), which is produced after interaction of bacterial cell wall components especially Lipid A with Toll-like receptors for the production of IL-12 and TNF-alpha [21]. Cell-mediated immunity plays a major role in recovery from brucellosis. However, innate and acquired humoral immunity also provide help to resist infection [22]. Acquired cellular resistance and hypersensitivity to *Brucella* may develop which contributes to some of the clinical outcomes of the disease [22].

Brucella melitensis, B. abortus, and *B. suis* cause abortion and infertility in animals [53]. Its case fatality rate is low and it can be persistent in subacute and chronic form. Human beings are susceptible in all age groups [41]. The persistent infection of the mammary gland and supra-mammary lymph nodes of carrier animals leads to permanent or intermediate shedding of the pathogen into milk throughout the lactation. *Brucella melitensis* is highly pathogenic to humans [54] and associated with acute illness whereas other species (*B. abortus, B. suis* and *B. canis*) predominantly cause subacute or chronic infection [55].

In female animals, the clinical signs are late-term abortion, stillbirth, infertility, low milk production, weight loss, lameness, and sometimes death in calves due to myocarditis. In addition, it can cause calving and breeding associated problems such as repeat breeding, metritis, retained fetal membranes, increased calving intervals, and weak infected calves [56]. In males, it can cause orchitis and epididymitis [57]. Other nonspecific signs include arthritis, bursitis, carpal hygroma, fistulous withers (in equines), fever and mastitis, loss in milk production, fertility, or even granulomas [56]. In humans, brucellosis is known to cause pyrexia of unknown origin [58]. Clinical manifestations of the disease include undulant fever, malaise, insomnia, anorexia, headache, arthralgia, constipation, sexual impotence, nervousness, and depression [59]. Musculoskeletal manifestations of brucellosis include sacroiliitis, spondylitis, arthritis, osteomyelitis, tenosynovitis, and bursitis [60]. Cutaneous involvement is infrequent with maculopapular rashes [61]. Due to the complexity of the clinical picture, human brucellosis still poses serious challenges to scientists and clinicians

across the globe regarding disease pathogenesis, diagnosis, and identification of the pathogen [62].

4. Potential risk factors

Host, environment, reservoirs, and management play an important role in the occurrence of brucellosis. Survival chances of *Brucella* are increased in an environment where aborted fetuses, fetal membranes and uterine discharges of infected animals are not disposed-off properly and are easy accessible to livestock, birds, and carnivores. The rearing of mixed livestock species with common watering and feeding points can facilitate disease spread [63]. Expansion of the animal industry, lack of hygienic conditions on the farm and improper food processing/handling make brucellosis a public health hazard. There is a risk of import to *Brucella* free regions due to international travel, import of dairy products and animals, and a risk of infection to slaughterhouse workers, hunters, farmers, and veterinarians [54]. Carrier animals can spread disease by contaminating the environment through reproductive material, feces, and shedding of the organism via milk [64]. Dogs, cats, and wild carnivores may act as mechanical disseminators by ingesting fetal and placental material, which was left undisposed [65]. Unvaccinated animals, herd size, population density, age, sexual maturity, and use of maternity pens are common factors, associated with the spread of brucellosis within the herd [66]. Brucellosis can be transmitted from herd to herd through the exchange of breeding stock and the introduction of newly purchased animals into the herd without quarantine [67,68]. Food consumption behavior, sanitation, occupational exposure, season, and health status of individuals are associated with the dissemination of brucellosis in human beings [69].

5. Diagnosis

The development of an accurate diagnostic tool for brucellosis is a challenging task. In field conditions, the most imperative diagnostic tools without laboratory aid are reports on history by the owner and clinical signs. Epidemiological surveillance, accurate, and correct diagnosis are the key features for effective control and eradication of brucellosis [4]. The diagnosis of brucellosis is still challenging and usually relies on serological tests [70], applied *in vitro* (milk or blood) or *in vivo* allergic tests. Isolation of *Brucella* spp. and detection of *Brucella* spp. DNA by PCR is the method that allows definite diagnosis [71]. Although confirmation of the disease is achieved by culture and isolation, *Brucella* is difficult to grow and culture is time-consuming. Additionally, this method poses a risk to laboratory personal and requires specific biosafety measures [72]. It is always recommended to use a combination of two or more techniques to avoid false negative/positive results [73,74].

5.1. Serological diagnosis

Serology remains a practical and the preferred method, as advanced laboratories are not ubiquitously available [55]. Under field conditions, animal screening may be performed using milk ring test (MRT), Rose Bengal plate agglutination test (RBPT) and serum agglutination test (SAT), while confirmation of the disease can be achieved by using enzyme-linked immunosorbent assay (ELISA) and complement fixation test (CFT) in the laboratory [75]. Evaluation of several serological tests for the diagnosis of brucellosis reveals high sensitivity and specificity for ELISAs. These tests can be used for screening and confirmation [76]. ELISAs are efficient, require little laboratory equipment and technical skills. Various ELISAs are used to diagnose the disease and important tests in the trade [77]. Sometimes cross-reactions to *Yersinia* LPS interfere with the diagnosis of brucellosis when i-ELISAs are used. To avoid this shortcoming c-ELISAs have been developed but loosing sensitivity [71]. Florescent Antigen-antibody assay (FAT) correlates well with other tests but requires specialized equipment and is relatively complicated to perform and interpret [78]. Fluorescent Polarization Assay (FPA) is another very specific test being very popular. It can be used for serum and milk sample analysis [79]. Milk ELISA is an efficient, sensitive, and specific method, especially for larger herds. Individual animals can be tested after positive bulk milk tank (BMT) analysis. Milk Ring Test (MRT) is a suitable alternative for testing bovine milk in the absence of milk ELISA [80]. Lateral Flow Assay (LFA) is easy to perform, sensitive and is a likely alternative to ELISAs and Coombs test in humans and probably in animals [81]. However, no test developed to date ensures 100% accuracy. Generally, serological diagnosis consists of testing sera by a screening test of high sensitivity followed by a confirmatory test of high specificity [75].

5.2. Bacteriological and biochemical assays

Bacterial isolation out of clinical specimens and identification of the causative agent remains the “gold standard” (with biochemical tests like CO_2 requirement, H_2S production, and dye sensitivity) [24]. Urease, oxidase, and catalase tests are also used for the identification of *Brucella* spp. [19,24]. Isolation is less than 20% sensitive due to secondary bacterial contaminants [78]. It needs advanced levels of biosafety, biosecurity, and training as live cultures may be disseminated through aerosols and may cause laboratory-acquired infections [82]. A comparatively new method, Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) emerged for microbiological identification [83]. It is an economical, easy, rapid, and accurate method based on automated analysis of the mass distribution of bacterial proteins [84]. A recently published study indicates that MALDI-TOF mass spectrometry can identify 99.5% and 97% of *Brucella* strains at the genus and species level, respectively, minimizing laboratory hazards of

Brucella infection. However, there are some limitations at sub-species level identification [85].

5.3. Molecular assays

Definite diagnosis of brucellosis is the recovery of causative agent from the host. But inherent problems with bacterial isolation like inefficiency, cost, and personal risk forced most of the laboratories to use safer and more cost-effective methods [86]. Molecular methods, currently accessible for most clinical laboratories, enable easy and rapid diagnosis of brucellosis without any risk to laboratory personnel while handling live bacteria [87]. Molecular detection, especially Polymerase Chain reaction (PCR) has proven to be more sensitive than culture and more specific than serology [82]. Depending on the purpose of the test, primers, dyes (e.g. SYBR Green and TaqMan® Probes) and laboratory conditions have been developed for both conventional and real-time PCR and Loop-mediated Isothermal Amplification (LAMP) [88-90]. Specific molecular targets allow rapid identification of *Brucella* species [91]. PCR-based assays are also useful in chronic patients, with low number of circulating bacteria and consequently often negative blood cultures [92]. Evaluation of PCR with serological assays gives results in nearly 100% sensitivity [93].

5.4. Genotyping of *Brucella* spp.

The species *B. melitensis*, *B. abortus*, and *B. suis* are further subdivided into biovars by phenotypic characteristics: Techniques used are serotyping, phage typing, sensitivity to dyes, or metabolic profiles [94]. These classic biotyping techniques have less discriminating power, pose personal risks, and are available in reference laboratories only [95]. Real-time PCR (RT-PCR) assays based on single nucleotide polymorphism (SNP) were developed for the rapid typing of *Brucella* isolates providing a solid basis for genotyping. Single nucleotide polymorphisms (SNPs) have been described and SNP assays were validated [96,97]. A robust SNP assay was also used to identify *Brucella* isolates at species level [98]. However, DNA-DNA hybridization confirms that *Brucella* species are highly genetically related (more than 90% relatedness of DNA) [14]. Thus, molecular epidemiology and typing of *Brucella* could be challenging owing to low genetic variations in its genome [99].

Recently, with the availability of reference genome sequences, various molecular typing methods have been developed. The most commonly used today are Multilocus Sequence Typing (MLST) and Multiple Loci VNTR (Variable Number of Tandem Repeats) Analysis (MLVA) [100,101]. MLVA based typing method has been applied on large collections of *Brucella* spp. worldwide [102]. Multi-locus sequence typing (MLST) is a discriminatory method of characterizing bacterial isolates based on the sequences of fragments of seven

housekeeping genes. MLST can also be a useful tool to identify specific genetic markers for diagnosis and differentiation [103]. Both, MLST and MLVA have proved to be useful to assess the genetic diversity and to identify and classify newly emerging *Brucella* strains [95]. However, core-genome multilocus sequence typing (cgMLST) based on whole-genome sequencing (WGS) has shown higher phylogenetic distance resolution compared to MLVA [104]. The combination of WGS and SNPs analysis for *Brucella* has higher resolution when compared to common typing approaches like MLVA [105].

Whole-genome sequencing provides the most comprehensive collection of an individual's genetic variation [106]. WGS based on next-generation sequencing technology adds an important method to complement epidemiological investigations in outbreak investigation and can provide high quality resolution in discriminating even highly genetically related bacteria like *Brucella* [104,105]. WGS is superior to conventional genotyping tools for studying outbreaks, geographical distribution, and newly emerging agents [107]. Next-generation sequencing technology gained popularity in disease diagnostics [108], therapeutics [109,110], antimicrobial susceptibility testing, and disease treatment [111]. WGS based sequencing has been successfully used to identify, genotype, in outbreak investigations, and antimicrobial resistance determination in *Brucella* species [104,112].

6. Prevention and control

The killing of the bacterium through effective heating is one of the methods to control the transmission of brucellosis. Timely and accurate diagnosis of infected animals and their isolation may control the spread. Thirdly, active immunization with available effective vaccines can protect bovine, ovine, and caprine herds from economic loss but not from infection [51]. The disease can be best prevented by eliminating all possible ways to introduce the infection in animal herds i.e. keeping the herd *Brucella* free by introduction of disease-free animals and insemination with *Brucella* free semen. Restriction of animal movement and quarantine measures in brucellosis endemic region is adviced. Vaccination of animals (preferably ≤1-year-old) with vaccine strains *B. abortus*-S19 (smooth form) and RB51 (rough form) in bovines and Rev-1 of *B. melitensis* in small ruminants may protect animals from symptom development [24,113]. In animal herds, where prevalence is low i.e. <2%, test and slaughter policy is effective for eradication of the infection [114,115]. So far, there is no licensed vaccine available for humans [116].

7. Treatment in humans

The intracellular location of brucellae in reticuloendothelial cells and their predilections sites (e.g. bones) limit the penetration of most of the antibiotics. Antimicrobial regimens with doxycycline, rifampicin, streptomycin, quinolones, and aminoglycoside alone or in

combination are used to treat brucellosis [117]. The combination of doxycycline with either aminoglycosides or rifampicin is a good choice to treat brucellosis [118]. Contraindications of tetracyclines (e.g. in pregnant women and children) may limit the treatment of acute brucellosis [119]. Regularly fail and numerous reports of relapses of brucellosis following therapy exist ranging from 5 to 15% in uncomplicated cases [120]. Relapses may be attributed to the delayed and inefficient treatment or development of antimicrobial resistance in brucellae against rationally used antimicrobials. The antimicrobial resistance in *Brucella* is emerging in endemic regions of the world (e.g. Egypt, Qatar, Iran, Malaysia, and China) [120]. Resistance to commonly used antimicrobials is mediated by mutations of *rpo*B gene (rifampicin), *gyr*A, *gyr*B, *par*C, *par*E genes (quinolones), *erm, mef, msr* (macrolides) or *tet* genes (tetracyclines), *mec*A (beta-lactams) and *fol*A (trimethoprim) [121]. Mutations in the *rpo*B and *gyr*A genes may occur naturally or can be induced *in vitro.* These mutants are phenotypically resistant to rifampicin/rifampin and quinolones [112,122,123].

8. Global epidemiological situation

Annually, more than 500,000 cases of brucellosis are emerging worldwide [5] and there are an estimated 2.4 billion people at risk [124]. Although its geographical incidence and distribution are limited by effective public and animal health control strategies, the prevalence of the disease varies in different countries of East Asia, South Central Asia, South East Asia, Western Asia, and the Middle East, Africa, the Mediterranean region and Latin America. It has been eradicated from many developed countries like northern, western, and central parts of Europe, some Asian and American countries, Australia, and New Zealand [2,5,125]. Overall, the frequency of brucellosis is higher in more agrarian societies and in places where handling of animal products and dairy products is less stringent. Incidence rates of 1.2-70 cases per 100,000 persons are reported (https://emedicine.medscape.com/) [2,5]. Brucellosis is a great risk for public health regionally and globally. Human brucellosis is found mostly in countries where animal brucellosis remains endemic or in the non-endemic countries where people returning from endemic areas after exposure [56,125,126]. According to the World Health Organization (WHO), 109,308,966 cases and 156 outbreaks are reported in South Asia. At present, 1-200 new human cases are emerging per 10 million individuals annually [127]. In humans, brucellosis is a non-sustainable disease and its source always resides in domestic or wild animal reservoirs [128].

9. Brucellosis in Egypt

At present, cattle and buffalo milk production in Egypt is ranked 11th worldwide [129]. The annual cattle import has been increased and it is estimated that around 36% cattle were

imported during the year 2019 [130]. At present, 27% of livestock-keeping households of which 21% are large animal producers, contributing about 40% to the agricultural GDP of Egypt. Livestock population includes cattle (4.9 million), buffaloes (3.7 million), sheep (5.46 million), goats (4.04 million), camels (1.52 million), asses (1.45 million) and horses (0.07 million) [131]. More than 50% of these animals are owned by poor livestock farmers [131]. More than 70% of households keep cattle and buffaloes in smallholdings mainly with a herd size of 2 or 1 animals. They face increasing challenges to improve their productivity [129,131,132]. The expansion in the livestock industry and import will challenge the disease spread and its control by vaccination [130]. Brucellosis incidence in humans reached 5.17% in 2016, with a considerable burden for both, farmers' livelihood and public health [131]. A serological survey conducted on domestic and imported animals in central Egypt categorized brucellosis among the top four zoonotic diseases [133]. *Brucella melitensis* and *B. abortus* have been identified in most countries in the Middle East [66].

Brucellosis is likely to be endemic disease in Egypt for thousands of years with high prevalence in animals [134]. Although brucellosis in Egypt has been reported since 1939 [129,135], it gained attention during the 1960s, as with import of cattle the incidence of *Brucella* infection became very high (38%) on some farms [136]. The average prevalence ranges from 2.47% to 26.66% in various livestock populations having a great socio-economic impact [137]. One study also reported the occurrence of anti-*Brucella* antibodies from rats (8%) present at cattle, sheep, and goat farms [138]. Sporadic cases of high prevalence have also been reported from various governorates in the country. A study conducted in seven governorates of Upper Egypt estimated that 0.2% of every household keeps at least one seropositive animal [139]. Samaha *et. al*. reported 4.48-4.98%, 3.37-3.52%, 4.8% and 2.19% seropositive cattle, buffaloes, sheep, and goats, respectively, in a multicentral study conducted in Beni-suef, Monofia, Qaliobia, Alexandria, Behera, Giza, and Assuit governorates indicating that Beni-suef had the highest number of seropositive animals [140].

Some studies identified the circulating *Brucella* spp. and biovars i.e. *B. melitensis* biovar 3, *B. abortus* biovar 1, and *B. suis* biovar 1 in Egypt [141-143]. *Brucella abortus* bv1 was identified from dogs and cats living in close contact with ruminants [65,144]. *Brucella melitensis* bv3 was also identified from freshwater Nile Catfish in Egypt [145]. *Brucella melitensis* bv3 and *B. abortus* bv1 were isolated from sheep and goats [142-144]. Although one study reported the identification of *B. abortus* DNA from camel milk [146], reliable data regarding camel brucellosis and circulating *Brucella* spp. in the camel population is scarce.

The presence of anti-*Brucella* antibodies was investigated using Buffered Acidified Plate Antigen Test (BAPAT), Rose Bengal Test (RBT), Rose Bengal plate test (RBPT), EDTA modified standard tube agglutination test (MSAT), Mercaptoethanol Test (MET), Tube Agglutination Test (TAT), Rivanol Test, Complement Fixation Test (CFT) and Enzyme-Linked Immunosorbent Assays (ELISA) in animals [70,134,143,147]. Fewer studies highlight the molecular identification and differentiation of *Brucella* spp. using *Brucella* genus-specific PCR, Bruce-ladder PCR, AMOS-PCR and MLVA analysis [65,141-144]. Some researchers have also used culture, Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF), and loop-mediated isothermal amplification (LAMP) for *Brucella* identification [70,141,148,149]. Real-time PCR detection identified *B. abortus* and *B. melitensis* circulating in diseased and apparently healthy animals [150].

Incidence of human brucellosis is ranging from 0.28-95 per 100,000 inhabitants per year [5,151]. *Brucella melitensis* predominately infects humans and was isolated from human blood samples [152]. A study revealed that 21% of human sera from patients with febrile illness were positive for brucellosis [129]. The predominance of smallholdings and rearing of mixed animal species are among the highlighted risk factors for the spread of brucellosis in the animal population [4,132,153]. Significantly, a higher prevalence was found in female animals [154]. Consumption of unpasteurized milk and dairy products, occupational contact with animals, and missing knowledge about the disease are risk factors associated with infection in humans [66,129,155,156]. Consumption of white cheese made from unpasteurized milk and the homemade Kareish cheese, native and famous in Egypt, can be a potential source of *Brucella* infection [129].

9.1. Brucellosis in cattle and buffaloes

Brucellosis in cattle and buffaloes is primarily caused by *B. abortus*, less frequently by *B. melitensis* and only occasionally by *B. suis* [24,157]. *Brucella melitensis* can cause bovine brucellosis when cattle is in close contact with infected sheep and goats [158,159]. Occasionally, *B. suis* may cause a chronic infection of the mammary gland of cattle, but it has not been reported to cause abortion or spread to other animals [24,160]. Clinically, bovine brucellosis is characterized by reproductive loss in terms of abortion, weak offspring, infertility, reduced milk production, and localization of the bacterium in the mammary glands that may result in mastitis. Occasionally, *Brucella* settles in joints and bones, and the male reproductive organs resulting in inflammation and other pathological lesions [24,161]. *Brucella* infection in non-pregnant cattle is likely to result in infection of the udder and excretion in milk [136].

In Egypt, *B. melitensis* is the predominant *Brucella* spp. causing infection in cattle and buffaloes [132] followed by *B. abortus* [142,162]. *Brucella abortus* was recovered from cattle as early as in the 1940s, and *B. melitensis* in 1970s [136]. Refai *et. al*., recovered *B. abortus* biotype 3 and 7, and *B. melitensis* biotype 3 from cattle and *B. abortus* biotype 3 from buffaloes [163]. Another study from Upper Egypt isolated *B. melitensis* biotype 3 from cattle [164]. Implementation of test and slaughter policy along with *B. abortus* S19 vaccination considerably reduces the *B. abortus* infection in bovines. However, efforts to control small ruminant brucellosis have been less intensive resulting in an increase of infections by *B. melitensis* in livestock [4]. *Brucella abortus* RB51 vaccine may cause abortion in cattle [141,165]. *Brucella suis* was isolated from cattle in Egypt suggesting a potential role of livestock as reservoir of several zoonotic *Brucella* species in the region [142]. *Brucella* spp. (*B. melitensis, B. abortus,* and *B. suis*) were isolated from milk, lymph nodes, spleen, and aborted fetuses of infected cattle and buffaloes from various regions in Egypt [142,143]. *Brucella melitensis* bv 3 and *B. abortus* bv 1 were also recovered from the uterine discharge of seronegative cattle [70]. *Brucella melitensis* bv 3 and *B. abortus* bv 1 were recovered from buffaloes in a study conducted on different farms from Upper Egypt [166]. Two studies from the same governorates conducted on a variety of samples collected from cattle showed the endemicity of *Brucella* species. *Brucella melitensis* bv 3 was isolated from 36% of specimens (supra-mammary lymph nodes, spleen, uterus, and mammary glands) of slaughtered animals and blood samples of cattle from private farms in Sharqia governorate [167]. Molecular identification and culture recovered *B. abortus* bv 1 from milk samples collected from Sharqia governorate [129].

Seroprevalence of bovine brucellosis ranges from 2.91% to 75.2%. The highest prevalence was recorded in cattle (32.8%, 32.8%, 37.9%-61.8% and 59.0%) compared to buffaloes (7.6%, 7.6%, 10.2-22.2% and 18.5%) by RBPT, Riv.T, TAT and MRT, respectively [163]. It has also been observed that cattle and buffaloes which were raised together with sheep and goats had 6.32 times the odds for testing seropositive for *Brucella* antibodies than raised alone [132]. A study conducted on apparently healthy animals from Monufia, Qalyubia, and Sharqia governorates of the Delta region showed 16% seropositive (milk i-ELISA) animals, and found *B. abortus* and *B. melitensis* DNA (RT-PCR) in these samples suggesting the shedding of brucellae in milk [150]. In Beni Suef Governorate from Upper Egypt, the incidence of brucellosis using BAPAT, RBPT, and CFT was 8.71%, 8.36% and 8.36% in cows and 8.23%, 7.86%, and 7.68% in buffaloes, respectively [168]. In Faiyum Governorate, seropositivity in cattle was 18.5 %, 14.8%, and 21.48% while in buffaloes it was 14.5%, 12.7%, and 5.5% by ELISA, SAT, and MPCR, respectively [169]. The incidence of brucellosis was 10.23% in cattle and 2.91% in buffaloes from animals at different

slaughterhouses in Asyut Governorate [170]. Another, seroprevalence study conducted on bovines at slaughterhouses in Asyut governorate revealed 2.14-4.0% seropositive animals [171]. 6% of cattle were found seropositive using RBPT in the Alexandria governorate [154]. Incidence of brucellosis in cattle and buffaloes was recorded to be 8.4% and 6.1%, respectively in Kafr El-Sheikh governorate [172]. Milk indirect ELISA conducted on milk samples of cattle and buffaloes taken from milk tanks in 40 villages at Kafr El-Sheikh governorate revealed 12.2% cattle and 12.0% buffaloes milk samples positive for anti-*Brucella* antibodies [153]. In another study, 7% of cattle were found seropositive by milk indirect ELISA in Kafr El-Sheikh governorate [173]. Buffaloes at Ismailia dairy farm were tested by Rose Bengal plate test (RBPT), Tube Agglutination Test (TAT), Mercaptoethanol Test (MET), Complement Fixation Test (CFT) and ELISA with 16.6 %, 11.76 %, 12.74%, 11.76 % and 13.72 % seropositive, respectively [174]. Seroprevalence in abattoir animals from Cairo and Ismailia governorates showed exceptionally high seropositivity of 53.9% and 75.2% by ELISA and RBPT, respectively [175]. At a buffalo farm in Ismailia governorate, 10.53% animals were found seropositive [176]. Prevalences of buffaloe brucellosis at 10 governorates in Upper Egypt were 34.7%, 37%, 28.2%, 23.7%, 32.3%, 33.8%, 8.20% by RBT, BAPA, Riv.test, modified SAT, indirect ELISA, AGPT and MRT, respectively [177]. A study conducted on private cattle farms in Sharqia governorate showed 4.42% and 8.91% seropositive animals at farms and individual level, respectively [167]. Another study from Sharqia governorates found 23.8% seropositive cattle [129]. In a study from Gharbia governorate, 5% buffaloes and 12% cattle were found positive for *Brucella* antibodies [155]. A comprehensive serological examination for detection of anti-*Brucella* antibodies by Rose Bengal test, Buffered acidified plate antigen test and Tube agglutination tests revealed that 17.8%, 8.9%, and 11.8% cattle were positive in Dakahlia, Damietta and Alexandria Governorates, respectively [178]. 13.3-16.6% cattle and 0-6.6% buffaloes were found positive for *Brucella* antibodies around El-Salam canal in North Sinai governorate [179]. A recent study comprehensively identified 16.7% anti-*Brucella* antibodies using i-ELISA in cattle from Alexandria, Gharbia, Suez, Monufia, and Qalyubia governorates [162].

9.2. Brucellosis in sheep and goats

Brucellosis in small ruminants is usually caused by *B. melitensis* [180,181]. *Brucella abortus* also causes infection in sheep and goats, particularly when raised with cattle [157,182]. Few sporadic infections with *B. suis* have been reported, but these cases are induced very rare [24,183]. Sharing of the same pasture, mixing of farm animal species and uncontrolled movement may spread infection outside its preferred hosts [182] and this trend has been observed [140]. Clinically, brucellosis in sheep and goats is very similar to *B. abortus* infection in cattle [24,184]. *Brucella ovis* causes infection in sheep characterized by genital

lesions and epididymitis in rams, placentitis, and rare abortions in ewes, and neonatal mortality in lambs [185]. Brucellosis epidemiology in small ruminants is complex because several extrinsic factors (flock management, ecological conditions, and socio-economic factors) play an important but poorly defined role [181].

Probably, *Zaki* (1948) published the first ovine brucellosis report in Egypt [186]. Researchers from Upper Egypt isolated *B. melitensis* biotype 3 from sheep and goats [164]. *Brucella melitensis* biovar 3 was isolated from sheep and goat milk [146]. *Brucella abortus* and *B. melitensis* strains were isolated from infected sheep and goats from tissue samples (lymph nodes, spleen, and liver) and aborted fetal stomach contents and genotyped using molecular assays [143,149,182]. Molecular assays identified *B. abortus* DNA in clinical specimens of infected sheep from Alexandria, Gharbia, Suez, Monufia, and Qalyubia governorates [162].

Few seroprevalence studies are available but 3.0-29.3% brucellosis cases were reported in sheep and goats. A study reported 26.66% and 18.88% prevalence of brucellosis in sheep and goats, respectively [187]. A diagnostic comparison study showed that 29.3%, 27.0%, 28.7% and 28.3% sheep blood samples were positive using Rose Bengal test (RBT), Serum Agglutination test (SAT), ELISA using both whole *Brucella* antigen (W-ELISA) and the periplasmic protein antigen (P-ELISA), respectively [188]. In Kafr El-Sheikh governorate, the cumulative incidence of brucellosis was higher in sheep (12%) than goats (6.4%) using RBPT, BPAT, ELISA, and CFT [172]. In another study, 12.2% of sheep and 11.3% of goats using RBPT and CFT were seropositive from Kafr El-Sheikh governorate, in the Nile Delta [153]. *Brucella melitensis* biovar 3 was identified in seropositive (3.0-5.0% by BAPAT, RBPT, TAT, SAT, CFT, and i-ELISA) Baladi goats by bacteriological and molecular assays in Kafr El-Sheikh governorate [189]. A comprehensive study focusing on knowledge, attitudes, and practices (KAPs) of sheep farming in brucellosis endemic regions at Kafr El-Sheikh governorate identified 17.95% seropositive sheep using RBPT [156]. In the Alexandria governorate, seroprevalence was 6% and 7% in sheep and goats, respectively [154]. A study conducted on various abattoirs located at different localities in Asyut governorate revealed anti-*Brucella* antibodies in 7.61% (sheep) and 5.08% (goats) when tested by BAPAT, RBPT, SAT, Rivanol test and ELISA and *B. melitensis* biovar 3 from spleen and lymph node was isolated [170]. The overall seroprevalence of brucellosis using RBPT was 15.87% in sheep from Asyut and Minya governorates in 2017 [190]. 8.5-20.7% sheep and 8.5-11.1% goats were found positive in Gharbia governorate [138,155]. Anti-*Brucella* antibodies were detected in 4-6% sheep and 8-12% goats from North Sinai governorate using various serological diagnostics [179]. A study on 2,883 serum samples collected from ewes and rams at sheep farms reported with sudden onset of abortions in Beheira governorate identified 12.1% cumulative sero-reactors using BAPAT, modified RBT, SAT,

CFT, and i-ELISA and isolation of *B. melitensis* biovar 3 from samples of spleens, supramammary lymph nodes and livers from aborted sheep [191]. A comprehensive study in Matruh governorate identified 11% and 10.56% seropositive sheep using RBPT and CFT, respectively [192]. A recent study comprehensively identified 16.5% anti-*Brucella* antibodies along with isolation and molecular identification of *B. abortus* DNA from clinical specimens of infected sheep from Alexandria, Gharbia, Suez, Monufia, and Qalyubia governorates [162].

9.3. Brucellosis in camels

Brucellosis in camels was first reported in 1931 [193]. Since then it has been reported from all camel rearing countries like Sudan, Ethiopia, Somalia, Kenya, Nigeria, Jordan, and Egypt, except Australia [194]. Camels are not primary host for *Brucella*, but they are susceptible to *B. abortus*, *B. melitensis* and *B. ovis* [147,195]. Published studies have shown that *B. abortus* and *B. melitensis* were fairly often isolated from infected camels [196]. Camels attract the infection by sharing pastures and drinking water with infected cattle, sheep, and goats [147,197,198]. Brucellosis in camels hardly provokes clinical signs, but symptoms may vary from i.e. abortion, retention of fetal membranes, weak offspring, impaired fertility and delayed sexual maturity in females and orchitis accompanied by lameness in males [147,193,199].

Brucella melitensis biovar 3 was isolated from camels in Egypt [147]. *Brucella abortus* serotype 1 and 7, and *B. melitensis* serotype 3 were recovered by bacteriological examination [200]. *Brucella melitensis* DNA was also amplified using PCR from camel milk [146]. *Brucella melitensis* biovar 3 was identified using Bruce-ladder PCR in samples from camel milk [197]. *Brucella melitensis* biovar 3 was isolated from the stomach content of an aborted camel fetus [195]. Biochemical and molecular assays also identified *B. abortus* infecting camels in Egypt [201]. However, detailed and comprehensive studies on the molecular characterization of *Brucella* species particularly *B. abortus* biovars circulating in camels in Egypt are lacking.

Previous reports from different regions have revealed seroprevalences of camel brucellosis ranging from 1.0 to 26.7% [147,198, 202]. Zaki in 1948 reported 20% seropositive camels using SAT for the first time in Egypt [186]. Salem *et. al.* (1990) reported the occurrence of 23.3% anti-*Brucella* antibodies using TAT in camel samples [203]. In camels, the prevalence in the Shalateen region of the Red Sea governorate was found to be 12.9%, 11.6%, and 11.5% using RBPT, BABAT, and CFT, respectively [195]. One study from the regions of Siwa Oasis, Asyut, and Cairo reported prevalences of 4.17% and 3.73% using RBPT and c-ELISA, respectively [197]. A similar study from Beheira district reveals prevalences of

8.74%, 9.53%, 9.92%, 8.09%, 8.87%, and 9.26 % using Rose Bengal test, Buffered acidified plate test, Tube agglutination test, Mercaptoethanol test, Rivanol test, and ELISA, respectively [204]. A comprehensive study conducted at slaughterhouses at different localities in Cairo, Giza, El Sharkyia, El Behira, and Matroh governorates revealed 9.5%, 8.8%, and 7.7% seropositive camels using BAPT, mRBPT, and TAT, respectively [201].

9.4. Brucellosis in pigs

Typically, swine brucellosis is caused by *B. suis* biovars 1-3 [205]. Sporadic cases of infection caused by *B. abortus* [206, 207] and *B. melitensis* [157, 208] were also reported in pigs. Hence, *B. abortus* [206, 207] and *B. melitensis* [157, 208] were isolated from pigs when kept together with infected ruminants and camels. Porcine brucellosis may be a serious, but presently unrecognized, a problem in Egypt. Brucellosis in pigs clinically results in abortion at any stage of gestation resulting in chronic lesions of the reproductive tract leading to infertility [209]. In males, it causes inflammation of the testes and joints with abscess formation [24].

Production of pigs in Egypt is found primarily in slums, rural, and peri-urban areas, especially in Cairo and Giza governorates. Christians, foreigners, and tourists to Egypt consume pork. Currently, the pig population is around two to three millions [210, 211]. In Egypt, pigs are kept in small groups in contact with other farm animals and humans [212]. However, brucellosis in pigs has not been noted in Egyptian surveillance reports. A Rose Bengal plate agglutination test (RBPT) was performed previously to quantify the risk for workers in slaughterhouses [212]. Although *B. suis* was isolated from cattle [142], no reports of isolation from pigs can be found in literature from Egypt. A recent study investigating brucellosis prevalence in pigs from Cairo and Giza governorates revealed 4.83% and 10.8% using i-ELISA and c-ELISA [213]. The study suggested that *Brucella suis* biovars are circulating in various other farm animal species in Egypt and need further investigation to get more details about the disease for concrete policy management to control the disease. DNA of *B. melitensis* and *B. suis* was amplified from pigs samples in Egypt using RT-PCR [213].

10. References

1. N Xavier, M.; A Paixao, T.; B den Hartigh, A.; M Tsolis, R.; L Santos, R. Pathogenesis of brucella spp. *The open veterinary science journal* **2010**, *4*.
2. Pappas, G.; Panagopoulou, P.; Christou, L.; Akritidis, N. Brucella as a biological weapon. *Cellular and molecular life sciences : CMLS* **2006**, *63*, 2229-2236.
3. Geering, W.A.; Forman, A.; Nunn, M. *Exotic diseases of animals: A field guide for australian veterinarians*. Australian Government Publishing Service: 1995.
4. Refai, M. Incidence and control of brucellosis in the near east region. *Veterinary microbiology* **2002**, *90*, 81-110.
5. Pappas, G.; Papadimitriou, P.; Akritidis, N.; Christou, L.; Tsianos, E. The new global map of human brucellosis. *Lancet Infect Dis* **2006**, *6*, 91-99.

6. Godfroid, J. Brucellosis in livestock and wildlife: Zoonotic diseases without pandemic potential in need of innovative one health approaches. *Arch Public Health.* **2017**, *75*, 6.
7. Cutler, S.J.; Whatmore, A.M.; Commander, N.J. Brucellosis--new aspects of an old disease. *J Appl Microbiol* **2005**, *98*, 1270-1281.
8. Doganay, G.D.; Doganay, M. Brucella as a potential agent of bioterrorism. *Recent patents on anti-infective drug discovery* **2013**, *8*, 27-33.
9. Guihot, A.; Bossi, P.; Bricaire, F. [bioterrorism with brucellosis]. *Presse medicale (Paris, France : 1983)* **2004**, *33*, 119-122.
10. Eduardo Gotuzzo; Pappas, G. Brucellosis. In *Tropical infectious diseases: Principles, pathogens and practice* 3rd ed.; Guerrant, R.L.; Walker, D.H.; Weller, P.F., Eds. Elsevier Health Sciences: MA, USA, 2011; p 1156.
11. Holt, J.G.; Krieg, N.R.; Sneath, P.H. Bergey's manual of determinative bacterology. **1994**.
12. Ficht, T. Brucella taxonomy and evolution. *Future Microbiol* **2010**, *5*, 859-866.
13. Whatmore, A.M. Current understanding of the genetic diversity of brucella, an expanding genus of zoonotic pathogens. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* **2009**, *9*, 1168-1184.
14. Paulsen, I.T.; Seshadri, R.; Nelson, K.E.; Eisen, J.A.; Heidelberg, J.F.; Read, T.D.; Dodson, R.J.; Umayam, L.; Brinkac, L.M.; Beanan, M.J.*, et al.* The brucella suis genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proceedings of the National Academy of Sciences of the United States of America* **2002**, *99*, 13148-13153.
15. Scholz, H.; Revilla-Fernandez, S.; Al Dahouk, S.; Hammerl, J.; Zygmunt, M.; Cloeckaert, A.; Koylass, M.; Whatmore, A.; Blom, J.; Vergnaud, G.*, et al. Brucella vulpis* sp. Nov., isolated from mandibular lymph nodes of red foxes (*vulpes vulpes*). *Int J Syst Evol Microbiol* **2016**, *66*, 2090-2098.
16. Gándara, B.; Merino, A.L.; Rogel, M.A.; Martínez-Romero, E. Limited genetic diversity of brucella spp. *J Clin Microbiol* **2001**, *39*, 235-240.
17. Jumas-Bilak, E.; Michaux-Charachon, S.; Bourg, G.; O'Callaghan, D.; Ramuz, M. Differences in chromosome number and genome rearrangements in the genus brucella. *Molecular microbiology* **1998**, *27*, 99-106.
18. Corbel, M.J. Brucellosis: An overview. *Emerging infectious diseases* **1997**, *3*, 213-221.
19. Percin, D. Microbiology of brucella. *Recent patents on anti-infective drug discovery* **2013**, *8*, 13-17.
20. Liu, D. Brucella. In *Molecular medical microbiology*, Elsevier: 2015; pp 1781-1788.
21. Baldwin, C.L.; Goenka, R. Host immune responses to the intracellular bacteria brucella: Does the bacteria instruct the host to facilitate chronic infection? *Critical reviews in immunology* **2006**, *26*, 407-442.
22. J.Young, E. Brucella species (brucellosis). In *Principles and practice of pediatric infectious diseases* 5th Edition ed.; Long, S.S.; Prober, C.G.; Fischer, M., Eds. Elsevier Health Sciences: 2018; pp 886-889.
23. Whatmore, A.M.; Davison, N.; Cloeckaert, A.; Al Dahouk, S.; Zygmunt, M.S.; Brew, S.D.; Perrett, L.L.; Koylass, M.S.; Vergnaud, G.; Quance, C.*, et al.* Brucella papionis sp. Nov., isolated from baboons (papio spp.). *International journal of systematic and evolutionary microbiology* **2014**, *64*, 4120-4128.
24. OIE. Brucellosis (*brucella abortus, b. Melitensis* and *b. Suis*) (infection with *b. Abortus, b. Melitensis* and *b. Suis*). In *In manual of diagnostic tests and vaccines for terrestrial animals* 7th ed.; OIE; World Health Organization for Animal Health: Paris, France, 2018; Vol. 1, pp 355-398.
25. Lucero, N.E.; Corazza, R.; Almuzara, M.N.; Reynes, E.; Escobar, G.I.; Boeri, E.; Ayala, S.M. Human brucella canis outbreak linked to infection in dogs. *Epidemiology and infection* **2010**, *138*, 280-285.
26. Nikokar, I.; Hosseinpour, M.; Asmar, M.; Pirmohbatei, S.; Hakeimei, F.; Razavei, M.T. Seroprevalence of brucellosis among high risk individuals in guilan, iran. *Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences* **2011**, *16*, 1366-1371.
27. Kato, Y.; Masuda, G.; Itoda, I.; Imamura, A.; Ajisawa, A.; Negishi, M. Brucellosis in a returned traveler and his wife: Probable person-to-person transmission of brucella melitensis. *Journal of travel medicine* **2007**, *14*, 343-345.
28. Pappas, G. The changing brucella ecology: Novel reservoirs, new threats. *Int J Antimicrob Agents.* **2010**, *36 Suppl 1*, S8-11. doi 10.1016/j.ijantimicag.2010.1006.1013. Epub 2010 Aug 1018.

29. Cross, P.; Maichak, E.; Brennan, A.; Scurlock, B.; Henningsen, J.; Luikart, G.J.R.S.e.T.-O.I.d.E. An ecological perspective on brucella abortus in the western united states. **2013**, *32*, 79-87.
30. Muma, J.B.; Samui, K.L.; Siamudaala, V.M.; Oloya, J.; Matop, G.; Omer, M.K.; Munyeme, M.; Mubita, C.; Skjerve, E. Prevalence of antibodies to brucella spp. And individual risk factors of infection in traditional cattle, goats and sheep reared in livestock-wildlife interface areas of zambia. *Tropical animal health and production* **2006**, *38*, 195-206.
31. Knight-Jones, T.J.; Mylrea, G.E.; Kahn, S. Animal production food safety: Priority pathogens for standard setting by the world organisation for animal health. *Revue scientifique et technique (International Office of Epizootics)* **2010**, *29*, 523-535.
32. Dadar, M.; Shahali, Y.; Whatmore, A.M. Human brucellosis caused by raw dairy products: A review on the occurrence, major risk factors and prevention. *International journal of food microbiology* **2019**, *292*, 39-47.
33. Tuon, F.F.; Gondolfo, R.B.; Cerchiari, N. Human to human transmission of *brucella* - a systematic review. *Trop Med Int Health.* **2017**, *22*, 539-546.
34. Ali, S.; Ali, Q.; Neubauer, H.; Melzer, F.; Elschner, M.; Khan, I.; Abatih, E.N.; Ullah, N.; Irfan, M.; Akhter, S. Seroprevalence and risk factors associated with brucellosis as a professional hazard in pakistan. *Foodborne pathogens and disease* **2013**, *10*, 500-505.
35. Galinska, E.M.; Zagorski, J. Brucellosis in humans--etiology, diagnostics, clinical forms. *Annals of agricultural and environmental medicine : AAEM* **2013**, *20*, 233-238.
36. Wareth, G.; Kheimar, A.; Neubauer, H.; Melzer, F. Susceptibility of avian species to brucella infection: A hypothesis-driven study. *Pathogens* **2020**, *9*.
37. Shome, R.; Kalleshamurthy, T.; Shome, B.; Sahay, S.; Natesan, K.; Bambal, R.; Sairiwal, L.; Mohandoss, N.; Barbuddhe, S. Lateral flow assay for brucellosis testing in multiple livestock species. *J Microbiol Methods* **2018**, *148*, 93-96.
38. Morgan, W. *Brucella* classification and regional distribution. *Dev Biol Stand* **1984**, *56*, 43-53.
39. Miller, M.; Burgess, T.; Dodd, E.; Rhyan, J.; Jang, S.; Byrne, B.; Gulland, F.; Murray, M.; Toy-Choutka, S.; Conrad, P.*, et al.* Isolation and characterization of a novel marine *brucella* from a southern sea otter (*enhydra lutris nereis*), california, USA. *J Wildl Dis* **2017**, *53*, 215-227.
40. Richomme, C.; Gauthier, D.; Fromont, E. Contact rates and exposure to inter-species disease transmission in mountain ungulates. *Epidemiology and infection* **2006**, *134*, 21-30.
41. Corbel, M.J. *Brucellosis in humans and animals*. World Health Organization: 2006.
42. B Lopes, L.; Nicolino, R.; PA Haddad, J. Brucellosis-risk factors and prevalence: A review. *The Open Veterinary Science Journal* **2010**, *4*.
43. Gul, H.C.; Erdem, H. Brucellosis (brucella species). In *Mandell, douglas, and bennett's principles and practice of infectious diseases*, Elsevier: 2015; pp 2584-2589. e2583.
44. Cooke, F.J.; Slack, M.P. Gram-negative coccobacilli. In *Infectious diseases*, Elsevier: 2017; pp 1611-1627. e1611.
45. Poester, F.P.; Samartino, L.E.; Santos, R.L. Pathogenesis and pathobiology of brucellosis in livestock. *Revue scientifique et technique (International Office of Epizootics)* **2013**, *32*, 105-115.
46. Lapaque, N.; Moriyon, I.; Moreno, E.; Gorvel, J.P. Brucella lipopolysaccharide acts as a virulence factor. *Current opinion in microbiology* **2005**, *8*, 60-66.
47. Amjadi, O.; Rafiei, A.; Mardani, M.; Zafari, P.; Zarifian, A. A review of the immunopathogenesis of brucellosis. *Infectious diseases (London, England)* **2019**, *51*, 321-333.
48. Franco, M.P.; Mulder, M.; Gilman, R.H.; Smits, H.L. Human brucellosis. *Lancet Infect Dis* **2007**, *7*, 775-786.
49. YAEGER, M.J.; HOLLER, L.D. Bacterial causes of bovine infertility and abortion. In *Current therapy in large animal theriogenology*, Robert S. Youngquist, W.R.T., Ed. Elsevier: 2007; pp 389-399.
50. Mantur, B.G.; Amarnath, S.K. Brucellosis in india - a review. *Journal of biosciences* **2008**, *33*, 539-547.
51. Moreno, E. Retrospective and prospective perspectives on zoonotic brucellosis. **2014**, *5*.
52. de Figueiredo, P.; Ficht, T.A.; Rice-Ficht, A.; Rossetti, C.A.; Adams, L.G. Pathogenesis and immunobiology of brucellosis: Review of brucella-host interactions. *The American journal of pathology* **2015**, *185*, 1505-1517.
53. Muflihanah, H.; Hatta, M.; Rood, E.; Scheelbeek, P.; Abdoel, T.H.; Smits, H.L. Brucellosis seroprevalence in bali cattle with reproductive failure in south sulawesi and brucella abortus biovar 1 genotypes in the eastern indonesian archipelago. *BMC veterinary research* **2013**, *9*, 233.

54. Robinson, A.; Production, A. *Guidelines for coordinated human and animal brucellosis surveillance*. FAO Rome, Italy: 2003.
55. Mantur, B.G.; Amarnath, S.K.; Shinde, R.S. Review of clinical and laboratory features of human brucellosis. *Indian journal of medical microbiology* **2007**, *25*, 188-202.
56. Hull, N.C.; Schumaker, B.A. Comparisons of brucellosis between human and veterinary medicine. *Infection ecology & epidemiology* **2018**, *8*, 1500846.
57. England, T.; Kelly, L.; Jones, R.D.; MacMillan, A.; Wooldridge, M. A simulation model of brucellosis spread in british cattle under several testing regimes. *Preventive veterinary medicine* **2004**, *63*, 63-73.
58. Kochar, D.K.; Gupta, B.K.; Gupta, A.; Kalla, A.; Nayak, K.C.; Purohit, S.K. Hospital-based case series of 175 cases of serologically confirmed brucellosis in bikaner. *The Journal of the Association of Physicians of India* **2007**, *55*, 271-275.
59. Megid, J.; Mathias, L.A.; Robles, C. Clinical manifestations of brucellosis in domestic animals and humans. *The Open Veterinary Science Journal* **2010**, 119-126.
60. Arkun, R.; Mete, B.D. Musculoskeletal brucellosis. *Seminars in musculoskeletal radiology* **2011**, *15*, 470-479.
61. Milionis, H.; Christou, L.; Elisaf, M. Cutaneous manifestations in brucellosis: Case report and review of the literature. *Infection* **2000**, *28*, 124-126.
62. Franco, M.; Mulder, M.; Gilman, R.; Smits, H. Human brucellosis. *The Lancet infectious diseases* **2007**, *7*, 775-786.
63. Radostitis, O.; Gay, C.; Hinchcliff, K.; Constable, P. Veterinary medicine. A text book of the diseases of cattle, horses, sheep, pigs and goats. 10th edn. Saunders elsevier; edinburgh, london, new york. Oxford, Philadelphia, St. Louis, Sydney, Toronto: 2007.
64. Earhart, K.; Vafakolov, S.; Yarmohamedova, N.; Michael, A.; Tjaden, J.; Soliman, A. Risk factors for brucellosis in samarqand oblast, uzbekistan. *Int J Infect Dis* **2009**, *13*, 749-753.
65. Wareth, G.; Melzer, F.; El-Diasty, M.; Schmoock, G.; Elbauomy, E.; Abdel-Hamid, N.; Sayour, A.; Neubauer, H. Isolation of brucella abortus from a dog and a cat confirms their biological role in re-emergence and dissemination of bovine brucellosis on dairy farms. *Transboundary and emerging diseases* **2017**, *64*, e27-e30.
66. Musallam, II; Abo-Shehada, M.N.; Hegazy, Y.M.; Holt, H.R.; Guitian, F.J. Systematic review of brucellosis in the middle east: Disease frequency in ruminants and humans and risk factors for human infection. *Epidemiology and infection* **2016**, *144*, 671-685.
67. Zeng, J.; Duoji, C.; Yuan, Z.; Yuzhen, S.; Fan, W.; Tian, L.; Cai, C.; Robertson, I. Seroprevalence and risk factors for bovine brucellosis in domestic yaks (bos grunniens) in tibet, china. *Tropical animal health and production* **2017**, *49*, 1339-1344.
68. Ahasan, M.S.; Rahman, M.S.; Rahman, A.K.; Berkvens, D. Bovine and caprine brucellosis in bangladesh: Bayesian evaluation of four serological tests, true prevalence, and associated risk factors in household animals. *Tropical animal health and production* **2017**, *49*, 1-11.
69. Liu, Q.; Cao, L.; Zhu, X. Major emerging and re-emerging zoonoses in china: A matter of global health and socioeconomic development for 1.3 billion. *Int J Infect Dis* **2014**, *25*, 65-72.
70. El-Diasty, M.; Wareth, G.; Melzer, F.; Mustafa, S.; Sprague, L.D.; Neubauer, H. Isolation of brucella abortus and brucella melitensis from seronegative cows is a serious impediment in brucellosis control. *Veterinary sciences* **2018**, *5*.
71. Godfroid, J.; Nielsen, K.; Saegerman, C. Diagnosis of brucellosis in livestock and wildlife. *Croat Med J.* **2010**, *51*, 296-305.
72. Mathew, C.; Stokstad, M.; Johansen, T.; Klevar, S.; Mdegela, R.; Mwamengele, G.; Michel, P.; Escobar, L.; Fretin, D.; Godfroid, J. First isolation, identification, phenotypic and genotypic characterization of *brucella abortus* biovar 3 from dairy cattle in tanzania. *BMC veterinary research* **2015**, *11*, 156.
73. Al, S.D.; Tomaso, H.; Nöckler, K.; Neubauer, H.; Frangoulidis, D. Laboratory-based diagnosis of brucellosis--a review of the literature. Part ii: Serological tests for brucellosis. *Clinical laboratory* **2003**, *49*, 577-589.
74. Al, S.; Tomaso, H.; Nöckler, K.; Neubauer, H.; Frangoulidis, D. Laboratory-based diagnosis of brucellosis--a review of the literature. Part i: Techniques for direct detection and identification of *brucella* spp. *Clinical laboratory* **2003**, *49*, 487-505.
75. Nielsen, K.; Yu, W.L. Serological diagnosis of brucellosis. *Prilozi* **2010**, *31*, 65-89.
76. Gómez, M.; Nieto, J.; Rosa, C.; Geijo, P.; Escribano, M.; Munoz, A.; López, C. Evaluation of seven tests for diagnosis of human brucellosis in an area where the disease is endemic. *Clinical and Vaccine Immunology* **2008**, *15*, 1031-1033.
77. Nielsen, K.; Smith, P.; Yu, W.; Elmgren, C.; Halbert, G.; Nicoletti, P.; Perez, B.; Conde, S.; Samartino, L.; Nicola, A. Validation of a second generation competitive enzyme

immunoassay (celisa) for the diagnosis of brucellosis in various species of domestic animals. *Veterinary immunology and immunopathology* **2008**, *125*, 246-250.
78. Nusrat, H. Disease specific diagnostic methods and lymphokines in human brucellosis. University of Karachi, Karachi, Pakistan, 2004.
79. Praud, A.; Durán-Ferrer, M.; Fretin, D.; Jaÿ, M.; O'Connor, M.; Stournara, A.; Tittarelli, M.; Travassos Dias, I.; Garin-Bastuji, B. Evaluation of three competitive elisas and a fluorescence polarisation assay for the diagnosis of bovine brucellosis. *Veterinary journal (London, England : 1997)* **2016**, *216*, 38-44.
80. Khan, M.Z.; Zahoor, M. An overview of brucellosis in cattle and humans, and its serological and molecular diagnosis in control strategies. *Trop Med Infect Dis.* **2018**, *3*, 14.
81. Christopher, S.; Umapathy, B.L.; Ravikumar, K.L. Brucellosis: Review on the recent trends in pathogenicity and laboratory diagnosis. *Journal of laboratory physicians* **2010**, *2*, 55-60.
82. Al Dahouk, S.; Nöckler, K. Implications of laboratory diagnosis on brucellosis therapy. *Expert review of anti-infective therapy* **2011**, *9*, 833-845.
83. Angeletti, S. Matrix assisted laser desorption time of flight mass spectrometry (maldi-tof ms) in clinical microbiology. *J Microbiol Methods* **2017**, *138*, 20-29.
84. Patel, R. Maldi-tof ms for the diagnosis of infectious diseases. *Clin Chem* **2015**, *61*, 100-111.
85. Sali, M.; De Maio, F.; Tarantino, M.; Garofolo, G.; Tittarelli, M.; Sacchini, L.; Zilli, K.; Pasquali, P.; Petrucci, P.; Marianelli, C.*, et al.* Rapid and safe one-step extraction method for the identification of *brucella* strains at genus and species level by maldi-tof mass spectrometry. *PLoS One* **2018**, *13*, e0197864.
86. Ulu-Kilic, A.; Metan, G.; Alp, E. Clinical presentations and diagnosis of brucellosis. *Recent patents on anti-infective drug discovery* **2013**, *8*, 34-41.
87. Sanjuan-Jimenez, R.; Colmenero, J.D.; Morata, P. Lessons learned with molecular methods targeting the bcsp-31 membrane protein for diagnosis of human brucellosis. *Clinica chimica acta; international journal of clinical chemistry* **2017**, *469*, 1-9.
88. Yu, W.L.; Nielsen, K. Review of detection of brucella spp. By polymerase chain reaction. *Croatian medical journal* **2010**, *51*, 306-313.
89. Ohtsuki, R.; Kawamoto, K.; Kato, Y.; Shah, M.M.; Ezaki, T.; Makino, S.I. Rapid detection of brucella spp. By the loop-mediated isothermal amplification method. *J Appl Microbiol* **2008**, *104*, 1815-1823.
90. Probert, W.S.; Schrader, K.N.; Khuong, N.Y.; Bystrom, S.L.; Graves, M.H. Real-time multiplex pcr assay for detection of brucella spp., b. Abortus, and b. Melitensis. *J Clin Microbiol* **2004**, *42*, 1290-1293.
91. Ratushna, V.G.; Sturgill, D.M.; Ramamoorthy, S.; Reichow, S.A.; He, Y.; Lathigra, R.; Sriranganathan, N.; Halling, S.M.; Boyle, S.M.; Gibas, C.J. Molecular targets for rapid identification of brucella spp. *BMC microbiology* **2006**, *6*, 13.
92. Shoukat, S.; Wani, H.; Ali, U.; Para, P.; Ara, S.; Ganguly, S. Brucellosis: A current review update on zoonosis. *Journal of Immunology and Immunopathology* **2017**, *19*, 61-69.
93. Mitka, S.; Anetakis, C.; Souliou, E.; Diza, E.; Kansouzidou, A. Evaluation of different pcr assays for early detection of acute and relapsing brucellosis in humans in comparison with conventional methods. *J Clin Microbiol* **2007**, *45*, 1211-1218.
94. Alton, G.G.; Jones, L.M.; Angus, R.; Verger, J. *Techniques for the brucellosis laboratory*. Institut National de la recherche Agronomique (INRA): 1988.
95. Vergnaud, G.; Hauck, Y.; Christiany, D.; Daoud, B.; Pourcel, C.; Jacques, I.; Cloeckaert, A.; Zygmunt, M.S. Genotypic expansion within the population structure of classical brucella species revealed by mlva16 typing of 1404 brucella isolates from different animal and geographic origins, 1974-2006. *Frontiers in microbiology* **2018**, *9*, 1545.
96. Foster, J.T.; Okinaka, R.T.; Svensson, R.; Shaw, K.; De, B.K.; Robison, R.A.; Probert, W.S.; Kenefic, L.J.; Brown, W.D.; Keim, P. Real-time pcr assays of single-nucleotide polymorphisms defining the major brucella clades. *J Clin Microbiol* **2008**, *46*, 296-301.
97. Scott, J.C.; Koylass, M.S.; Stubberfield, M.R.; Whatmore, A.M. Multiplex assay based on single-nucleotide polymorphisms for rapid identification of brucella isolates at the species level. *Applied and environmental microbiology* **2007**, *73*, 7331-7337.
98. Gopaul, K.K.; Koylass, M.S.; Smith, C.J.; Whatmore, A.M. Rapid identification of brucella isolates to the species level by real time pcr based single nucleotide polymorphism (snp) analysis. *BMC microbiology* **2008**, *8*, 86.
99. Foster, J.T.; Beckstrom-Sternberg, S.M.; Pearson, T.; Beckstrom-Sternberg, J.S.; Chain, P.S.; Roberto, F.F.; Hnath, J.; Brettin, T.; Keim, P. Whole-genome-based phylogeny and divergence of the genus brucella. *Journal of bacteriology* **2009**, *191*, 2864-2870.

100. Le Fleche, P.; Jacques, I.; Grayon, M.; Al Dahouk, S.; Bouchon, P.; Denoeud, F.; Nockler, K.; Neubauer, H.; Guilloteau, L.A.; Vergnaud, G. Evaluation and selection of tandem repeat loci for a brucella mlva typing assay. *BMC microbiology* **2006**, *6*, 9.
101. Whatmore, A.M.; Perrett, L.L.; MacMillan, A.P. Characterisation of the genetic diversity of brucella by multilocus sequencing. *BMC microbiology* **2007**, *7*, 34.
102. Singh, M.; Malik, M.A.; Singh, D.K.; Doimari, S.; Bhavna; Sharma, R. Multilocus variable number tandem repeat analysis (mlva)-typing of brucella abortus isolates of india reveals limited genetic diversity. *Tropical animal health and production* **2020**, *52*, 1187-1194.
103. Ma, J.Y.; Wang, H.; Zhang, X.F.; Xu, L.Q.; Hu, G.Y.; Jiang, H.; Zhao, F.; Zhao, H.Y.; Piao, D.R.; Qin, Y.M.*, et al.* Mlva and mlst typing of brucella from qinghai, china. *Infectious diseases of poverty* **2016**, *5*, 26.
104. Janowicz, A.; De Massis, F.; Ancora, M.; Cammà, C.; Patavino, C.; Battisti, A.; Prior, K.; Harmsen, D.; Scholz, H.; Zilli, K.*, et al.* Core genome multilocus sequence typing and single nucleotide polymorphism analysis in the epidemiology of brucella melitensis infections. *J Clin Microbiol* **2018**, *56*.
105. Georgi, E.; Walter, M.C.; Pfalzgraf, M.T.; Northoff, B.H.; Holdt, L.M.; Scholz, H.C.; Zoeller, L.; Zange, S.; Antwerpen, M.H. Whole genome sequencing of brucella melitensis isolated from 57 patients in germany reveals high diversity in strains from middle east. *PLoS One* **2017**, *12*, e0175425.
106. Ng, P.C.; Kirkness, E. Whole genome sequencing. In *Genetic variation*, Springer: 2010; pp 215-226.
107. Roetzer, A.; Diel, R.; Kohl, T.A.; Rückert, C.; Nübel, U.; Blom, J.; Wirth, T.; Jaenicke, S.; Schuback, S.; Rüsch-Gerdes, S.*, et al.* Whole genome sequencing versus traditional genotyping for investigation of a mycobacterium tuberculosis outbreak: A longitudinal molecular epidemiological study. *PLoS medicine* **2013**, *10*, e1001387.
108. Simner, P.; Miller, S.; Carroll, K. Understanding the promises and hurdles of metagenomic next-generation sequencing as a diagnostic tool for infectious diseases. *Clinical Infectious Diseases* **2017**.
109. Meldrum, C.; Doyle, M.; Tothill, R. Next-generation sequencing for cancer diagnostics: A practical perspective. *The Clinical Biochemist Reviews* **2011**, *32*, 177.
110. Voelkerding, K.; Dames, S.; Durtschi, J. Next-generation sequencing: From basic research to diagnostics. *Clinical chemistry* **2009**, *55*, 641-658.
111. Owen, J.; Noyes, N.; Young, A.; Prince, D.; Blanchard, P.; Lehenbauer, T.; Aly, S.; Davis, J.; O'Rourke, S.; Abdo, Z. Whole-genome sequencing and concordance between antimicrobial susceptibility genotypes and phenotypes of bacterial isolates associated with bovine respiratory disease. *G3: Genes, Genomes, Genetics* **2017**, *7*, 3059-3071.
112. Johansen, T.B.; Scheffer, L.; Jensen, V.K.; Bohlin, J.; Feruglio, S.L. Whole-genome sequencing and antimicrobial resistance in brucella melitensis from a norwegian perspective. *Scientific reports* **2018**, *8*, 8538.
113. Ficht, T.A.; Kahl-McDonagh, M.M.; Arenas-Gamboa, A.M.; Rice-Ficht, A.C. Brucellosis: The case for live, attenuated vaccines. *Vaccine* **2009**, *27 Suppl 4*, D40-43.
114. Islam, M.A.; Khatun, M.M.; Werre, S.R.; Sriranganathan, N.; Boyle, S.M. A review of brucella seroprevalence among humans and animals in bangladesh with special emphasis on epidemiology, risk factors and control opportunities. *Veterinary microbiology* **2013**, *166*, 317-326.
115. Martins, H.; Garin-Bastuji, B.; Lima, F.; Flor, L.; Pina Fonseca, A.; Boinas, F. Eradication of bovine brucellosis in the azores, portugal-outcome of a 5-year programme (2002-2007) based on test-and-slaughter and rb51 vaccination. *Preventive veterinary medicine* **2009**, *90*, 80-89.
116. Avila-Calderón, E.D.; Lopez-Merino, A.; Sriranganathan, N.; Boyle, S.M.; Contreras-Rodríguez, A. A history of the development of brucella vaccines. *BioMed research international* **2013**, *2013*, 743509.
117. Ariza, J.; Bosilkovski, M.; Cascio, A.; Colmenero, J.D.; Corbel, M.J.; Falagas, M.E.; Memish, Z.A.; Roushan, M.R.; Rubinstein, E.; Sipsas, N.V.*, et al.* Perspectives for the treatment of brucellosis in the 21st century: The ioannina recommendations. *PLoS Med.* **2007**, *4*.
118. Solera, J.; Beato, J.L.; Martinez-Alfaro, E.; Segura, J.C.; de Tomas, E. Azithromycin and gentamicin therapy for the treatment of humans with brucellosis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **2001**, *32*, 506-509.
119. Solera, J.; Martinez-Alfaro, E.; Espinosa, A. Recognition and optimum treatment of brucellosis. *Drugs* **1997**, *53*, 245-256.

120. del Pozo, J.S.G.; Solera, J. Treatment of human brucellosis-review of evidence from clinical trials. In *Updates on brucellosis*, InTech.: London, UK, 2015.
121. Biswas, S.; Raoult, D.; Rolain, J.M. A bioinformatic approach to understanding antibiotic resistance in intracellular bacteria through whole genome analysis. *Int J Antimicrob Agents.* **2008**, *32*, 207-220.
122. Marianelli, C.; Ciuchini, F.; Tarantino, M.; Pasquali, P.; Adone, R. Genetic bases of the rifampin resistance phenotype in *brucella* spp. *J Clin Microbiol.* **2004**, *42*, 5439-5443.
123. Ravanel, N.; Gestin, B.; Maurin, M. In vitro selection of fluoroquinolone resistance in *brucella melitensis*. *Int J Antimicrob Agents.* **2009**, *34*, 76-81.
124. Jennings, G.J.; Hajjeh, R.A.; Girgis, F.Y.; Fadeel, M.A.; Maksoud, M.A.; Wasfy, M.O.; El-Sayed, N.; Srikantiah, P.; Luby, S.P.; Earhart, K.*, et al.* Brucellosis as a cause of acute febrile illness in egypt. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **2007**, *101*, 707-713.
125. Moreno, E. Retrospective and prospective perspectives on zoonotic brucellosis. *Front Microbiol* **2014**, *5*, 213.
126. Lewis, J.M.; Folb, J.; Kalra, S.; Squire, S.B.; Taegtmeyer, M.; Beeching, N.J. Brucella melitensis prosthetic joint infection in a traveller returning to the uk from thailand: Case report and review of the literature. *Travel medicine and infectious disease* **2016**, *14*, 444-450.
127. Grace, D.; Mutua, F.; Ochungo, P.; Kruska, R.; Jones, K.; Brierley, L.; Lapar, L.; Said, M.; Herrero, M.; Phuc, P. Mapping of poverty and likely zoonoses hotspots. Zoonoses project 4. Report to the uk department for international development. *International Livestock Research Institute, Nairobi, Kenya* **2012**.
128. Godfroid, J.; Cloeckaert, A.; Liautard, J.P.; Kohler, S.; Fretin, D.; Walravens, K.; Garin-Bastuji, B.; Letesson, J.J. From the discovery of the malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Veterinary research* **2005**, *36*, 313-326.
129. El-Diasty, M.M.; Ahmed, H.A.; Sayour, A.E.; El Hofy, F.I.; Tahoun, A.B.; Shafik, S.M. Seroprevalence of brucella spp. In cattle, molecular characterization in milk, and the analysis of associated risk factors with seroprevalence in humans, egypt. *Vector borne and zoonotic diseases (Larchmont, N.Y.)* **2016**, *16*, 758-764.
130. Abdi, A. *Livestock and products, egypt.* ; Agricultural Ministery, Cairo, Egypt, 2018; pp 1-10.
131. FAO. *Africa sustainable livestock 2050, country brief egypt.* ; 2017; pp 1-10.
132. Holt, H.R.; Eltholth, M.M.; Hegazy, Y.M.; El-Tras, W.F.; Tayel, A.A.; Guitian, J. Brucella spp. Infection in large ruminants in an endemic area of egypt: Cross-sectional study investigating seroprevalence, risk factors and livestock owner's knowledge, attitudes and practices (kaps). *BMC public health* **2011**, *11*, 341.
133. Horton, K.C.; Wasfy, M.; Samaha, H.; Abdel-Rahman, B.; Safwat, S.; Abdel Fadeel, M.; Mohareb, E.; Dueger, E. Serosurvey for zoonotic viral and bacterial pathogens among slaughtered livestock in egypt. *Vector borne and zoonotic diseases (Larchmont, N.Y.)* **2014**, *14*, 633-639.
134. Wareth, G.; Hikal, A.; Refai, M.; Melzer, F.; Roesler, U.; Neubauer, H. Animal brucellosis in egypt. *J Infect Dev Ctries* **2014**, *8*, 1365-1373.
135. Refai, M. Brucellosis in animal in egypt. Updated review. *J. Egypt. Vet Med. Assoc* **1994**, *54*, 173-183.
136. Refai, M. Brucellosis in animals and man in egypt. *Egypt J Vet Sci* **2003**, *37*, 1-31.
137. Eltholth, M.; Hegazy, Y.; El-Tras, W.; Bruce, M.; Rushton, J. Temporal analysis and costs of ruminant brucellosis control programme in egypt between 1999 and 2011. *Transboundary and emerging diseases* **2017**, *64*, 1191-1199.
138. Salem, L.; Khalifa, N.O.; Moustafa, S.M.J.B.V.M.J. Sero-diagnosis of brucellosis in gharbiya governorate, egypt. **2016**, *31*, 10-16.
139. Hegazy, Y.M.; Molina-Flores, B.; Shafik, H.; Ridler, A.L.; Guitian, F.J. Ruminant brucellosis in upper egypt (2005-2008). *Preventive veterinary medicine* **2011**, *101*, 173-181.
140. Samaha, H.; Al-Rowaily, M.; Khoudair, R.M.; Ashour, H.M. Multicenter study of brucellosis in egypt. *Emerging infectious diseases* **2008**, *14*, 1916-1918.
141. Wareth, G.; Melzer, F.; Bottcher, D.; El-Diasty, M.; El-Beskawy, M.; Rasheed, N.; Schmoock, G.; Roesler, U.; Sprague, L.D.; Neubauer, H. Molecular typing of isolates obtained from aborted foetuses in brucella-free holstein dairy cattle herd after immunisation with brucella abortus rb51 vaccine in egypt. *Acta tropica* **2016**, *164*, 267-271.
142. Menshawy, A.M.; Perez-Sancho, M.; Garcia-Seco, T.; Hosein, H.I.; Garcia, N.; Martinez, I.; Sayour, A.E.; Goyache, J.; Azzam, R.A.; Dominguez, L.*, et al.* Assessment of genetic

diversity of zoonotic brucella spp. Recovered from livestock in egypt using multiple locus vntr analysis. *BioMed research international* **2014**, *2014*, 353876.
143. Khan, A.U.; Shell, W.S.; Melzer, F.; Sayour, A.E.; Ramadan, E.S.; Elschner, M.C.; Moawad, A.A.; Roesler, U.; Neubauer, H.; El-Adawy, H. Identification, genotyping and antimicrobial susceptibility testing of brucella spp. Isolated from livestock in egypt. *Microorganisms* **2019**, *7*, 603.
144. Wareth, G.; El-Diasty, M.; Melzer, F.; Murugaiyan, J.; Abdulmawjood, A.; Sprague, L.D.; Neubauer, H. Trueperella pyogenes and brucella abortus coinfection in a dog and a cat on a dairy farm in egypt with recurrent cases of mastitis and abortion. *Veterinary medicine international* **2018**, *2018*, 2056436.
145. El-Tras, W.F.; Tayel, A.A.; Eltholth, M.M.; Guitian, J. Brucella infection in fresh water fish: Evidence for natural infection of nile catfish, clarias gariepinus, with brucella melitensis. *Veterinary microbiology* **2010**, *141*, 321-325.
146. Hamdy, M.E.; Amin, A.S. Detection of brucella species in the milk of infected cattle, sheep, goats and camels by pcr. *Veterinary journal (London, England : 1997)* **2002**, *163*, 299-305.
147. Gwida, M.; El-Gohary, A.; Melzer, F.; Khan, I.; Rosler, U.; Neubauer, H. Brucellosis in camels. *Research in veterinary science* **2012**, *92*, 351-355.
148. Amin, A.S.; Hamdy, M.E.; Ibrahim, A.K. Detection of brucella melitensis in semen using the polymerase chain reaction assay. *Veterinary microbiology* **2001**, *83*, 37-44.
149. El-Midany, S.-A.; El-Tras, W.F.; Eltholth, M.M.; Seada, A.S.; Zaki, H.M.J.W. Identification of brucella spp. And assessing impact of brucellosis control programme on ruminants and human in gharbia governorate, egypt. **2016**, *6*, 156-165.
150. Wareth, G.; Melzer, F.; Elschner, M.C.; Neubauer, H.; Roesler, U. Detection of brucella melitensis in bovine milk and milk products from apparently healthy animals in egypt by real-time pcr. *J Infect Dev Ctries* **2014**, *8*, 1339-1343.
151. Dean, A.S.; Crump, L.; Greter, H.; Schelling, E.; Zinsstag, J. Global burden of human brucellosis: A systematic review of disease frequency. *PLoS Negl Trop Dis* **2012**, *6*, e1865.
152. Abdel-Maksoud, M.; House, B.; Wasfy, M.; Abdel-Rahman, B.; Pimentel, G.; Roushdy, G.; Dueger, E. In vitro antibiotic susceptibility testing of brucella isolates from egypt between 1999 and 2007 and evidence of probable rifampin resistance. *Annals of clinical microbiology and antimicrobials* **2012**, *11*, 24.
153. Hegazy, Y.M.; Moawad, A.; Osman, S.; Ridler, A.; Guitian, J. Ruminant brucellosis in the kafr el sheikh governorate of the nile delta, egypt: Prevalence of a neglected zoonosis. *PLoS Negl Trop Dis* **2011**, *5*, e944.
154. Haggag, Y.N.; Samaha, H.A.; Nossair, M.A.; Mohammad, H.S.J.A.J.f.V.S. Monitoring of ruminant sera for the presence of brucella antibodies in alexandria province. **2016**, *51*.
155. El Sherbini, A.; Kabbash, I.; Schelling, E.; El Shennawy, S.; Shalapy, N.; Elnaby, G.H.; Helmy, A.A.; Eisa, A. Seroprevalences and local variation of human and livestock brucellosis in two villages in gharbia governorate, egypt. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **2007**, *101*, 923-928.
156. Hegazy, Y.; Elmonir, W.; Abdel-Hamid, N.H.; Elbauomy, E.M. Seroprevalence and "knowledge, attitudes and practices" (kaps) survey of endemic ovine brucellosis in egypt. *Acta veterinaria Scandinavica* **2016**, *58*, 1.
157. De Massis, F.; Zilli, K.; Di Donato, G.; Nuvoloni, R.; Pelini, S.; Sacchini, L.; D'Alterio, N.; Di Giannatale, E. Distribution of brucella field strains isolated from livestock, wildlife populations, and humans in italy from 2007 to 2015. *PLoS One* **2019**, *14*, e0213689.
158. Saeed, U.; Ali, S.; Khan, T.M.; El-Adawy, H.; Melzer, F.; Khan, A.U.; Iftikhar, A.; Neubauer, H. Seroepidemiology and the molecular detection of animal brucellosis in punjab, pakistan. *Microorganisms* **2019**, *7*.
159. Godfroid, J.; Scholz, H.C.; Barbier, T.; Nicolas, C.; Wattiau, P.; Fretin, D.; Whatmore, A.M.; Cloeckaert, A.; Blasco, J.M.; Moriyon, I.*, et al.* Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. *Preventive veterinary medicine* **2011**, *102*, 118-131.
160. Szulowski, K.; Iwaniak, W.; Weiner, M.; Złotnicka, J. Brucella suis biovar 2 isolations from cattle in poland. *Annals of agricultural and environmental medicine : AAEM* **2013**, *20*, 672-675.
161. Olsen, S.; Tatum, F. Bovine brucellosis. *Vet Clin North Am Food Anim Pract* **2010**, *26*, 15.
162. Selim, A.; Attia, K.; Ramadan, E.; Hafez, Y.M.; Salman, A. Seroprevalence and molecular characterization of brucella species in naturally infected cattle and sheep. *Preventive veterinary medicine* **2019**, *171*, 104756.

163. Refai, M.; El-Gibaly, S.; Salem, T.F. In *Brucellosis in cows and buffaloes in egypt*, Proceedings of the second world buffalo congress, India, 1988; pp 12-16.
164. Affi, M.; Abdul-Raouf, U.; El-Bayoumy, E.; Montasser, A.; Mohamad, H.J.J.o.A.S. Isolation and biotyping of brucella melitensis from upper egypt. **2011**, *7*, 50-60.
165. Gwida, M.; El-Ashker, M.; Melzer, F.; El-Diasty, M.; El-Beskawy, M.; Neubauer, H. Use of serology and real time pcr to control an outbreak of bovine brucellosis at a dairy cattle farm in the nile delta region, egypt. *Irish veterinary journal* **2015**, *69*, 3.
166. KA, A.-G.J.J.o.V.M.R. Seroprevalence and bacteriological identification of brucellosis in buffaloes in upper egypt. **2018**, *25*, 148-156.
167. El-Hady, A.; Ahmed, M.; Saleh, M.; Younis, E. Seroprevalence and molecular epidemiology of brucellosis in cattle in egypt. *Adv. Dairy Res* **2016**, *4*, 1-4.
168. Ramadan, E.S.; Nassar, N.R.; Ibrahim, I.G.; Zayed, A.F.J.A.J.f.V.S. Epidemiological and zoonotic surveillance of brucellosis in beni-suef governorate. **2019**, *61*.
169. Ghoneim, N.; Zeedan, G.; Ghazy, A.; Abdalhamed, A.J.I. Molecular and serological detection of the most common brucella species infection in bovine and human in egypt. **2014**, *2*, 217-226.
170. Al-Habaty, S.; Abuo-Gazia, K.; Ammar, M. Prevalence study on brucellosis in some ruminants slaughtered out of abattoir in assuit governorate. *Assiut Vet. Med. J.* **2015**, *61*, 65-72.
171. Koriem, A.; AL-Habaty, S.; Makar, N.; Abd-El-kader, H.J.A.V.M.J. Seroprevalence of brucellosis in slaughtered animals at assiut governorate. **2013**, *59*, 72-76.
172. Selim, A.; Gaber, A.; Moustafa, A. Diagnosis of brucellosis in ruminants in kafr el-sheikh governorate, egypt. *International Journal of Advanced Research* **2015**, *3*, 345-350.
173. Elmonir, W.; Hegazy, Y.; Abdel-Hamid, N.H.; Elbauomy, E.M.J.A.J.f.V.S. Brucellosis at the human-animal interface in kafrelsheikh governorate, egypt. **2016**, *50*.
174. Madboly, A.; Ghazi, Y.; Sokkar, S.M.; Desouky, H.; Ahmed, Y. Serological, bacteriological, molecular and immunohistochemical techniques for diagnosis of brucellosis in buffaloes. *Middle East J. Appl. Sci* **2014**, *4*, 762-770.
175. Zakaria, A.M.; Ahmed, S.F.; Motawae, M.S. Seroprevalence and real-time pcr detection of brucellosis in abattoirs animals as a potential route of infection in egypt. *European Journal of Medical and Health Sciences* **2019**, *1*, 1-5.
176. Hosein, H.I.; Zaki, H.M.; Safwat, N.M.; Menshawy, A.M.S.; Rouby, S.; Mahrous, A.; Madkour, B.E. Evaluation of the general organization of veterinary services control program of animal brucellosis in egypt: An outbreak investigation of brucellosis in buffalo. *Veterinary world* **2018**, *11*, 748-757.
177. Ragaa, M.; El-Seedy, F.; Abou-Gazia, K.A. Seroprevalence and bacteriological identification of brucellosis in buffaloes in upper egypt. *Journal of Veterinary Medical Research* **2018**, *25*, 148-156.
178. Shalaby, H.; El-Diasty, M.; Dhryg, R.; El-Shrbyny, M.J.A.V.M.J. Some epidemiological, haematological and biochemical studies on brucellosis in cattle and human contacts in some egypt governorates. **2013**, *59*, 71-79.
179. Wassif, I.; Mohamed, R.; El-Kattan, A. Studies on ruminant brucellosis in el salam canal area, egypt. *Benha Journal of Applied Sciences* **2017**, *2*, 151-155.
180. Castelo, C.; Simoes, J. Risk factors of brucellosis (re-)incidence in sheep and goat flocks in an endemic area of portugal. *Tropical animal health and production* **2019**, *51*, 487-490.
181. Reviriego, F.J.; Moreno, M.A.; Dominguez, L. Risk factors for brucellosis seroprevalence of sheep and goat flocks in spain. *Preventive veterinary medicine* **2000**, *44*, 167-173.
182. Wareth, G.; Melzer, F.; Tomaso, H.; Roesler, U.; Neubauer, H. Detection of brucella abortus DNA in aborted goats and sheep in egypt by real-time pcr. *BMC research notes* **2015**, *8*, 212.
183. Cao, X.; Li, Z.; Liu, Z.; Fu, B.; Liu, Y.; Shang, Y.; Zhou, J.; Jing, Z. Molecular epidemiological characterization of brucella isolates from sheep and yaks in northwest china. *Transboundary and emerging diseases* **2018**, *65*, e425-e433.
184. Garin-Bastuji, B.; Blasco, J.M.; Grayon, M.; Verger, J.M. Brucella melitensis infection in sheep: Present and future. *Veterinary research* **1998**, *29*, 255-274.
185. Elderbrook, M.; Schumaker, B.; Cornish, T.; Peck, D.; Sondgeroth, K. Seroprevalence and risk factors of brucella ovis in domestic sheep in wyoming, USA. *BMC veterinary research* **2019**, *15*, 246.
186. Ragheb, Z. Brucella infection among ewes, camels and pigs in egypt. *The Journal of comparative pathology and therapeutics* **1948**, *58*, 145-151.
187. Kaoud, H.; Zaki, M.M.; El-Dahshan, A.; Nasr, S.A.J.N.; Science. Epidemiology of brucellosis among farm animals. **2010**, *8*, 190-197.

188. Hafez, S.M.A.; El-Razik, K.A.A.; Hassan, H.M.; Gad, I.J.A.J.o.M.R. Comparative diagnosis of ovine brucellosis using single step blood-pcr with old and new serological tools. **2011**, *5*, 3976-3980.
189. El-Razik, K.; Desouky, H.; Ahmed, W.J.P.J.B.S. Investigations on brucellosis in egyptian baladi does with emphasis on evaluation of diagnostic techniques. **2007**, *10*, 342-348.
190. Abdelbaset, A.E.; Abushahba, M.F.N.; Hamed, M.I.; Rawy, M.S. Sero-diagnosis of brucellosis in sheep and humans in assiut and el-minya governorates, egypt. *International journal of veterinary science and medicine* **2018**, *6*, S63-s67.
191. Elbauomy, E.M.; Abdel-Hamid, N.H.; Abdel-Haleem, M.H.J.A.H.R.J. Epidemiological situation of brucellosis in five related ovine farms with suggestion of appropriate programmes for disease control. **2014**, *2*, 129-142.
192. Diab, M.S.; Elnaker, Y.F.; Ibrahim, N.A.; Sedeek, E.K.; Zidan, S.A.A. Seroprevalence and associated risk factors of brucellosis in sheep and human in four regions in matrouh governorate, egypt. *World Vet J* **2018**, *8*, 65-72.
193. Fatima, S.; Khan, I.; Nasir, A.; Younus, M.; Saqib, M.; Melzer, F.; Neubauer, H.; El-Adawy, H. Serological, molecular detection and potential risk factors associated with camel brucellosis in pakistan. *Tropical animal health and production* **2016**, *48*, 1711-1718.
194. Gyuranecz, M.; Wernery, U.; Kreizinger, Z.; Juhasz, J.; Felde, O.; Nagy, P. Genotyping of *brucella melitensis* strains from dromedary camels (*camelus dromedarius*) from the united arab emirates with multiple-locus variable-number tandem repeat analysis. *Veterinary microbiology* **2016**, *186*, 8-12.
195. El-Sayed, A.M.; Sayed-Ahmed, M.Z.; El-Diasty, M.M.; El-Beskawy, M.A.; Shoieb, S.M.; Younis, E.E. Sero-prevalence of camel brucellosis (*camelus dromedarius*) and phenotypic characteristics of *brucella melitensis* biovar 3 in shalateen city, red sea governorate, egypt. *Afr. J. Microbiol. Res.* **2017**, *11*, 1259-1266.
196. Salisu, U.S.; Kudi, C.A.; Bale, J.O.O.; Babashani, M.; Kaltungo, B.Y.; Saidu, S.N.A.; Asambe, A.; Baba, A.Y. Seroprevalence of *brucella* antibodies in camels in katsina state, nigeria. *Tropical animal health and production* **2017**, *49*, 1041-1046.
197. Hosein, H.I.; Rouby, S.; Menshawy, A.; Ghazy, N. Seroprevalence of camel brucellosis and molecular characterization of *brucella melitensis* recovered from dromedary camels in egypt. *Res. J. Vet. Pract* **2016**, *4*, 17-24.
198. Wernery, U. Camelid brucellosis: A review. *Revue scientifique et technique (International Office of Epizootics)* **2014**, *33*, 839-857.
199. Sprague, L.D.; Al-Dahouk, S.; Neubauer, H. A review on camel brucellosis: A zoonosis sustained by ignorance and indifference. *Pathog Glob Health* **2012**, *106*, 144-149.
200. El-Seedy, F.; Radwan, A.; El-Shabrawy, M.J.V.M.J.G. Serological and bacteriological investigations on brucella infection in one humped camels (camelus dromedarius) in egypt. **2000**, *48*, 83-89.
201. Abd El Tawab, A.; Agag, M.; Khoudair, R.; Anwer, B.J.B.V.M.J. Some studies on brucella among camels with reference to isolated strains. **2018**, *35*, 12-21.
202. Gwida, M.; Al Dahouk, S.; Melzer, F.; Rosler, U.; Neubauer, H.; Tomaso, H. Brucellosis - regionally emerging zoonotic disease? *Croatian medical journal* **2010**, *51*, 289-295.
203. Salem, A.; EL-Gibaly, M.; Shawkat, E.; Ibrahim, I.; Nada, R.J.A.V.M.J. Some studies on brucellosis in camels. **1990**, *23*, 139-143.
204. Moghney, A.R.F.A. A preliminary study on brucellosis on camels at behira province. *Ass. Univ. Bull. Environ. Res.* **2004**, *7*, 39-43.
205. Olsen, S.; Tatum, F. Swine brucellosis: Current perspectives. *Vet Med (Auckl)* **2017**, *8*, 1-12.
206. Higgins, J.; Stuber, T.; Quance, C.; Edwards, W.H.; Tiller, R.V.; Linfield, T.; Rhyan, J.; Berte, A.; Harris, B. Molecular epidemiology of brucella abortus isolates from cattle, elk, and bison in the united states, 1998 to 2011. *Applied and environmental microbiology* **2012**, *78*, 3674-3684.
207. Nagalingam, M.; Shome, R.; Balamurugan, V.; Shome, B.R.; NarayanaRao, K.; Vivekananda; Isloor, S.; Prabhudas, K. Molecular typing of brucella species isolates from livestock and human. *Tropical animal health and production* **2012**, *44*, 5-9.
208. Lucero, N.E.; Ayala, S.M.; Escobar, G.I.; Jacob, N.R. Brucella isolated in humans and animals in latin america from 1968 to 2006. *Epidemiology and infection* **2008**, *136*, 496-503.
209. Pilo, C.; Tedde, M.T.; Orrù, G.; Addis, G.; Liciardi, M. Brucella suis infection in domestic pigs in sardinia (italy). *Epidemiology and infection* **2015**, *143*, 2170-2177.
210. Abdelwhab, E.; Abdel-Moneim, A.S.J.V. Epidemiology, ecology and gene pool of influenza a virus in egypt: Will egypt be the epicentre of the next influenza pandemic? **2015**, *6*, 6-18.

211. Gomaa, M.R.; Kandeil, A.; El-Shesheny, R.; Shehata, M.M.; McKenzie, P.P.; Webby, R.J.; Ali, M.A.; Kayali, G. Evidence of infection with avian, human, and swine influenza viruses in pigs in cairo, egypt. *Archives of virology* **2018**, *163*, 359-364.
212. Ashraf, M.B.; Hassan, A.; Raafat, M.; Fathia, A.J.J.o.A.S. Occupational health hazard of egyptian employees in contact with wastage nourished swine. **2011**, *7*.
213. Khan, A.U.; Melzer, F.; El-Soally, S.; Elschner, M.C.; Mohamed, S.A.; Sayed Ahmed, M.A.; Roesler, U.; Neubauer, H.; El-Adawy, H. Serological and molecular identification of brucella spp. In pigs from cairo and giza governorates, egypt. *Pathogens* **2019**, *8*.

C H A P T E R 2.

Swine brucellosis in Egypt

Serological and molecular identification of *Brucella* spp. in pigs from Cairo and Giza governorates, Egypt

Pathogens. 2019 Dec; 8(4): 248

https://doi.org/10.3390/pathogens8040248

pathogens

MDPI

Article

Serological and Molecular Identification of *Brucella* spp. in Pigs from Cairo and Giza Governorates, Egypt

Aman Ullah Khan[1,2,3], Falk Melzer [1], Sherif Abdel Ghafar Elsayed El-Soally [4], Mandy C. Elschner [1], Shereen Aziz Mohamed [5], Mohamed Abdelmonem Sayed Ahmed [4], Uwe Roesler [2], Heinrich Neubauer [1] and Hosny El-Adawy [1,6,*]

1 Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, 07743 Jena, Germany; AmanUllah.Khan@fli.de (A.U.K.); falk.melzer@fli.de (F.M.); mandy.elschner@fli.de (M.C.E.); 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 (Sub-Campus UVAS-Lahore), Jhang 35200, Pakistan
4 Veterinary Service Department, Armed Forces Logistics Authority, Egyptian Armed Forces, 11765 Nasr City, Egypt; dr.sherifelsoaaly@gmail.com (S.A.G.E.E-S); moh_vet2001@hotmail.com (M.A.S.A.)
5 Veterinary Serum and Vaccine Research Institute, 11517 Abbasaia, Cairo, Egypt; shereenibraheem1968@gmail.com
6 Faculty of Veterinary Medicine, Kafrelsheikh University, 33516 Kafr El-Sheikh, Egypt
* Correspondence: Hosny.eladawy@fli.de.

Received: 23 October 2019; Accepted: 16 November 2019; Published: 20 November 2019

Abstract: Brucellosis is considered as endemic disease of animals and humans since thousands of years in Egypt. However, brucellosis in pigs has never been reported in Egypt. Thus, serological and molecular assays were applied to detect anti-*Brucella* antibodies and DNA in serum samples collected from pigs. In total 331 blood samples collected from male and female pigs at slaughterhouses of Cairo and Giza governorates were investigated using *Brucella* c- and i-ELISA and *Brucella* real-time PCR. Anti-*Brucella* antibodies were detected in 16 (4.83%) and 36 (10.8%) sera by i-ELISA and c-ELISA, respectively. *Brucella* DNA was detected in 10 (3.02%) seropositive samples and identified as *Brucella melitensis* (7/10) *and Brucella suis* (3/10). A higher prevelance was found in boars. This is the first study investigating pig brucellosis in Egypt. The results of this study will raise awareness for brucellosis in these farm animals and will help to develop effective control strategies.

Keywords: brucellosis; swine; Egypt; ELISA; real-time PCR

1. Introduction

Brucellosis is a zoonotic disease of public health importance affecting livestock, wildlife, and humans globally. The *Brucella* genus includes eleven recognized species with varying host preferences, pathogenicity, and epidemiology [1,2]. The disease is well controlled in developed countries but is still endemic in many others with the highest records in humans in Middle East and Central Asian regions [3].

Brucellosis is one of the major livestock production constraints in Egypt [4]. It is likely that it has been endemic in Egypt for thousands of years [5,6]. The disease has been detected with increasing prevalence in livestock species but predominantly in ruminants [7,8]. Prevalences ranging from 2.47% to 26.66% were found in various animal populations [9]. *Brucella abortus* and *Brucella melitensis* were

isolated from livestock and humans and *Brucella suis* was identified in cattle [6,10]. Brucellosis proved to be a serious occupational health hazard to livestock handlers, especially abattoir workers in Egypt [11].

World pig production has increased fourfold over the last five decades to meet protein requirements globally and is expected to continue growing [12]. Production of pigs in Egypt is found primarily in slums, rural, and peri-urban areas especially in Cairo and Giza governorates. Pork is consumed by Christians, foreigners, and tourists in Egypt. Currently the pig population is around two to three millions [13,14]. In Egypt, pigs are kept in small groups in contact with other farm animals and humans sharing pathogens with each other [15].

Typically, the infection is caused by *B. suis* biovars 1–3 [16]. The disease occurs in many countries where pigs are raised. Generally, the prevalence is low, but in some parts of the world, especially in the Southeast Asia and the South America, the prevalence may be much higher. *B. suis* bv1 infection has been reported in feral pigs in some parts of the southern states of USA and in Queensland, Australia. In these regions, a number of human brucellosis cases have been reported in hunters and handlers of materials of feral pigs. *B. suis* bv2 outbreaks have also been reported in Europe in wild boars, which were implicated in transmission of *B. suis* bv2 to domestic outdoor pigs [2]. Human pathogenic biovars (*B. suis* biovar 1–4) pose a sever hazard to humans [16]. Hence, *B. abortus* [17,18] and *B. melitensis* [19,20] were also isolated from pigs when kept together with infected ruminants and camels.

Brucellosis in pigs is a contagious disease characterized by infertility, production of small litters, and abortion in sows and orchitis and infection of secondary sex organs in boars [21]. The clinical manifestations are not pathognomonic. A diagnosis of brucellosis can be made mainly by the isolation and identification of *Brucella*, but in situations where bacteriological examination is not practicable, diagnosis should be based on immunological methods [2]. Serological tests are preferred for screening as they are comparatively sensitive and specific compared to bacterial cultivation to minimize the risk of laboratory acquired infections [2].

"Pig" brucellosis in humans is frequently a disease of slaughterhouse workers, farmers, and veterinarians [16]. Direct contact with infected animals or aborted materials may lead to human infection. In humans, brucellosis is generally a chronic illness manifested by intermittent fever, malaise, night sweats, and musculoskeletal and neurological signs [2,16].

For serological testing various tests, usually a screening test of high sensitivity, followed by a confirmatory test of high specificity are used [22]. Generally, c-ELISAs are more specific than i-ELISAs but less sensitive [23]. Sensitivities and specificities of ELISAs were evaluated previously. 100% sensitivity and specificity were found for c-ELISAs, and i-ELISA showed 99.1% specificity and 100% sensitivity, respectively [24]. c-ELISAs proved to be highly sensitive and specific when compared to other commonly used serological tests, i.e., Rose Bengal test, fluorescence polarization assay, i-ELISA for diagnosis of swine brucellosis [25].

Although confirmation of the disease is achieved by bacterial culture and isolation of the etiological agent, *Brucella* is difficult to grow and bacterial culturing and biochemical identification are time consuming. Additionally, this method poses risk to laboratory personnel and requires specific biosafety measures [26]. Hence, detection of DNA by PCR in clinical samples is considered a preferred tool for definitive diagnosis of brucellosis [27].

Although brucellosis in pigs has not been noted in Egyptian surveillance reports, a Rose Bengal plate agglutination assay (RBPT) was performed previously to quantify the risk for workers in slaughterhouses [15].

Considering public health concerns and the zoonotic importance of brucellosis, the present study was aimed to identify seropositive pigs at slaughterhouses and to characterize subsequently the *Brucella* species involved in swine brucellosis in Egypt.

2. Materials and Methods

2.1. Study Area and Sera Collection

The study was conducted from March 2017 to July 2019. The serum samples were collected from abattoirs of Cairo and Giza governorates in Egypt. The data for each sample including origin, sex, and date of sampling were recorded. In total 331 blood samples (116 from males and 215 from females) were collected in sterile vacutainer tubes without anticoagulant. The serum was harvested and stored at −20 °C. The serum was shipped to Friedrich–Loeffler Institut, Jena, Germany for further analysis.

2.2. Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide of the Egyptian Network of Research Ethics Committees (ENREC), which complies with the international laws and regulation regarding ethical considerations in research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Detection of Anti-Brucella Antibodies

Antibody detection was carried out using the IDVet indirect ELISA kit (ID Screen® Brucellosis Serum Indirect Multi-species) (IDVet Innovative Diagnostics Grabels, France) and the SVANOVIR® *Brucella*-Ab c-ELISA kit (Uppsala, Sweden) according to the manufacturer's instructions.

2.4. Molecular Detection of Brucella DNA

DNA was extracted from all collected serum samples by using the QIAamp DNA Mini Kit (QIAGEN, Germany) according to the instructions of the manufacturer.

Genus- (*Brucella*) and species-specific (*B. abortus*, *B. melitensis*, and *B. suis*) multiplex real-time PCRs were used for detection of *Brucella* DNA. PCR was performed using the primer and probe sets given in Table 1 (Jena Bioscience GmbH, Germany). Briefly, the PCR reaction was done in a 15 μL multiplex PCR mixture with 2× TaqMan™ Environmental master mix (Applied Biosystems®, Germany), 0.2 μM of each primer, 0.1 μM of each probe, and 5 μL of template DNA. Amplification and real-time fluorescence detection was carried out on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Germany). The reaction conditions were decontamination at 50 °C for 2 minutes, initial denaturation at 95 °C for 10 minutes followed by 50 cycles of denaturing at 95 °C for 25 seconds and annealing/elongation at 60 °C for 1 minute. Sample data scores were confirmed by visual inspection of graphical plots and cycle threshold (CT) values for each sample were obtained. CT values below 38 were considered positive. Reference strains of *B. abortus* S-99 (ATCC 23448), *B. melitensis* 16M (ATCC 23456), and *B. suis* biovar 1 (ATCC 23444) were used as positive controls for each PCR reaction to ensure no cross-contamination.

Table 1. Primer and probe sequences used in real-time multiplex PCR assay for the detection of *Brucella* spp., *B. abortus*, *B. melitensis*, and *B. suis*.

Target	Primer sequences		Reference
Brucella	5′-GCT CGG TTG CCA ATA TCA ATG C-3′	Forward	[28]
	5′-GGG TAA AGC GTC GCC AGA AG-3′	Reverse	
	FAM-AAATCTTCCACCTTGCCCTTGCCATCA-MGB	Probe	
B. abortus	5′-GCG GCT TTT CTA CGG TAT TC-3′	Forward	
	5′-CAT GCG CTA TGA TCT GGT TAC G-3′	Reverse	
	Joe-CGCTCATGCTCGCCAGACTTCAATG-BHQ-1	Probe	
B. melitensis	5′-AAC AAG CGG CAC CCC TAA AA-3′	Forward	
	5′-CAT GCG CTA TGA TCT GGT TAC G-3′	Reverse	
	NED-CAGGAGTGTTTCGGCTCAGAATAATCCACA-MGB	Probe	
B. suis	5′-GCCAAATATCCATGCGGGAAG-3′	Forward	[29]
	5′-TGG GCA TTC TCT ACG GTG TG-3′	Reverse	
	VIC-TTGCGCTTTTGTGATCTTTGCTTATGG-MGB	Probe	

3. Results

3.1. Anti-Brucella Antibodies in Pig Sera

Out of 331 sera samples, 16 (4.83%) were positive for anti-*Brucella* antibodies by i-ELISA and 36 (10.8%) were positive by c-ELISA, respectively. In the Cairo governorate, 1.21% and 9.75% sera were positive while, in the Giza governorate, 6.02% and 11.2% were positive by i-ELISA and c-ELISA, respectively. Anti-*Brucella* antibodies were detected in 12.9% and 21.5% of boars by i-ELISA and c-ELISA, respectively. Only 1 (0.46%) female animal was seropositive by i-ELISA while 5.11% were positive by c-ELISA (Table 2). Only three sera samples were positive with both ELISAs.

Table 2. Seroprevalence and molecular identification of *Brucella*-DNA in pig sera collected from Cairo and Giza governorates, Egypt.

Governorates	Sex	Number of Samples	Number of Brucellosis Positive Sera		Molecular Identification		
			i-ELISA No. (%)	c-ELISA No. (%)	Real-Time PCR No. (%)	*Brucella* spp. DNA	*Ct* value
Cairo	Male	82	1 (1.21)	8 (9.75)	3 (3.65)	*B. melitensis*	34
						B. melitensis	34
						B. melitensis	36
Giza	Male	34	14 (41.1)	17 (50.0)	4 (11.7)	*B. melitensis*	36
						B. melitensis	29
						B. suis	36
						B. suis	34
	Female	215	1 (0.46)	11 (5.11)	3 (1.39)	*B. melitensis*	36
						B. melitensis	36
						B. suis	36
Total		331	16 (4.83)	36 (10.8)		10 (3.02)	

3.2. Detection of Brucella DNA in Pig Sera

Brucella-specific DNA was detected in 10 (3.02%) samples and typed as *B. melitensis* (7/10) *and B. suis* (3/10) (Table 2). In Cairo, 3.65% sera were positive, and in Giza it was 2.81%. In 6.03% boars *Brucella*-specific DNA was detected, in female pigs it was 1.39%. Boars originating from Giza governorate were more often positive (11.7%) than those from the Cairo governorate (3.65%). Only three sera were positive with all tested assays, while *Brucella* DNA was detected in all c-ELISA positive serum samples.

4. Discussion

This study is the first investigation of swine brucellosis using serological and molecular tools in Egypt. Despite the endemicity of *Brucella* infection in humans and ruminants for many years [7], pig brucellosis has never been reported. Many published studies highlighted the identification of *Brucella* in various animal species (cattle, buffalo, sheep, goat, bison, African buffalo, Alpine ibex) to define their potential role in disease spread [30–33]. The pigs investigated in this study were raised in slums, rural, and per-urban areas likely having close contact with other livestock (cattle, sheep, and goats) which may lead to sharing of pathogens with each other as described previously in Egypt [15].

Swine brucellosis is a zoonotic disease and is widely prevalent in many pig-rearing countries [16]. The proof of the existence of swine brucellosis in Egypt may now raise awareness and can help to tailor control strategies to improve human health.

Usually, brucellosis is diagnosed by using serological screening tests of high sensitivity followed by highly specific tests due to the false–positive reactions which probably arise from cross-reactions with other bacteria and mainly with *Yersinia enterocolitica* O:9. Swine serum may sometimes contain nonspecific antibodies, probably IgM, that reduce the specificity of conventional tests, especially for serum agglutination tests. Moreover, the swine complement interacts with the guinea-pig complement to produce pro-complementary activity that reduces the sensitivity of the complement

fixation test (CFT) [34]. The c-ELISA is more sensitive and specific in swine brucellosis serology [25]. Both serological tests applied showed different results. Previous studies on pig brucellosis found 100% sensitivity and specificity for c-ELISA, and 99.1% specificity and 100% sensitivity for the i-ELISA, respectively [24].

In this study, 331 samples of pigs collected at slaughterhouses of Cairo and Giza governorates that have the highest swine populations in Egypt were investigated. A higher number of seropositive pigs was recorded by c-ELISA (10.8%) when compared to the i-ELISA (4.83%). Although these samples were not taken following the sampling plan of the Egyptian prevalence study plan of ruminants, the ranges are in agreement with the previous prevalence reports of brucellosis in cattle, buffaloes, sheep, and goats in Egypt [7,10,35].

Quantitative real-time PCR for *Brucella* DNA detection has proved highly specific and sensitive when compared to other conventional PCR assays and serology [36]. In the current study, *Brucella* DNA was detected in 3.02% of pig samples. Qualitative multiplex real-time PCR confirmed seven *B. melitensis* and three *B. suis* DNAs. Detection of *B. melitensis* DNA in the present study in pigs reared in Cairo and Giza governorates was expected as previous reports showed the endemicity of *B. melitensis* in these regions in Egypt [7]. The identification of *B. suis* in this study is not unexpected as previously *B. suis* was isolated from cattle [6], ensuring the presence of these species in Egypt. The detection of a higher number of *B. melitensis* DNA samples as compared to *B. suis* DNA in this study is expected as these pigs are in close contact with free grazing sheep and goat flocks. It is common in extensive livestock farming to share pastures and watering. Such type of mixing of animals is an important risk factor to spread the disease from infected to healthy animals or other livestock species [37]. Most sheep and goat flocks are mobile in Egypt. Movements of infected animals can contaminate feeding and grazing areas and may spread infection to other animals (e.g., cattle, buffalo, camel) [10]. The prevalence of *B. melitensis* and *B. suis* in swine may be attributed to the cross-contamination or co-rearing of pigs with other animals [6,15,19,20].

In this study, *Brucella* DNA was detected in 10 (3.02%) of the seropositive samples. Out of 10 positive DNAs, three samples were found positive with both ELISAs (i-ELISA and c-ELISA), while seven DNAs were found positive in samples which were only positive with c-ELISA. The higher number of *Brucella* DNA was identified in c-ELISA positives sera. It was proven that c-ELISA has shown higher sensitivities and specificities for the diagnosis of swine brucellosis [25].

Sex dependent prevalence has been documented in cattle and small ruminants, i.e., it is especially in the case of *B. melitensis* as it is more often found in females. Hence, this phenomenon in pigs has not fully been investigated, it may affect both sexes (male and female) equally [38]. In this study, higher prevalence was found in male pigs than in female pigs. Significantly higher molecular prevalence of brucellosis in males (27.7%) than in females (8.09%) were previously reported from India [21]. Higher prevalence of anti-*Brucella* antibodies in boars was reported also 11.11% vs 3.29% from Nepal, previously [39].

The endemic nature of the disease, particularly the identification of *B. melitensis* and *B. suis* DNA from swine sera suggests a complex underlying epidemiological situation in Egypt.

5. Conclusions

To the best of our knowledge, this is the first study reporting the presence of anti-*Brucella* antibodies and *Brucella* DNA in serum collected from pigs in Egypt. This study, although performed on a limited number of samples and focusing on two governorates only, gives an insight on the situation of brucellosis and *Brucella* species prevalent in pig holdings in Egypt. As the investigated pigs in this study were apparently healthy and admitted for slaughtering, we believe that pigs can be carriers of brucellosis and present a risk to livestock or even humans or may act as a dead-end host, unlikely to be involved in the transmission. Further investigation is needed to assess the prevalence of *Brucella* species particularly *B. suis* in swine to discover ways of cross-contamination and the risk for consumers.

Author Contributions: Data curation, A.U.K., S.A.G.E.E.-S, S.A.M., M.A.S.A. and H.E.-A.; Investigation, A.U.K., H.E.-A. and F.M.; Methodology, A.U.K., F.M. and H.E.-A.; Resources, H.E.-A.; Supervision, F.M., M.E., U.R.,

H.N. and H.E.-A.; Writing – original draft, A.U.K., F.M. and H.E.-A.; Writing – review & editing, F.M., M.C.E., U.R., H.N. and H.E.-A.

Funding: This research received no external funding.

Acknowledgments: The authors thank Michaela Ganss and Katja Fischer at the Institute of Bacterial Infections and Zoonoses, Friedrich–Loeffler Institut (FLI) for their cooperation and technical assistance. This research work was supported by the International Research Project as part of the "German Biosecurity Program" funded by the Federal Foreign Office, Germany. The authors thank Islamic Development Bank (IDB), Jeddah, Saudi Arabia for PhD grant.

Conflicts of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. El-Sayed, A.; Awad, W. Brucellosis: Evolution and expected comeback. *Int. J. Vet. Sci. Med*. **2018**, 6, 31–35. [CrossRef] [PubMed]
2. OIE. Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*) (infection with *B. abortus, B. melitensis* and *B. suis*).In Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019, OIE; World Health Organization for Animal Health: Paris, France, **2019**; pp. 355–398.
3. Kirk, M.D.; Pires, S.M.; Black, R.E.; Caipo, M.; Crump, J.A.; Devleesschauwer, B.; Dopfer, D.; Fazil, A.; Fischer-Walker, C.L.; Hald, T.; et al. World health organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: A data synthesis. *PLoS Med*. **2015**, 12, e1001921.
4. Hosein, H.I.; Zaki, H.M.; Safwat, N.M.; Menshawy, A.M.S.; Rouby, S.; Mahrous, A.; Madkour, B.E. Evaluation of the general organization of veterinary services control program of animal brucellosis in egypt: An outbreak investigation of brucellosis in buffalo. *Vet. World* **2018**, 11, 9. [CrossRef] [PubMed]
5. Refai, M. Incidence and control of brucellosis in the near east region. *Vet. Microbiol*. **2002**, 90, 81–110. [CrossRef]
6. Menshawy, A.M.; Perez-Sancho, M.; Garcia-Seco, T.; Hosein, H.I.; Garcia, N.; Martinez, I.; Sayour, A.E.; Goyache, J.; Azzam, R.A.; Dominguez, L.; et al. Assessment of genetic diversity of zoonotic *Brucella* spp. Recovered from livestock in egypt using multiple locus vntr analysis. *BioMed Res. Int.* **2014**, 2014, 353876. [CrossRef] [PubMed]
7. Wareth, G.; Hikal, A.; Refai, M.; Melzer, F.; Roesler, U.; Neubauer, H. Animal brucellosis in egypt. *J. Infect. Dev. Ctries.* **2014**, 8, 1365–1373. [CrossRef]
8. Hegazy, Y.M.; Molina-Flores, B.; Shafik, H.; Ridler, A.L.; Guitian, F.J. Ruminant brucellosis in upper egypt (2005–2008). *Prev. Vet. Med.* **2011**, 101, 9. [CrossRef]
9. Eltholth, M.M.; Hegazy, Y.M.; El-Tras, W.F.; Bruce, M.; Rushton, J. Temporal analysis and costs of ruminant brucellosis control programme in egypt between 1999 and 2011. *Transbound. Emerg. Dis.* **2017**, 64, 1191–1199. [CrossRef]
10. Samaha, H.; Al-Rowaily, M.; Khoudair, R.M.; Ashour, H.M. Multicenter study of brucellosis in egypt. Emerg. *Infect. Dis.* **2008**, 14, 1916–1918. [CrossRef]
11. Zakaria, A.M.; Ahmed, S.F.; Motawae, M.S. Seropositivity in animals and risk of occupational brucellosis among abattoirs personnel associated with poor work practices and absence of safety policy in egypt. *Int. J. Occup. Environ. Health* **2018**, 24, 55–60. [CrossRef]
12. Lassaletta, L.; Estelles, F.; Beusen, A.H.W.; Bouwman, L.; Calvet, S.; van Grinsven, H.J.M.; Doelman, J.C.; Stehfest, E.; Uwizeye, A.; Westhoek, H. Future global pig production systems according to the shared socioeconomic pathways. *Sci. Total Environ*. **2019**, 665, 739–751. [CrossRef]
13. Abdelwhab, E.M.; Abdel-Moneim, A.S. Epidemiology, ecology and gene pool of influenza a virus in egypt: Will egypt be the epicentre of the next influenza pandemic? *Virulence* **2015**, 6, 6–18. [CrossRef]
14. Gomaa, M.R.; Kandeil, A.; El-Shesheny, R.; Shehata, M.M.; McKenzie, P.P.; Webby, R.J.; Ali, M.A.; Kayali, G. Evidence of infection with avian, human, and swine influenza viruses in pigs in cairo, egypt. *Arch. Virol.* **2018**, 163, 359–364. [CrossRef]
15. Ashraf, M.B.; Hassan, A.; Raafat, M.; Fathia, A.M. Occupational health hazard of egyptian employees in contact with wastage nourished swine. *J. Am. Sci.* **2011**, 7, 6.
16. Olsen, S.C.; Tatum, F.M. Swine brucellosis: Current perspectives. *Vet. Med.* **2017**, 8, 1–12. [CrossRef]

17. Nagalingam, M.; Shome, R.; Balamurugan, V.; Shome, B.R.; NarayanaRao, K.; Vivekananda; Isloor, S.; Prabhudas, K. Molecular typing of *Brucella* species isolates from livestock and human. *Trop. Anim. Health Prod.* **2012**, 44, 5–9. [CrossRef]
18. Higgins, J.; Stuber, T.; Quance, C.; Edwards, W.H.; Tiller, R.V.; Linfield, T.; Rhyan, J.; Berte, A.; Harris, B. Molecular epidemiology of *Brucella abortus* isolates from cattle, elk, and bison in the united states, 1998 to 2011. *Appl. Environ. Microbiol*. **2012**, 78, 10. [CrossRef]
19. Lucero, N.E.; Ayala, S.M.; Escobar, G.I.; Jacob, N.R. *Brucella* isolated in humans and animals in latin america from 1968 to 2006. *Epidemiol. Infect*. **2008**, 136, 496–503. [CrossRef]
20. De Massis, F.; Zilli, K.; Di Donato, G.; Nuvoloni, R.; Pelini, S.; Sacchini, L.; D'Alterio, N.; Di Giannatale, E. Distribution of *Brucella* field strains isolated from livestock, wildlife populations, and humans in italy from 2007 to 2015. *PLoS ONE* **2019**, 14, 16. [CrossRef]
21. Shome, R.; Kalleshamurthy, T.; Natesan, K.; Jayaprakash, K.R.; Byrareddy, K.; Mohandoss, N.; Sahay, S.; Shome, B.R.; Hiremath, J.; Rahman, H.; et al. Serological and molecular analysis for brucellosis in selected swine herds from southern india. *J. Infect. Public Health,* **2019**, 12, 247–251. [CrossRef]
22. Nielsen, K.; Yu, W.L. Serological diagnosis of brucellosis. *Prilozi* **2010**, 31, 65–89.
23. Godfroid, J.; Nielsen, K.; Saegerman, C. Diagnosis of brucellosis in livestock and wildlife. *Croat. Med. J.* **2010**, 51, 296–305. [CrossRef] [PubMed]
24. Di Febo, T.; Luciani, M.; Portanti, O.; Bonfini, B.; Lelli, R.; Tittarelli, M. Development and evaluation of diagnostic tests for the serological diagnosis of brucellosis in swine. *Vet. Ital.* **2012**, 48, 133–156. [PubMed]
25. Praud, A.; Gimenez, O.; Zanella, G.; Pozzi, N.; Antras, V.; Meyer, L.; Garin-Bastuji, B. Evaluation of five serological tests for the diagnosis of porcine brucellosis in french polynesia. *Trop. Anim. Health Prod.* **2013**, 45, 931–933. [CrossRef] [PubMed]
26. Mathew, C.; Stokstad, M.; Johansen, T.B.; Klevar, S.; Mdegela, R.H.; Mwamengele, G.; Michel, P.; Escobar, L.; Fretin, D.; Godfroid, J. First isolation, identification, phenotypic and genotypic characterization of *Brucella abortus* biovar 3 from dairy cattle in tanzania. *BMC Vet. Res.* **2015**, 11, 9. [CrossRef] [PubMed]
27. Ulu-Kilic, A.; Metan, G.; Alp, E. Clinical presentations and diagnosis of brucellosis. Recent Pat. *Anti Infect. Drug Discov.* **2013**, 8, 34–41. [CrossRef]
28. Probert, W.S.; Schrader, K.N.; Khuong, N.Y.; Bystrom, S.L.; Graves, M.H. Real-time multiplex pcr assay for detection of *Brucella* spp., *B. abortus*, and *B. melitensis*. *J. Clin. Microbiol*. **2004**, 42, 1290–1293. [CrossRef]
29. Hansel, C.; Mertens, K.; Elschner, M.C.; Melzer, F. Novel real-time pcr detection assay for *Brucella suis*. *Vet. Rec. Open* **2015**, 2, 7. [CrossRef]
30. Sanogo, M.; Abatih, E.; Thys, E.; Fretin, D.; Berkvens, D.; Saegerman, C. Importance of identification and typing of brucellae from west african cattle: A review. *Vet. Microbiol*. **2013**, 164, 202–211. [CrossRef]
31. Godfroid, J.; Al Dahouk, S.; Pappas, G.; Roth, F.; Matope, G.; Muma, J.; Marcotty, T.; Pfeiffer, D.; Skjerve, E. A "one health" surveillance and control of brucellosis in developing countries: Moving away from improvisation. *Comp. Immunol. Microbiol. Infect. Dis.* **2013**, 36, 8. [CrossRef]
32. Machavarapu, M.; Poonati, R.; Mallepaddi, P.C.; Gundlamadugu, V.; Raghavendra, S.; Polavarapu, K.K.B.; Polavarapu, R. Endemic brucellosis in indian animal and human populations: A billion dollar issue. *J. Curr. Trends Biotechnol. Pharm.* **2019**, 13, 112–123.
33. Godfroid, J. Brucellosis in livestock and wildlife: Zoonotic diseases without pandemic potential in need of innovative one health approaches. *Arch. Public Health* **2017**, 75, 34. [CrossRef] [PubMed]
34. El-Hady, A.M.; Sayed-Ahmed, M.; Saleh, M.E.; Younis, E.E. Seroprevalence and molecular epidemiology of brucellosis in cattle in egypt. *Adv. Dairy Res*. **2016**, 4, 1–4. [CrossRef]
35. Al-Ajlan, H.H.; Ibrahim, A.S.; Al-Salamah, A.A. Comparison of different pcr methods for detection of *Brucella* spp. In human blood samples. *Pol. J. Microbiol*. **2011**, 60, 27–33. [CrossRef] [PubMed]
36. Diaz Aparicio, E. Epidemiology of brucellosis in domestic animals caused by *Brucella melitensis*, *Brucella suis* and *Brucella abortus*. *Rev. Sci. Tech*. **201**3, 32, 53–60. [CrossRef]
37. Coelho, A.C.; Díez, J.G.; Coelho, A.M. Risk factors for *Brucella* spp. In domestic and wild animals. In *Updates on Brucellosis*; Intech Open: London, UK, 2015.

38. Sharma, S.; Khanal, D.R.; Panth, Y. Seroprevalence of brucellosis in pigs of bhaktapur, kavre and banke districts of nepal. *Int. J. Appl. Sci. Biotechnol.* **2017**, 5, 466–469. [CrossRef]

C H A P T E R 3.

Epidemiology of camel brucellosis in Egypt

Seroprevalence and molecular identification of *Brucella* spp. in camels in Egypt

Microorganisms 2020, 8(7): 1035
https://doi.org/10.3390/microorganisms8071035

microorganisms

MDPI

Article

Seroprevalence and molecular identification of *Brucella* spp. in camels in Egypt

Aman Ullah Khan[1,2,3], Ashraf E. Sayour[4], Falk Melzer[1], Sherif Abdel Ghafar Elsayed El-Soally[5], Mandy C. Elschner[1], Waleed S. Shell[6], Amira A. Moawad[1,7], Shereen Aziz Mohamed[8], Ashraf Hendam[9], Uwe Roesler[2], Heinrich Neubauer[1] and Hosny El-Adawy[1,10]*

1 Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, 07743 Jena, Germany; AmanUllah.Khan@fli.de (A.U.K.); falk.melzer@fli.de (F.M.); mandy.elschner@fli.de (M.C.E.); Heinrich.neubauer@fli.de (H.N.); hosny.eladawy@fli.de (H. E-A)
2 Institut for Animal Hygiene and Environmental Health, Free University of Berlin, 14163, Berlin, Germany; uwe.roesler@fu-berlin.de (U.R);
3 Department of Pathobiology, College of Veterinary and Animal Sciences, Jhang 35200, Pakistan
4 Department of Brucellosis, Animal Health Research Institute, Agricultural Research Center, 12618 Dokki - Giza, Egypt; sayourashraf@gmail.com (A. E. S.)
5 Veterinary Service Department, Armed Forces Logistics Authority, Egyptian Armed Forces, 11765 Nasr City, Egypt; dr.sherifelsoaaly@gmail.com (S.A.G.E.E-S)
6 Central Laboratory for Evaluation of Veterinary Biologics, Agricultural Research Center, 11517 Abbasaia-Cairo , Egypt; tarikwaleedshell@hotmail.com (W. S. S.)
7 Provincial Laboratory, Institute of Animal Health Research, Agricultural Research Center, 35516 Mansoura, Egypt. amira.moawad@fli.de (A. A. M.)
8 Veterinary Serum and Vaccine Research Institute, Agricultural Research Center, 11517 Abbasaia- Cairo, Egypt; shereenibraheem1968@gmail.com (S. A. M.)
9 Climate Change Information Center, Renewable Energy and Expert Systems (CCICREES), Agricultural Research Center, 9 Algamaa Street, 12619 Giza , Egypt, a_hendam@hotmail.com (A. H.)
10 Faculty of Veterinary Medicine, Kafrelsheikh University, 33516 Kafr El-Sheikh, Egypt
* Correspondence: Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, 07743 Jena, Germany; Hosny.eladawy@fli.de

Received: 21 April 2020; Accepted: 9 July 2020; Published: 13 July 2020

Abstract: Brucellosis is one of the most important worldwide zoonoses of many countries including Egypt. Camel brucellosis has not gained much attention in Egypt yet. This study is focused on the three governorates with the highest camel populations and the largest camel markets in the country to determine the disease seroprevalence and identify the *Brucella* species in local camel holdings. In total, 381 serum samples were collected from male and female camels from Giza, Aswan, and Al-Bahr Al-Ahmar (The Red Sea) governorates. Samples were serologically examined using the Rose–Bengal plate test (RBPT), indirect ELISA (i-ELISA), competitive ELISA (c-ELISA) and complement fixation test (CFT). *Brucella* antibodies were detected in 59 (15.5%), 87 (22.8%), 77 (20.2%) and 118 (31.0%) of sera by RBPT, i-ELISA, c-ELISA and CFT, respectively. Using real-time PCR, *Brucella* DNA was amplified in 32 (8.4%) seropositive samples including *Brucella abortus* (25/32), *Brucella suis* (5/32) and *Brucella melitensis* (2/32), defining a complex epidemiological status. To the best of our knowledge, this is the first study reporting *Brucella suis* DNA in camel serum. The risk-associated factors including age, sex, breed and geographical distribution were statistically analyzed, showing non-significant association with seroprevalence. The results of this study will raise awareness for camel brucellosis and help develop effective control strategies.

Keywords: brucellosis; camel; *B. suis*; Egypt; seroprevalence; real-time PCR

1. Introduction

Brucellosis is a global zoonotic disease affecting cattle, sheep, goats, camels, pigs and wildlife as well as humans. It is well controlled in many countries but is still endemic in many others with high records in humans in the Middle East and central Asian regions [1]. Brucellosis in camels was first reported in 1931 [2]. Since then, it has been testified by all camel rearing countries like Sudan, Ethiopia, Somalia, Kenya, Nigeria, Jordan and Egypt but not Australia [3]. There is no separate specific species of *Brucella* that displays a preference for camels as they can be infected by those that have already been shown to be prevalent in bovines, ovines, caprines and swines [4–6]. The clinical picture of brucellosis in camels can vary from asymptomatic to abortion, retention of fetal membranes, weak offspring, impaired fertility and delayed sexual maturity in females and orchitis accompanied by lameness in males [2,4,7].

The 120000 camels kept in Egypt represent 1.1% and 0.9% of the total number of camels in Arab countries and Africa, respectively [8]. Higher numbers of camels are raised in countries of the Horn of Africa (Djibouti, Eritrea, Somalia and Ethiopia) as well as parts of Kenya, Sudan, and Uganda [2]. Camels are usually imported from Sudan to Egypt. About half of the camel population lives in the Shalateen area of Al-Bahr Al-Ahmar (The Red Sea) governorate.

Brucellosis has been endemic in Egypt for thousands of years [9,10]. The disease has been detected in livestock predominantly in ruminants with prevalences from 2.47% to 26.66% [11–13]. *Brucella abortus* and *B. melitensis* were isolated from all livestock species and humans but *B. suis* was identified in cattle and pigs only [10,14,15]. There are few seroprevalence reports on camel brucellosis in Egypt, as the disease has not received much attention.

For serological testing, a screening test of high sensitivity is usually followed by a confirmatory test of high specificity [16]. Rose-Bengal plate test (RBPT), complement fixation test (CFT), standard agglutination test (SAT), competitive enzyme-linked immunosorbent assay (c-ELISA), fluorescence polarization assay (FPA) and indirect ELISA (i-ELISA) have been used for detection of anti-*Brucella* antibodies in camel sera [2]. Culture of brucellae is sometimes difficult and time consuming. Additionally, this method poses a risk to laboratory personnel and requires specific biosafety measures [17]. Thus, detection of *Brucella* DNA by PCR in clinical samples is considered a preferred tool for definitive diagnosis of brucellosis [18]. Combination of PCR with at least one of the conventionally used serological tests (e.g., RBPT, SAT, ELISA) was recommended for developing countries [19].

Infection in humans may occur by direct contact with infected animals or consumption of contaminated raw camel milk [2]. Outbreaks of human brucellosis by consumption of infected raw camel milk have been reported in Qatar, Israel and countries of the African Horn [20–22]. Brucellosis proved to be a serious occupational health hazard to livestock handlers especially abattoir workers in Egypt [23].

Considering public health concerns and zoonotic importance of brucellosis, the present study aimed at serological monitoring of camelid brucellosis with molecular identification of *Brucella* species involved in Egypt, filling a gap in knowledge of the disease epidemiology.

2. Materials and Methods

2.1. Study area and sera collection

The study was conducted from March 2017 to November 2019. Sera of the Arabian one-humped or dromedary camel (*Camelus dromedarius*) were collected from Giza, Aswan and Al-Bahr Al-Ahmar (The Red Sea) governorates in Egypt. These governorates house the highest camel populations and the largest camel markets, *viz*. Birqash market in Imbaba (Giza), Daraw market in Aswan and Shalateen International Market in Al-Bahr Al-Ahmar (The Red Sea). The main portals of entry for camels imported from Sudan and Somalia, as well as the main local routes of camel transport to central markets, are shown in Figure 1. Camels usually live for some years on farms to produce milk, to be used for cheesemaking, tourism, etc.

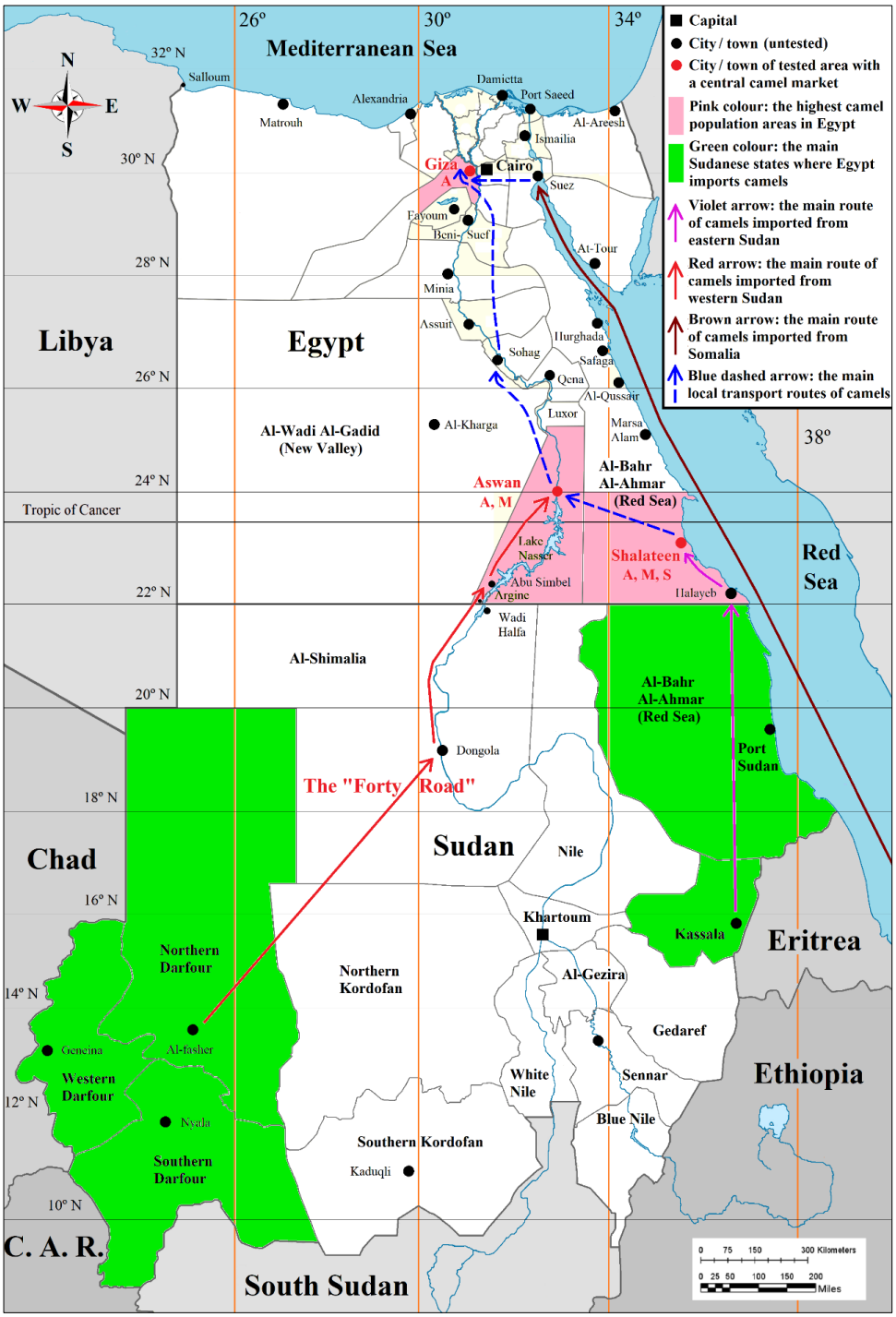

Figure 1. Map of Egypt showing the geographic distribution and the main portals of entry of camels imported from Sudan and Somalia, as well as the main local routes of camel transport to central markets.

The data for each sample including origin, sex, breed and age were recorded. In total, 381 serum samples (106 from Giza, 186 from Aswan, and 89 from Al-Bahr Al-Ahmar (the Red Sea)) from domestic camels were collected in sterile vacutainer tubes without anticoagulant. The serum was separated and stored at −20 °C.

2.2. Ethics statement

This study was carried out in strict accordance with the Guidelines of the Egyptian Network of Research Ethics Committees (ENREC), which complies with the international laws and regulations regarding the ethical considerations in research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Detection of anti-Brucella antibodies

All sera were screened for anti-*Brucella* antibodies by RBPT (IDEXX, Westbrook, ME, USA), indirect ELISA (ID Screen® Brucellosis Serum Indirect Multi-species (protein G-HRP conjugate), IDVet Innovative Diagnostics, Grabels, France), c-ELISA (SVANOVIR® *Brucella*-Ab c-ELISA kit, Boehringer Ingelheim Animal Health International GmbH, Ingelheim, Germany) and complement fixation test (CFT) according to the manufacturers' instructions.

These tests are mainly standardized for use in cattle, but the OIE recommends their use in camels as well after validation [24]. The RBPT antigen was standardized against the OIE International Standard Serum (OIEISS) to give a positive reaction at a dilution of 1:45 and a negative reaction at a dilution of 1:55. The CFT followed the range of recommendations by the OIE [24]. This included an antigen standardized to give 2:200 of the OIEISS (one 50% hemolytic unit), 2% sheep RBCs, two full (100% hemolysis) units of complement and four full units of hemolysin. Serum showing a value ≥20 ICFTU/mL of the OIEISS was considered positive for CFT. ELISA methods conducted and results were calculated in accordance with the manufacturer's instructions.

2.4. Molecular detection of Brucella spp. DNA

DNA was extracted automatically from serum samples by QIAcube machine (QIAGEN, Hilden, Germany) using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer. Reference strains of *Escherichia coli* (ATCC 25922) and serum form non infected animals were used as negative extraction control in each cycle. Genus *Brucella* and species-specific (*B. abortus*, *B. melitensis* and *B. suis*) real-time PCRs were used for detection of *Brucella* DNA. PCR was performed using primer and probe (Jena Bioscience GmbH, Jena, Germany) sets as given in Table 1.

The PCR protocol was modified (volume and temperature) than previously published [25,26] to obtain most optimal results as DNA used in this study as template was extracted from serum not from bacterial colonies. Briefly, PCR reaction was performed in 15 µl multiplex PCR mixture with 2× TaqMan™ environmental master mix (Applied Biosystems®, Darmstadt, Germany), 0.2 µM of each primer, 0.1 µM of each probe and 5 µl of template DNA. Amplification and real-time fluorescence detection were carried out on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The reaction conditions were: decontamination at 50°C for 2 minutes, initial denaturation at 95°C for 10 minutes followed by 50 cycles of denaturing at 95°C for 25 seconds and anealing/ elongation at 57°C (*B. abortus* and *B. melitensis*) [25] and 60°C (*B. suis*) [26] for one minute. Sample data scores were confirmed by visual inspection of graphical plots and Cycle Threshold (CT) values for each sample were obtained. CT values ≤ 38 were considered positive after in house validation to avoid false positive results. The CT values of negative extraction controls were either > 38 or not detected. Reference strains of *B. abortus* S-99 (ATCC 23448), *B. melitensis* 16M (ATCC 23456) and *B. suis* biovar 1 (ATCC 23444) were used as positive controls. Reference strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Ochrobactrum intermedium* (DSM 17986) were used as negative controls.

Table 1. Primer and probe sequences used in real-time PCR assays for the detection of *Brucella* spp., *B. abortus, B. melitensis* and *B. suis* in camel sera, Egypt.

Target	Primer and probe sequences		Reference
Brucella Spp.	**5′-**GCT CGG TTG CCA ATA TCA ATG C-**3′** **5′-**GGG TAA AGC GTC GCC AGA AG-**3′** 6-FAM-AAA TCT TCC ACC TTG CCC TTG CCA TCA-MGB	Forward Reverse Probe	[25]
B. abortus	**5′-**GCG GCT TTT CTA CGG TAT TC-**3′** **5′-**CAT GCG CTA TGA TCT GGT TAC G-**3′** Hex-CGC TCA TGC TCG CCA GAC TTC AAT G-BHQ1	Forward Reverse Probe	
B. melitensis	**5′-**AAC AAG CGG CAC CCC TAA AA-**3′** **5′-**CAT GCG CTA TGA TCT GGT TAC G-**3′** Cy5-CAG GAG TGT TTC GGC TCA GAA TAA TCC ACA-BHQ2	Forward Reverse Probe	
B. suis	**5′-**GCC AAA TAT CCA TGC GGG AAG-**3′** **5′-**TGG GCA TTC TCT ACG GTG TG-**3′** VIC-TTG CGC TTT TGT GAT CTT TGC TTA TGG-MGB	Forward Reverse Probe	[26]

2.5. Statistical analysis

The agreement of positive camel results of serological tests and real-time PCR was expressed using Venn diagrams (Figure 2).

Correlation of potential risk factors (geographical location, breed type, sex and age) with seroprevalence and molecular detection of 381 camels was analyzed using Pearson`s Chi-squared test (X^2) and odds ratio (OR). The estimation of X^2 was done using RStudio Version 1.1.463.

3. Results

3.1. Seroprevalence of anti-Brucella antibodies in camel sera

Out of 381 camel serum samples, 59 (15.5%), 87 (22.8%), 77 (20.2%) and 118 (31.0%) were found positive for *Brucella* antibodies by RBPT, i-ELISA, c-ELISA and CFT, respectively (Table 2). Higher numbers of seropositive animals, i.e. 17.7%, 25.8%, 22.0% and 31.7% were detected from Aswan governorate using RBPT, i-ELISA, c-ELISA and CFT, respectively. The corresponding values from Giza governorate were 14.2%, 22.6%, 14.2% and 31.1% and from Al-Bahr Al-Ahmar (the Red Sea) governorate were 12.4%, 16.9%, 23.6% and 29.2% in that order. Only 16 serum samples were found seropositive by all serological tests.

Higher seroprevalences were recorded in male animals (17.6%, 24.1%, 23.4% and 34.9%) than female animals (8.1%, 18.6%, 12.8% and 17.4%) using RBPT, i-ELISA, c-ELISA and CFT, respectively, in the investigated governorates (Table 2).

Correlation of potential risk factors (geographical location, breed, sex and age) with seroprevalence and molecular detection of *Brucella* spp. in 381 camels is shown in Table 3. Using i-ELISA, 25.8% of seropositive samples were found in Aswan, 22.6% in Giza and 16.9% in Al-Bahr Al-Ahmar (the Red Sea) (Table 3).

Table 2. Seroprevalence and molecular identification of *Brucella* DNA in camel sera collected from Giza, Aswan and Al-Bahr Al-Ahmar (The Red Sea) governorates, Egypt.

Governorate	Sex	Number of Samples	Seroprevalence *n* (%)				Molecular Identification		
			RBPT *	i-ELISA *	c-ELISA *	CFT *	Real-Time-PCR n (%)	*Brucella* spp. DNA Identification	*Cq/Ct-Values* **
Giza	male	55	11 (20.0)	17 (30.9)	7 (12.7)	28 (50.9)	3 (5.5)	3 *B. abortus*	37, 36, 35
	female	51	4 (7.8)	7 (13.7)	8 (15.7)	5 (9.8)	6 (11.8)	6 *B. abortus*	30, 32, 33, 36, 37
Sub-total		106	15 (14.2)	24 (22.6)	15 (14.2)	33 (31.1)		9 (8.5)	
Aswan	male	161	30 (18.6)	41 (25.5)	39 (24.2)	51 (31.7)	7 (4.4)	6 *B. abortus*	37, 37, 36, 36, 36, 36
								1 *B. melitensis*	34
	female	25	3 (12.0)	7 (28.0)	2 (8.0)	8 (32.0)	1 (4.0)	1 *B. abortus*	36
Sub-total		186	33 (17.7)	48 (25.8)	41 (22.0)	59 (31.7)		8 (4.3)	
Al-Bahr Al-Ahmar (the Red Sea)	male	79	11(13.9)	13 (16.5)	20 (25.3)	24 (30.4)	14 (17.7)	8 *B. abortus*	37, 35, 31, 37, 35, 32, 36, 35
								1 *B. melitensis*	36
								5 *B. suis*	37, 36, 33, 28, 37
	female	10	0 (0.0)	2 (20.0)	1 (10.0)	2 (20.0)	1 (10.0)	1 *B. abortus*	37
Sub-total		89	11 (12.4)	15 (16.9)	21 (23.6)	26 (29.2)		15 (16.9)	
Grand-total		381	59 (15.5)	87 (22.8)	77 (20.2)	118 (31.0)		32 (8.4)	

* RBPT: Rose-Bengal plate test; i-ELISA: indirect ELISA; c-ELISA: competitive ELISA; CFT: complement fixation test.

** Cq/Ct-values: cycle quantification/ cycle threshold values.

Table 3. Relation of the risk factors with the seroprevalence and molecular detection of brucellosis in 381camels, Egypt.

Variable	Seroprevalence *n* (%)				Molecular Identification	
	RBPT *	i-ELISA *	c-ELISA *	CFT *	Real-Time-PCR *n* (%)	*Brucella* DNA Identification
Geographical location						
Aswan (*n* = 186)	33 (17.7)	48 (25.8)	41 (22.0)	59 (31.7)	8 (4.3)	7 *B. abortus* 1 *B. melitensis*
Giza (*n* =106)	15 (14.2)	24 (22.6)	15 (14.2)	33 (31.1)	9 (8.5)	9 *B. abortus*
Al-Bahr Al-Ahmar (the Red Sea) (*n* = 89)	11 (12.4)	15 (16.9)	21 (23.6)	26 (29.2)	15 (16.9)	9 *B. abortus* 5 *B. suis* 1 *B. melitensis*
p-value **	0.4688	0.3205	0.4171			
X2	1.5153	2.2757	1.7489			
Df		2		NA		NA
95% CI	-	-	-			
OR	-	-	-			
Breed						
Al-Beshary (*n* = 89)	11 (12.4)	15 (16.9)	21 (23.6)	26 (29.2)	15 (16.9)	9 *B. abortus* 5 *B. suis* 1 *B. melitensis*
Al-Ebadi (*n* = 93)	16 (17.2)	26 (28.0)	22 (23.7)	30 (32.3)	4 (4.3)	3 *B. abortus* 1 *B. melitensis*
Al-Zemkly (*n* = 106)	15 (14.2)	24 (22.6)	15 (14.2)	33 (31.1)	9 (8.5)	9 *B. abortus*
Al-Zubaidi (*n* = 93)	17 (18.3)	22 (23.7)	19 (20.4)	29 (31.2)	4 (4.3)	4 *B. abortus*
p-value **	0.6775	0.4658	0.5823			
X2	1.5205	2.5532	1.9524			
Df		3		NA		NA
95% CI	-	-	-			
OR	-	-	-			
Sex						
Females (*n* = 86)	7 (8.1)	16 (18.6)	11 (12.8)	15 (17.4)	8 (9.3)	8 *B. abortus*

Males (*n* = 295)	52 (17.6)	71 (24.1)	66 (23.4)	103 (34.9)	24 (8.3)	17 *B. abortus* 5 *B. suis* 2 *B. melitensis*
p-value **	0.7177	0.3515	0.9164	NA		NA
X2	0.13075	0.86806	0.011028			
Df		1				
95% CI	0.2819–2.2582	0.6333–3.1044	0.4215–2.4729			
OR	0.8438063	1.4091	1.0436			
			Age			
<8 years (*n* = 227)	39 (17.2)	52 (22.9)	46 (20.2)	81 (35.7)	21 (9.3)	15 *B. abortus* 5 *B. suis* 1 *B. melitensis*
≥8–11 years (*n* = 68)	13 (19.1)	19 (27.9)	20 (29.4)	22 (32.4)	3 (4.4)	2 *B. abortus* 1 *B. melitensis*
11–13 years (*n* = 51)	4 (7.8)	7 (13.7)	8 (15.7)	5 (9.8)	6 (11.8)	6 *B. abortus*
>13–15 years (*n* = 35)	3 (8.6)	9 (25.71)	3 (8.6)	10 (28.6)	2 (5.7)	2 *B. abortus*
p-value **	0.7844	0.5792	0.1672	NA		NA
X2	1.0699	1.9674	5.0641			
Df		3				
95% CI	-	-	-			
OR	-	-	-			

* The Univariate analysis was based on the RBPT, i-ELISA and cELISA results; OR: odds ratio; CI: confidence interval; df: degree of freedom; X2: Pearson's Chi-squared test; Ref: reference. ** (Statistical value of significance: *p*-value ≤ 0.05).

The seroprevalence in different breeds using i-ELISA was 16.9% (15 out of 89) in Al-Beshary, 28.0% (26 out of 93) in Al-Ebadi, 22.6% (24 out of 106) in Al-Zemkly and 23.7% (22 out of 93) in Al-Zubaidi breeds (Table 3).

The results of this study showed relatively higher seropositive males (17.6%, 24.1% and 23.4%) than females (8.1%, 18.6% and 12.8%) using RBPT, iELISA and cELISA, respectively with confidence intervals (95%CI) 0.2819-2.2582, 0.6333-3.1044 and 0.4215-2.4729, respectively.

Seroprevalences of age groups were 22.9% (52/227), 27.9% (19/68), 13.7% (7/51) and 25.7% (9/35) in animals of < 8 years, ≥ 8 - 11 years, 11- 13 years and > 13-15 years using iELISA, respectively. In the univariate analysis based on iELISA, all variables (geographical location, breed, age and sex) showed no association with seroprevalence (Table 3).

3.2. Detection of Brucella spp. DNA in camel sera

Brucella DNA was detected in serum samples positive by either RBPT, i-ELISA, c-ELISA or CFT. *Brucella* DNA was detected in 32 (8.4%) samples and was typed as *B. abortus* (25/32), *B. suis* (5/32) and *B. melitensis* (2/32) (Table 2). *Brucella* DNA was detected in 4.3%, 8.49% and 16.8% of camels from Aswan, Giza and Al-Bahr Al-Ahmar (The Red Sea), respectively. *Brucella* DNA was amplified in 14.7% (24 out 295; 17 *B. abortus*, 5 *B. suis* and 2 *B. melitensis*) and 9.3% (8 out 86; *B. abortus*) samples of male and female camels, respectively. The DNA concentration of positive samples was not sufficient for optimal sequencing.

Sera from Al-Bahr Al-Ahmar (The Red Sea) governorate were more often positive in PCR (16.8%) than those of the Giza governorate (8.5%) and Aswan governorate (4.3%).

Brucella DNA was identified in all camel breeds (Table 3). Identification of *B. suis* DNA from camel sera is a new finding of this study. *B. suis* was only identified in 5 seropositive male animals of breed Al-Beshary in Al-Bahr Al-Ahmar (The Red Sea) governorate.

The *Brucella* DNA extracted from seronegative samples by either RBPT, i-ELISA, c-ELISA or CFT was either not amplified or showed CT values >38 by real-time PCR.

3.3. Statistical analysis

Investigating the agreement of the results of positive cases detected by serological tests and real-time PCR, the Venn diagram (Figure 2) reveals that 60, 18, 13 and 5 camels were identified as positives by CFT, c-ELISA, i-ELISA and RBPT only. There were only 4 animals classified as positive by all serological assays and real-time PCR. The CFT agreed with real-time PCR, i-ELISA, c-ELISA and RBPT in 12, 34, 33 and 32 positive animals, respectively. Indirect ELISA and c-ELISA had 40 positive results (Figure 2).

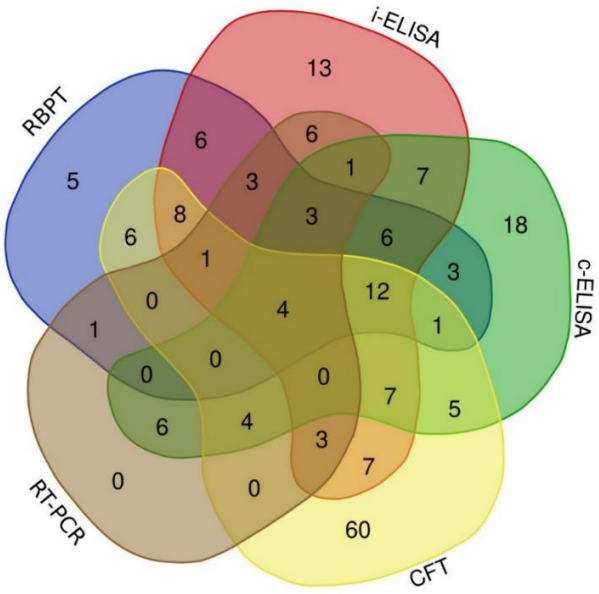

Figure 2. Venn diagram showing the agreement of positive results of serological tests and real-time PCR.

4. Discussion

Despite the available data on *Brucella* infection in humans and ruminants, little is known about the status of camel brucellosis in Egypt. The identification of *Brucella* spp. in various farm animals and wildlife species (*viz*. cattle, buffalo, sheep, goat, camel, bison, African buffalo, Alpine ibex) highlights their role in disease spread [27–31]. Consumption of raw milk and dairy products of infected camels was associated with brucellosis in humans [20–22]. This study is investigating camel brucellosis in three Egyptian governorates with the highest number of camels, *viz*. Giza, Aswan and Al-Bahr Al-Ahmar (the Red Sea). The latter two governorates are the main entry portals for camels imported from Sudan. Giza receives imported Sudanese camels from Aswan as well as Somali camels shipped to the port of Suez. Apart from camels smuggled through the desert, Egypt has been importing camels officially from east Africa where brucellosis is enzootic in ruminants including camels [19,32,33]. The fact that these camels are imported do not preclude the possibility of acquiring brucellosis from a local source.

In this study, 381 camel sera were investigated serologically and 59 (15.5%), 87 (22.8%), 77 (20.2%) and 118 (31.0%) were found positive for anti-*Brucella* antibodies by RBPT, i-ELISA, c-ELISA and CFT, respectively (Table 2). Previous report from camel-keeping countries have revealed seroprevalence of camel brucellosis ranging from 1.0 to 23.3% [4]. The seroprevalence of brucellosis in apparently healthy Sudanese camels were 79.3%, 71.4%, 70.7%, 70.6% and 68.8% using FPA, CFT, RBT, SAT and c-ELISA [19]. In camels, the prevalence was 12.9% in the Shalateen region of the Red Sea governorate [5]. One study from the regions of Siwa Oasis, Asyut and Cairo reported a prevalence of 4.17% using RBPT [34]. A similar study from Beheira district revealed a prevalence of 8.74% and 9.26% using RBPT and ELISA, respectively [35]. The general consistency of seroprevalence data from all the governorates as revealed by i-ELISA (25.8%, 22.6% and 16.9%) in Aswan, Giza and Al-Bahr Al-Ahmar respectively) is a reflection of the continuous flow and regular distribution of imported camels from the same source countries.

In the current study, *Brucella* DNA was detected in 32 (8.4%) of all investigated camel sera (Table 2). Quantitative multiplex real-time PCR confirmed the presence of *Brucella* DNAs of 25 *B. abortus*, 5 *B. suis* and 2 *B. melitensis*. Detection of *B. abortus* DNA in the three target governorates in addition to *B. melitensis* in camels reared in Aswan and Al-Bahr Al-Ahmar (the Red Sea) governorates was expected as previous reports showed the endemicity of *B. melitensis* and *B. abortus* in these regions

already [10]. Previously, *B. melitensis* was isolated from camel stomach contents of an aborted fetus [5] as well as from whole citrated blood samples from Al-Bahr Al-Ahmar (the Red Sea) governorate [36]. Similar studies reported the identification of *B. melitensis* DNA from camel milk from Giza and Aswan [34,37]. The source of *B. melitensis* in camels might be attributed to small ruminants as camels are usually reared in herds with sheep and goats in mobile flocks [31,38].

As *B. abortus* is enzootic in Egypt, the detection of a relevant number of camel sera containing *B. abortus* DNA might indicate that *B. abortus* may be the predominant spp. in camels in this region but more results are needed to confirm this.

B. abortus has been isolated from camels in Sudan and it can be speculated that camels were infected by cattle, the primary hosts of *B. abortus* [39]. These data do not allow to speculate if camels were already infected when imported or that they got infected in Egypt. Interestingly, more positive serum samples were collected from Al-Bahr Al-Ahmar (the Red Sea) governorate sharing common borders with Sudan. This governorate hosts about half of the Egyptian camels, a fact that may favour the spread of brucellosis in these regions [8]. The very low amount of *Brucella* DNA extracted from camel sera hindered biotyping. Further investigation of the Egyptian *B. abortus* strain is necessary to prove or deny the epidemiological relation with the Sudanese *B. abortus* (biovar 6) strains detected in cattle in Darfour [40] and sheep in Kassala [41] as well as *B. abortus* biovar 3 from camels in Eastern Sudan [42]. Camel herds move between the states of North Sudan [43] and they are reared with cattle, sheep and goats [31]. Camels from the whole Darfour sector usually gather at Southern Darfour during the autumn months seeking water of the tropical heavy rain season. There, they also mingle intensely with cattle again. Unlike camels, Sudanese cattle do cross the Sudanese borders during the dry season to South Sudan, Central African Republic and Congo reaching as far as Niger and Mali to the West.

The detection of *B. suis* DNA is a new finding of this study probably attributable to the first use of recently developed highly sensitive and specific primer for *B. suis* biovars 1 to 4 to test camel sera [26]. However, the identification of *B. suis* in the present study is not unexpected as *B. suis* has previously been isolated from cattle [10] and *B. suis* or its DNA was identified in pigs in Egypt [15,44].

The source of *B. suis* in camels could be traceable to either domestic or wild pigs, e.g., the wild boars (*Sus scrofa*) of the adjacent Eastern Desert. Being a border governorate with Sudan, Al-Bahr Al-Ahmar (the Red Sea), is also likely to have *B. suis* imported from Sudan, where some pig farms in Khartoum state to the west of Kassala state exist. The uncontrolled transboundary movement of Sudanese cattle to adjacent African countries, i.e., South Sudan and Central African Republic may contribute to the spread of *B. suis* as both states have domestic and wild pigs [45]. The countries of the Horn of Africa, with huge camel populations have similar restricted pig populations comparable to Egypt in contrast to Uganda [45]. The exact source of B. suis should be traced to stop further transmission as camels could have acquired the disease from a local source.

Identification of risk factors is crucial for control of brucellosis. Animal related risk factors (age, sex, breed and species), farm management, geographical distribution, herd management and farmers' awareness of brucellosis have been associated with the prevalence of brucellosis [46].

In this study, the seroprevalences of age groups were 22.9% (52/227), 27.9% (19/68), 13.7% (7/51) and 25.7% (9/35) in animals of <8 years, ≥8–11 years, 11–13 years and >13–15 years using iELISA, respectively. In previous study, the seroprevalence was significantly higher (29.4%) in camels brought for slaughtering at Akaki abattoir, Ethiopia of the 5–9 years age group when compared to other age groups (0–4.8%) using RBPT [47]. However, known risk factors (age, sex, breed and locality) were found unrelated, which is consistent with a previously published report [48]. Rearing of camels with other farm animals might also identified as important risk factors of camel brucellosis as previously described [2,47,49,50].

None of the tests can differentiate among *B. abortus*, *B. suis* and *B. melitensis*. Many immunoassays are available with different sensitivity and specificity but they must be used in accordance with strict standardization rules and meet the requirements laid down by the OIE [24]. An obvious discrepancy among the tests used in this study was seen: (CFT (31.0%), i-ELISA (22.8%), c-ELISA (20.2%), RBPT (15.5%) and real-time PCR (8.4%)). Although these samples were not taken

according to the sampling plan of the Egyptian surveillance policy of ruminants, the ranges were in agreement with the previously published reviews and reports of camel brucellosis (ranging from 1.0% to 24.0%) in Egypt [4,5,34,51].

Interestingly, the results of this study showed relatively higher seropositive males (17.6%, 24.1% and 23.4%) than females (8.1%, 18.6% and 12.8%) using RBPT, iELISA and cELISA, respectively. This may be due to the fact that the vast majority of imported camels are males for slaughter with some females that farmers usually keep for breeding.

Of the 32 real-time PCR positive cases, the i-ELISA identified 21 followed by the c-ELISA (18) and the CFT and the RBPT each detected 12. In terms of positive camel recognition, the CFT revealed the highest number (118), followed by the i-ELISA (87), the c-ELISA (77), the RBPT (59), and finally the real-time PCR (32), with exclusive detections of 60, 18, 13, 5 and 0 by every single test, respectively. It is noteworthy that the 60 CFT positive samples that were negative by all other tests revealed low titers of 1:10 or 1:20 and rarely 1:40.

The nature of camelid humoral immune response and the unique nature of their heavy chain antibodies might be an explanation for these puzzling results. A reduced sensitivity of c-ELISA has been reported previously [52]. These findings call for validation and standardization of tested kits for camel brucellosis and in the worst case scenario for the development of new "camelid" diagnostics.

5. Conclusion

Under conditions of this investigation, DNA of three *Brucella* species was identified in 32 camel sera. *B. abortus* was the most common (25 camels), followed by *B. suis* in 5 camels and *B. melitensis* in only 2 camels. To the best of our knowledge, this is the first study reporting the identification of *B. suis* DNA in serum from camels. As camels in this study were apparently healthy, we believe that camels can act both as a reservoir of brucellosis and as a source of infection to other camels. The relative high seropositive camels in this study might reflect that the camels were imported from brucellosis infected herds.

The endemic nature of the disease together with the DNA identification of the three classic *Brucella* species in camel sera demonstrate complicated epidemiological situation that needs careful handling. Further investigation is needed to assess the prevalence of *Brucella* species particularly *B. suis* in camels as well as biovar and genotype identification. More attention should be paid to the standardization of serological tests for brucellosis diagnosis in camels.

Author Contributions: Data curation, S.A.G.E.E.-S. and H.E.-A.; Investigation, A.U.K., F.M., S.A.G.E.E.-S., W.S.S., A.A.M. and H.E.-A.; Methodology, A.U.K., A.E.S., F.M., A.H., H.N. and H.E.-A.; Supervision, F.M., M.C.E., U.R., H.N. and H.E.-A.; Writing–original draft, A.U.K. and H.E-A.; Writing–review & editing, A.U.K., A.E.S., F.M., S.A.G.E.E-S., M.C.E.,W.S.S., A.A.M., S.A.M., A.H., U.R., H.N. and H.E.-A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors thank Michaela Ganss and Katja Fischer at the Institute of Bacterial Infections and Zoonoses, Friedrich–Loeffler Institut (FLI) for their cooperation and technical assistance. This research work was supported by the International Research Project as part of the "German Biosecurity Program" funded by the Federal Foreign Office, Germany. The authors thank the Islamic Development Bank (IDB), Jeddah, Saudi Arabia for PhD grant.

Conflicts of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. Kirk, M.D.; Pires, S.M.; Black, R.E.; Caipo, M.; Crump, J.A.; Devleesschauwer, B.; Döpfer, D.; Fazil, A.; Fischer-Walker, C.L.; Hald, T.; et al. World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. *PLoS Med.* **2015**, *12*, e1001921, doi:10.1371/journal.pmed.1001921.

2. Fatima, S.; Khan, I.; Nasir, A.; Younus, M.; Saqib, M.; Melzer, F.; Neubauer, H.; El-Adawy, H. Serological, molecular detection and potential risk factors associated with camel brucellosis in Pakistan. *Trop. Anim. Heal. Prod.* **2016**, *48*, 1711–1718, doi:10.1007/s11250-016-1148-9.
3. Gyuranecz, M.; Wernery, U.; Kreizinger, Z.; Juhász, J.; Felde, O.; Nagy, P. Genotyping of Brucella melitensis strains from dromedary camels (*Camelus dromedarius*) from the United Arab Emirates with multiple-locus variable-number tandem repeat analysis. *Veter Microbiol.* **2016**, *186*, 8–12, doi:10.1016/j.vetmic.2016.02.009.
4. Gwida, M.; Elgohary, A.; Melzer, F.; Khan, I.; Rösler, U.; Neubauer, H. Brucellosis in camels. *Res. Veter Sci.* **2012**, *92*, 351–355, doi:10.1016/j.rvsc.2011.05.002.
5. Sayed-Ahmed, M.; Mohamed, Z.S.-A.; Mohamed, M.E.-D.; Mohamed, A.E.-B.; Sherif, M.S.; Emad, E.Y.; El-Sayed, A.M.; El-Diasty, M.M.; El-Beskawy, M.A.; Shoieb, S.M.; et al. Sero-prevalence of camel brucellosis (*Camelus dromedarius*) and phenotypic characteristics of Brucella melitensis biovar 3 in Shalateen City, Red Sea Governorate, Egypt. *Afr. J. Microbiol. Res.* **2017**, *11*, 1259–1266, doi:10.5897/ajmr2017.8629.
6. Salisu, U.; Kudi, C.; Bale, J.; Babashani, M.; Kaltungo, B.; Saidu, S.; Asambe, A.; Baba, A. Seroprevalence of *Brucella* antibodies in camels in Katsina State, Nigeria. *Trop. Anim. Heal. Prod.* **2017**, *49*, 1041–1046, doi:10.1007/s11250-017-1297-5.
7. Sprague, L.D.; al Dahouk, S.; Neubauer, H. A review on camel brucellosis: A zoonosis sustained by ignorance and indifference. *Pathog. Glob. Heal.* **2012**, *106*, 144–149, doi:10.1179/2047773212y.0000000020.
8. Othman, O.E.; El-Kader, H.A.A.; Alam, S.S.; El-Aziem, S.H.A. Cytochrome b conservation between six camel breeds reared in Egypt. *J. Genet. Eng. Biotechnol.* **2017**, *15*, 1–6, doi:10.1016/j.jgeb.2017.04.006.
9. Refai, M. Incidence and control of brucellosis in the Near East region. *Veter Microbiol.* **2002**, *90*, 81–110, doi:10.1016/s0378-1135(02)00248-1.
10. Menshawy, A.; Perez-Sancho, M.; García-Seco, T.; Hosein, H.I.; García, N.; Martínez, I.; Sayour, A.; Goyache, J.; Azzam, R.A.A.; Domínguez, L.; et al. Assessment of Genetic Diversity of Zoonotic *Brucella* spp. Recovered from Livestock in Egypt Using Multiple Locus VNTR Analysis. *BioMed Res. Int.* **2014**, *2014*, 1–7, doi:10.1155/2014/353876.
11. Wareth, G.; Hikal, A.; Refai, M.; Melzer, F.; Roesler, U.; Neubauer, H. Animal brucellosis in Egypt. *J. Infect. Dev. Ctries.* **2014**, *8*, 1365–1373, doi:10.3855/jidc.4872.
12. Hegazy, Y.M.; Molina-Flores, B.; Shafik, H.; Ridler, A.; Guitian, F.; Guitian, J. Ruminant brucellosis in Upper Egypt (2005–2008). *Prev. Veter Med.* **2011**, *101*, 173–181, doi:10.1016/j.prevetmed.2011.05.007.
13. Eltholth, M.M.; Hegazy, Y.M.; El-Tras, W.F.; Rushton, J.; Bruce, M. Temporal Analysis and Costs of Ruminant Brucellosis Control Programme in Egypt Between 1999 and 2011. *Transbound. Emerg. Dis.* **2016**, *64*, 1191–1199, doi:10.1111/tbed.12491.
14. Samaha, H.; Al-Rowaily, M.; Khoudair, R.M.; Ashour, H.M. Multicenter Study of Brucellosis in Egypt. *Emerg. Infect. Dis.* **2008**, *14*, 1916–1918, doi:10.3201/eid1412.071452.
15. Khan, A.U.; Melzer, F.; El-Soally, S.A.G.E.; Elschner, M.C.; Mohamed, S.A.; Ahmed, M.A.S.; Roesler, U.; Neubauer, H.; El-Adawy, H.; Khan; et al. Serological and Molecular Identification of *Brucella* spp. in Pigs from Cairo and Giza Governorates, Egypt. *Pathogens* **2019**, *8*, 248, doi:10.3390/pathogens8040248.
16. Nielsen, K.; Yu, W.L. Serological diagnosis of brucellosis. *Prilozi* **2010**, *31*, 65–89.
17. Mathew, C.; Stokstad, M.; Johansen, T.B.; Klevar, S.; Mdegela, R.H.; Mwamengele, G.; Michel, P.; Escobar, L.; Fretin, D.; Godfroid, J. First isolation, identification, phenotypic and genotypic characterization of *Brucella abortus* biovar 3 from dairy cattle in Tanzania. *BMC Veter Res.* **2015**, *11*, 156, doi:10.1186/s12917-015-0476-8.
18. Ulu-Kilic, A.; Metan, G.; Alp, E. Clinical presentations, and diagnosis of brucellosis. *Recent Pat. Antiinfect Drug Dis.* **2013**, *8*, 34–41.
19. Gwida, M.; Elgohary, A.; Melzer, F.; Tomaso, H.; Roesler, U.; Wernery, U.; Wernery, R.; Elschner, M.; Khan, I.; Eickhoff, M.; et al. Comparison of diagnostic tests for the detection of *Brucella* spp. in camel sera. *BMC Res. Notes* **2011**, *4*, 525, doi:10.1186/1756-0500-4-525.
20. Garcell, H.G.; Garcia, E.G.; Pueyo, P.V.; Martín, I.R.; Arias, A.V.; Serrano, R.N.A. Outbreaks of brucellosis related to the consumption of unpasteurized camel milk. *J. Infect. Public Heal.* **2016**, *9*, 523–527, doi:10.1016/j.jiph.2015.12.006.
21. Rhodes, H.M.; Williams, D.N.; Hansen, G.T. Invasive human brucellosis infection in travelers to and immigrants from the Horn of Africa related to the consumption of raw camel milk. *Travel Med. Infect. Dis.* **2016**, *14*, 255–260, doi:10.1016/j.tmaid.2016.03.013.

22. Ben-Shimol, S.; Dukhan, L.; Belmaker, I.; Bardenstein, S.; Sibirsky, D.; Barrett, C.; Greenberg, D. Human brucellosis outbreak acquired through camel milk ingestion in southern Israel. *Isr. Med Assoc. J. IMAJ* **2012**, *14*, 475–478.
23. Zakaria, A.M.; Ahmed, S.F.; Motawae, M.S. Seropositivity in animals and risk of occupational brucellosis among abattoirs personnel associated with poor work practices and absence of safety policy in Egypt. *Int. J. Occup. Environ. Heal.* **2018**, *24*, 55–60, doi:10.1080/10773525.2018.1516839.
24. OIE. Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*) (infection with *B. abortus, B. melitensis* and *B. suis*) In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019, OIE*; World Health Organization for Animal Health World Organisation for Animal Health (Office International des Épizooties): Paris, France: 2019; pp. 355–398.
25. Probert, W.S.; Schrader, K.N.; Khuong, N.Y.; Bystrom, S.L.; Graves, M.H. Real-Time Multiplex PCR Assay for Detection of *Brucella* spp., *B. abortus*, and *B. melitensis*. *J. Clin. Microbiol.* **2004**, *42*, 1290–1293, doi:10.1128/jcm.42.3.1290-1293.2004.
26. Hänsel, C.; Mertens, K.; Elschner, M.C.; Melzer, F. Novel real-time PCR detection assay for *Brucella suis*. *Veter Rec. Open* **2015**, *2*, doi:10.1136/vetreco-2014-000084.
27. Sanogo, M.; Abatih, E.; Thys, E.; Fretin, D.; Berkvens, D.; Saegerman, C. Importance of identification and typing of *Brucellae* from West African cattle: A review. *Vet. Microbiol.* **2013**, *164*, 202–211, doi:10.1016/j.vetmic.2013.02.009.
28. Godfroid, J.; al Dahouk, S.; Pappas, G.; Roth, F.; Matope, G.; Muma, J.; Marcotty, T.; Pfeiffer, D.U.; Skjerve, E. A "One Health" surveillance and control of brucellosis in developing countries: Moving away from improvisation. *Comp. Immunol. Microbiol. Infect. Dis.* **2013**, *36*, 241–248, doi:10.1016/j.cimid.2012.09.001.
29. Machavarapu, M.; Poonati, R.; Mallepaddi, P.C.; Gundlamadugu, V.; Raghavendra, S.; Polavarapu, K.K.B.; Polavarapu, R. Endemic brucellosis in Indian animal and human populations: A billion-dollar issue. *J. Curr. Trends Biotechnol. Pharm.* **2019**, *13*, 112–123.
30. Godfroid, J. Brucellosis in livestock and wildlife: Zoonotic diseases without pandemic potential in need of innovative one health approaches. *Arch. Public Heal.* **2017**, *75*, 34, doi:10.1186/s13690-017-0207-7.
31. Musa, M.; Eisa, M.; el Sanousi, E.; Wahab, M.A.; Perrett, L. Brucellosis in Camels (*Camelus dromedarius*) in Darfur, Western Sudan. *J. Comp. Pathol.* **2008**, *138*, 151–155, doi:10.1016/j.jcpa.2007.10.005.
32. Chisholm, K.; Dueger, E.; Fahmy, N.T.; Samaha, H.A.T.; Zayed, A.; Abdel-Dayem, M.S.; Villinski, J.T. Crimean-Congo Hemorrhagic Fever Virus in Ticks from Imported Livestock, Egypt. *Emerg. Infect. Dis.* **2012**, *18*, 181–182, doi:10.3201/eid1801.111071.
33. Wakene, W.Z. Review on Epidemiology of Camel and Human Brucellosis in East Africa, Igad Member Countries. *Sci. J. Clin. Med.* **2017**, *6*, 109, doi:10.11648/j.sjcm.20170606.13.
34. Ibrahim, H.H.; Rouby, S.; Menshawy, A.; Ghazy, N. Seroprevalence of Camel Brucellosis and Molecular Characterization of *Brucella melitensis* Recovered from Dromedary Camels in Egypt. *Res. J. Vet. Pr.* **2016**, *4*, 17–24, doi:10.14737/journal.rjvp/2016/4.1.17.24.
35. Moghney, A.R.F.A. A preliminary study on brucellosis on camels at Behira Province. *Ass. Univ. Bull. Environ. Res.* **2004**, *7*, 39–43.
36. Sayour, A.E.; Elbauomy, E.; Abdel-Hamid, N.H.; Mahrous, A.; Carychao, D.; Cooley, M.B.; Elhadidy, M. MLVA fingerprinting of *Brucella melitensis* circulating among livestock and cases of sporadic human illness in Egypt. *Transbound. Emerg. Dis.* **2020**, doi:10.1111/tbed.13581.
37. Hamdy, M.; Amin, A. Detection of *Brucella* Species in the Milk of Infected Cattle, Sheep, Goats and Camels by PCR. *Vet. J.* **2002**, *163*, 299–305, doi:10.1053/tvjl.2001.0681.
38. Abdel-Hamid, N.H.; Elbauomy, E.M.; Ghobashy, H.M.; Sayour, A.E.; Ismail, R.I.; Soliman, H.S.; Abdel-Haleem, M.H. Role of sheep and goat mobile flocks in the transmission of brucellosis to the household ruminants and the disease prevalence in these flocks. *Anim. Health Res. J.* **2017**, *5*, 95–105.
39. Omer, M.; Musa, M.; Bakhiet, M.; Perrett, L. Brucellosis in camels, cattle, and humans: Associations and evaluation of serological tests used for diagnosis of the disease in certain nomadic localities in Sudan. *Rev. Sci. Tech. OIE* **2010**, *29*, 663–669, doi:10.20506/rst.29.3.2003.
40. Musa, M.; Jahans, K.; Fadalla, M. *Brucella* Biovars isolated from nomadic cattle in the Southern Darfur Province of Western Sudan. *J. Comp. Pathol.* **1990**, *102*, 49–54, doi:10.1016/s0021-9975(08)80006-0.

41. Gumaa, M.; Osman, H.; Omer, M.; el Sanousi, E.; Godfroid, J.; Ahmed, A. Seroprevalence of brucellosis in sheep and isolation of *Brucella abortus* biovar 6 in Kassala State, Eastern Sudan. *Rev. Sci. Tech. OIE* **2014**, *33*, 957–965, doi:10.20506/rst.33.3.2333.
42. Agab, H.; Abbas, B.; el Jack Ahmed, H.; Maoun, I.E. First report on the isolation of *Brucella abortus* biovar 3 from camel (*Camelus dromedarius*) in the Sudan. *Rev. Elev. Med. Vet. Pays. Trop.* **1994**, *47*, 361–363.
43. Faye, B.; Abdelhadi, O.M.A.; Ahmed, A.I.; Bakheit, S.A. Camel in Sudan: Prospects. *Livestock Res. Rural Develop*. **2011**, *23*, 1–11.
44. Ibrahim, S.I. Studies on swine brucellosis in Egypt. *J. Egypt Vet. Med. Ass*. **1996**, *56*, 1–12.
45. Gilbert, M.; Nicolas, G.; Cinardi, G.; van Boeckel, T.P.; Vanwambeke, S.O.; Wint, G.R.W.; Robinson, T.P. Global distribution data for cattle, buffaloes, horses, sheep, goats, pigs, chickens, and ducks in 2010. *Sci. Data* **2018**, *5*, 180227, doi:10.1038/sdata.2018.227.
46. Coelho, A.; Díez, J.G.; Coelho, A.M. Risk Factors for Brucella spp. in Domestic and Wild Animals. In *Updates on Brucellosis*; IntechOpen: London, UK, 2015.
47. Abebe, G.; Worku, Y.; Mamo, G.; Nazir, S. Sero-prevalence and Associated Risk Factors of Brucellosis in Camel at Akaki Abattoir, Central Ethiopia. *J. Anim. Res.* **2017**, *7*, 617, doi:10.5958/2277-940x.2017.00094.8.
48. Ullah, S. Prevalence of Brucellosis among Camels in District Muzaffargarh, Pakistan. *J. Infect. Mol. Boil.* **2015**, *3*, 52–56, doi:10.14737/journal.jimb/2015/3.2.52.56.
49. Bayasgalan, C.; Chultemdorj, T.; Roth, F.; Zinsstag, J.; Hattendorf, J.; Badmaa, B.; Argamjav, B.; Schelling, E. Risk factors of brucellosis seropositivity in Bactrian camels of Mongolia. *BMC Veter Res.* **2018**, *14*, 342, doi:10.1186/s12917-018-1664-0.
50. Al-Majali, A.M.; Al-Qudah, K.; Al-Tarazi, Y.H.; Al-Rawashdeh, O.F. Risk factors associated with camel brucellosis in Jordan. *Trop. Anim. Heal. Prod.* **2007**, *40*, 193–200, doi:10.1007/s11250-007-9080-7.
51. Wernery, U. Camelid brucellosis: A review. *Rev. Sci. Tech. OIE* **2014**, *33*, 839–857, doi:10.20506/rst.33.3.2322.
52. Sayour, A.E.; Elbauomy, E.M.; Shehata, A.A.; El-Kholi, M.K. Brucellosis prevalence and serological profile of male one-humped camels reared in Somaliland and eastern Ethiopia for meat production. *Global Vet*. **2015**, *14*, 67–76.

C H A P T E R 4.

Antimicrobial resistance of *Brucella* spp. in Egypt

Identification, genotyping and antimicrobial susceptibility testing of *Brucella* spp. isolated from livestock in Egypt

Microorganisms. 2019 Dec; 7(12): 603.
https://doi.org/10.3390/microorganisms7120603

microorganisms

MDPI

Article

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
2 Institute for Animal Hygiene and Environmental Health, Free University of Berlin, 14163, Berlin, Germany
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 Abbasaia- Cairo, Egypt
5 Department of Brucellosis, Animal Health Research Institute, Agricultural Research Center, 12618 Dokki - Giza, Egypt.
6 Animal Reproduction Research Institute, Agricultural Research Center, 12556 Al Ahram -Giza, Egypt
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

Received: 23 October 2019; Accepted: 16 November 2019; Published: 22 November 2019

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 *rpo*B gene associated with rifampicin resistance were identified in all phenotypically resistant isolates. Mutations in *gyr*A and *gyr*B 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 *rpo*B 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 CO_2 requirement, H_2S 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 *rpo*B gene (rifampicin), *gyr*A, *gyr*B, *par*C, *par*E genes (quinolones), *erm*, *mef*, *msr* (macrolides) or the presence of *tet* genes (tetracyclines), *mecA* (beta-lactams) and *fol*A (trimethoprim) [50]. Mutations in the *rpo*B and *gyr*A 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.

2.2. 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% CO_2. 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.

2.3. 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).

2.4. Molecular Identification and Differentiation

The presence of the *Brucella* genus-specific *bscp31* gene [55] and *Brucella*-specific insertion sequence 711 (IS*711*) [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 HPLC water, 12.5 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).

2.5. 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% CO_2 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), MIC_{50} and MIC_{90}. 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).

2.6. 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., *cat*B, *gyr*A and *gyr*B, *rpo*B, *Aac* genes and *tet* genes for chloramphenicol, ciprofloxacin, rifampicin, streptomycin, gentamicin and tetracycline, respectively (Supplementary Table S1). The primers used for amplification of the *rpo*B gene were designed by using submitted sequences for the *rpo*B 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).

2.7. PCR Amplicon Sequencing and Data Analysis

Amplified PCR products for *gyr*A, *gyr*B and *rpo*B 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 *gyr*A (CP034103 and AE017223), *gyr*B (CP007760 and SDWB01000001) and *rpo*B (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 MIC_{50} and MIC_{90} 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 (*cat*B, *Aac* and *tet* (*tet*A, *tet*B, *tet*M and *tet*O) conferring resistance to chloramphenicol, streptomycin/gentamicin and tetracycline, respectively)

were not identified either in resistant or sensitive isolates. The *gyr*A, *gyr*B and *rpo*B genes were amplified in all isolates.

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

Mutations in *gyr*A gene associated with phenotypic-ciprofloxacin resistance were detected at positions 167 (ATG to AGG/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 *gyr*B 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 CTC/tyrosine to leucine) and 1435 (AAG to CAG/lysine to glutamine) in the *rpo*B gene of phenotypic resistant *B. melitensis* isolates while the same was recorded at position 2890 (CGT to GGT/arginine to glycine) in the *rpo*B gene of *B. abortus* isolates. No mutation was detected in *gyr*A and *gyr*B gene of *B. abortus* strains.

Table 1. Microbiological and molecular identification of Brucella spp. isolated from animal species in Egypt.

Sample ID	Animal Species	Origin of Sample	Type of Sample	Growth with CO_2			Slide Agglutination A-M-R-Serum			MALDI-TOF MS	Molecular Identification
				[c]Bruc	[d]Brusel	[e]BBA	A	M	R	Result	
18RB17227	Cattle	Giza	Lymph node	+	+	+	[a]+ve	+ve	[b]−ve	*B. melitensis 3*	*Brucella* spp. *(B. abortus)*	*B. melitensis*
18RB17228	Cattle	Giza	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. abortus)*	*B. melitensis*
18RB17229	Cattle	Giza	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella melitensis*	*B. melitensis*
18RB17230	Cattle	Giza	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. melitensis)*	*B. melitensis*
18RB17231	Cattle	Giza	Lymph node	+	+	+	−ve	−ve	−ve	[*] NA	*Achromobacter* spp.	-ve
18RB17232	Cattle	Giza	Lymph node	+	+	+	−ve	−ve	−ve	NA	*Achromobacter* spp.	-ve
18RB17233	Cattle	Giza	Lymph node	+/−	+/−	+/−	+ve	−ve	−ve	*B. abortus 1*	*B. abortus*	*B. abortus*
18RB17234	Cattle	Giza	Lymph node	+	+	+	−ve	−ve	−ve	NA	*Achromobacter* spp.	-ve
18RB17235	Cattle	Giza	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp *(B. microti)*	*B. melitensis*
18RB17236	Cattle	Giza	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. melitensis)*	*B. melitensis*
18RB17237	Cattle	Giza	Lymph node	+	+	+	−ve	−ve	−ve	NA	*Achromobacter* spp.	-ve
18RB17238	Cattle	Giza	Lymph node	+	+	+	+ve	−ve	−ve	*B. abortus 1*	*Brucella* spp. *(B. microti)*	*B. melitensis*
18RB17239	Cattle	Giza	Lymph node	+	+	+	−ve	−ve	−ve	NA	*Achromobacter* spp.	-ve
18RB17240	Cattle	Beheira	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. microti)*	*B. melitensis*
18RB17241	Cattle	Beheira	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. microti)*	*B. melitensis*
18RB17242	Cattle	Beheira	Lymph node	+/−	+/−	+/−	+ve	−ve	−ve	*B. abortus 1*	*B. abortus*	*B. abortus*
18RB17243	Cattle	Beheira	Lymph node	+/−	+/−	+/−	+ve	−ve	−ve	*B. abortus 1*	*B. abortus*	*B. abortus*
18RB17244	Buffalo	Asyut	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. abortus)*	*B. melitensis*
18RB17245	Buffalo	Asyut	Lymph node	+/−	+/−	+/−	+ve	−ve	−ve	*B. abortus 1*	*B. abortus*	*B. abortus*
18RB17246	Goat	Beni-Suef	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. microti)*	*B. melitensis*
18RB17247	Cattle	Asyut	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. melitensis)*	*B. melitensis*
18RB17248	Cattle	Qalyubia	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. microti)*	*B. melitensis*
18RB17249	Cattle	Qalyubia	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. melitensis)*	*B. melitensis*
18RB17250	Sheep	Beni-Suef	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. melitensis)*	*B. melitensis*
18RB17251	Cattle	Beni-Suef	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp *(B. microti)*	*B. melitensis*
18RB17252	Cattle	Ismailia	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp *(B. melitensis)*	*B. melitensis*
18RB17253	Cattle	Ismailia	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp *(B. abortus)*	*B. melitensis*
18RB17254	Cattle	Ismailia	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp	*B. melitensis*

18RB17255	Cattle	Beheira	Fetal stomach content	+/−	+/−	+/−	+ve	−ve	−ve	*B. abortus 1*	*B. abortus*	*B. abortus*
18RB17256	Cattle	Dakahlia	Lymph node	+/−	+/−	+/−	+ve	−ve	−ve	*B. abortus 1*	*B. abortus*	*B. abortus*
18RB17257	Cattle	Monufia	Lymph node	+/−	+/−	+/−	+ve	−ve	−ve	*B. abortus 1*	*B. abortus*	*B. abortus*
18RB17258	Cattle	Monufia	Milk	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. abortus)*	*B. melitensis*
18RB17259	Cattle	Qalyubia	Lymph node	+/−	+/−	+/−	+ve	−ve	−ve	*B. abortus 1*	*B. abortus*	*B. abortus*
18RB17260	Buffalo	Qalyubia	Lymph node	+	+	+	+ve	+ve	−ve	*B. melitensis 3*	*Brucella* spp. *(B. microti)*	*B. melitensis*

[*] 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**	**Breakpoints**			***B. melitensis***			***B. abortus***		
		Sensitive (mg/L)	**Intermedium (mg/L)**	**Resistant (mg/L)**	**R (%)**	**MIC_{50} (mg/L)**	**MIC_{90} (mg/L)**	**R (%)**	**MIC_{50} (mg/L)**	**MIC_{90} (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	0.06	0.06
Erythromycin	Macrolides	−	−	≥16	19.04	4	8	87.5	32	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 *rpo*B 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 GTT	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	
18RB17229	*B. melitensis*	4	676, 677	TAC to CTC	Tyrosine to leucine	MN544030, MN544044, MN544058, MN544072, MN544086
			1011	AAC to AGC	Asparagine to serine	
			1456, 1458	GAA to AAG	Glutamic acid to lysine	
			1787	AAG to ACG	Lysine to threonine	
			2491	ACC to CCC	Threonine to proline	
18RB17230	*B. melitensis*	8	676, 677	TAC to CTC	Tyrosine to leucine	MN544031, MN544045, MN544059, MN544073, MN544087
			1435	AAG to CAG	Lysine to glutamine	
			1798, 1799	GGC to AAC	Glycine to asparagine	
			1801, 1802	AAG to GGG	Lysine to glycine	
			1804, 1806	GTG to CTT	Valine to leucine	
			1807	ACG to TCG	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	
18RB17236	*B. melitensis*	8	676, 677	TAC to CTC	Tyrosine to leucine	MN544033, MN544047, MN544061, MN544075, MN544089
18RB17238	*B. melitensis*	16	677	TAC to TTC	Tyrosine to phenylalanine	MN544034, MN544048, MN544062, MN544076, MN544090
			1780	TAT to GAT	Tyrosine to aspartic acid	
			1786, 1788	AAG to CAA	Lysine to glutamine	
			2869, 2871	CGT to GGG	Arginine to glycine	
18RB17240	*B. melitensis*	16	2494, 2496	TCG to CTC	Serine to leucine	MN544035, MN544049, MN544063, MN544077, MN544091
18RB17241	*B. melitensis*	6(8)	1435	AAG to CAG	Lysine to glutamine	MN544036, MN544050, MN544064, MN544078, MN544092
			2870, 2871	CGT to CCG	Arginine to proline	
18RB17246	*B. melitensis*	4	676, 678	TAC to CTT	Tyrosine to leucine	MN544037, MN544051, MN544065, MN544079, MN544093
			1436, 1437	AAG to ACA	Lysine to threonine	
			2870	CGT to CCT	Arginine to proline	
			3898	TAC to AAC	Tyrosine to asparagine	
			3901	ACG to CCG	Threonine to proline	
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 toAGC	Arginine to serine	
18RB17253	*B. melitensis*	4	1435	AAG to CAG	Lysine to glutamine	MN544039, MN544053, MN544067, MN544081,
			1745	GCC to GGC	Alanine to glycine	MN544095
18RB17258	*B. melitensis*	6	676, 677	TAC to CTC	Tyrosine to leucine	MN544040, MN544054, MN544068, MN544082,
			2501, 2502	CAC to CCA	Histidine to proline	MN544096
18RB17260	*B. melitensis*	4	1435	AAG to CAG	Lysine to glutamine	MN544041, MN544055, MN544069, MN544083,
			3670, 3672	CAG to TAT	Glutamine to tyrosine	MN544097
18RB17233	*B. abortus*	4	703, 704	ACT to CTT	Threonine to leucine	
			709, 710	ACC to CAC	Threonine to histidine	
			1457, 1458	AAG to ACA	Lysine to threonine	MN544013,
			1460	GAA to GGA	Glutamic acid to glycine	MN544016,
			2512	ACC to CCC	Threonine to proline	MN544019,
			2515, 2517	TCG to CTC	Serine to leucine	MN544022,
			2890, 2892	CGT to GGG	Arginine to glycine	MN544025
			3123	GAC to GAG	Aspartic acid to glutamic acid	
			3124, 3125	GAC to ATC	Aspartic acid to isoleucine	
18RB17242	*B. abortus*	>4	698, 699	TAC to TTT	Tyrosine to phenylalanine	
			1457, 1458	AAG to ACA	Tyrosine to threonine	MN544014,
			1460	GAA to GGA	Glutamic acid to glycine	MN544017,
			1789	ATC to GTC	Isoleucine to valine	MN544020,
			1801	TAT to GAT	Tyrosine to aspartic acid	MN544023,
			2887	GAG to AAG	Glutamic acid to lysine	MN544026
			2890	CGT to GGT	Arginine to glycine	
18RB17245	*B. abortus*	4	709	ACC to CCC	Threonine to proline	MN544015, MN544018, MN544021, MN544024,
			2890	CGT to GGT	Arginine to glycine	MN544027

Table 4. Detection of mutations in *gyr*A and *gyr*B 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	*gyr*A	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		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	Asparagine to threonine	MN536680
18RB17230	*B. melitensis*	0.5		1144	ATC to CTC	Isoleucine to leucine	MN536681
18RB17244	*B. melitensis*	0.25	*gyr*B	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 *B. abortus* isolates from

cattle in brazil [62]. All *B. melitensis* and *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 *Brucella* as well as in other bacteria is the beta-subunit of the DNA dependent RNA polymerase (RNAP) encoded by *rpo*B gene [47,51]. In this study, mutations were identified in *rpo*B gene associated with phenotypic rifampicin resistant *Brucella* strains isolated from clinical specimens of animals in Egypt. Mutations were detected in all phenotypically resistant brucellae. 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 *rpo*B gene of phenotypically resistant *B. melitensis* were detected. These mutations are different from previously reported mutations (in vitro mutations) associated with rifampicin resistance in *Brucella* [47].

Johansen et al. reported mutations in phenotypic rifampicin resistant or intermediately resistant *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 *B. melitensis*.

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

Fluoroquinolone/quinolone resistance in *Brucella* is multifactorial by nature in addition to obvious mutations of the *gyr*A, *gyr*B, *par*C and *par*E genes [51,52]. In this study, the mutations in *gyr*A and *gyr*B genes in phenotypically resistant *B. melitensis* and *B. abortus* to ciprofloxacin were investigated. The mutations in *gyr*A did not correspond with fluoroquinolone resistance mutations described by Turkmani et al. [49], although they investigated mutations in vitro selected fluoroquinolone resistant *Brucella* mutants. The mutations in the *gyr*B 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 *B. melitensis* considered as novel findings of this study. None of these mutations was detected in *B. abortus* strains in *gyr*A or *gyr*B genes. However, the role of *par*C, *par*E and efflux systems cannot be ruled out for fluoroquinolone resistance [51] as we did not investigate the changes in *par*C and *par*E genes.

Genes responsible for resistance against chloramphenicol (*cat*B), gentamicin (*Aac*) and tetracycline (*tet*A, *tet*B, *tet*M and *tet*O) were not detected in all investigated *Brucella* isolated in this study, which in accordance with the phenotypic antimicrobial susceptibility results of isolated *Brucella* isolates. It is also worth mentioning that all resistant *Brucella* strains were isolated from animals and they showed resistance to antimicrobials clinically used in humans practice, suggesting that the source of these *Brucella* strains may be of human origin. These findings point to the fact that inter-species and intra-host species *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 *Brucella* [33]. Higher phenotypic resistance in *Brucella* against these antimicrobials is likely to limits the treatment effectiveness, owing to the increased number of infections. Emergence of multidrug resistance *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 *Brucella* species, the situation with respect to hospital care may worsen and limits the treatment options in public health settings.

5. Conclusion

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 when 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 www.mdpi.com/xxx/s1, Table S1: List of primers and primer sequences used for detection of antimicrobial associated resistance mechanism.

Author Contributions: Data curation, A.U.K., W.S.S. and H.E-A.; Investigation, A.U.K., W.S.S., A.E.S., E.S.R., A.A.M. and H.E-A.; Methodology, A.U.K., W.S.S., A.E.S., E.S.R. and H.E-A.; Resources, H.N., F.M. and H.E-A.; Supervision, F.M., M.C.E., U.R., H.N. and H.E-A.; Writing – original draft, A.U.K., A.A.M. and H.E-A.; Writing – review & editing, F.M., M.C.E., U.R., H.N. and H.E-A.

Funding: This research received no external funding.

Acknowledgments: The authors thank Michaela Ganss and Katja Fischer at the Institute of Bacterial Infections and Zoonoses, Friedrich-Loeffler-Institut (FLI) for their cooperation and technical assistance. This research work was supported by the International Research Project as part of the "German biosecurity program" funded by Federal Foreign Office, Germany. The authors thank Islamic Development Bank (IDB), Jeddah, Saudi Arabia for PhD grant.

Conflicts of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. Moreno, E. Retrospective and prospective perspectives on zoonotic brucellosis. *Front. Microbiol.* **2014**, *5*, 18.
2. Georgi, E.; Walter, M.C.; Pfalzgraf, M.T.; Northoff, B.H.; Holdt, L.M.; Scholz, H.C.; Zoeller, L.; Zange, S.; Antwerpen, M.H. Whole genome sequencing of *brucella melitensis* isolated from 57 patients in germany reveals high diversity in strains from middle east. *PLoS ONE.* **2017**, *12*, e0175425.
3. Whatmore, A.M.; Davison, N.; Cloeckaert, A.; Al Dahouk, S.; Zygmunt, M.S.; Brew, S.D.; Perrett, L.L.; Koylass, M.S.; Vergnaud, G.; Quance, C.; et al. *Brucella papionis* sp. Nov., isolated from baboons (*papio* spp.). *Int. J. Sys.t Evol. Microbiol.* **2014**, *64*, 4120–4128.
4. Scholz, H.C.; Revilla-Fernandez, S.; Al Dahouk, S.; Hammerl, J.A.; Zygmunt, M.S.; Cloeckaert, A.; Koylass, M.; Whatmore, A.M.; Blom, J.; Vergnaud, G.; et al. *Brucella vulpis* sp. Nov., isolated from mandibular lymph nodes of red foxes (*vulpes vulpes*). *Int. J. Syst. Evol. Microbiol.* **2016**, *66*, 2090–2098.
5. Shome, R.; Kalleshamurthy, T.; Shome, B.R.; Sahay, S.; Natesan, K.; Bambal, R.G.; Sairiwal, L.; Mohandoss, N.; Barbuddhe, S.B. Lateral flow assay for brucellosis testing in multiple livestock species. *J. Microbiol. Methods* **2018**, *148*, 93–96.
6. Morgan, W.J. *Brucella* classification and regional distribution. *Dev. Biol. Stand.* **1984**, *56*, 43–53.
7. Miller, M.A.; Burgess, T.L.; Dodd, E.M.; Rhyan, J.C.; Jang, S.S.; Byrne, B.A.; Gulland, F.M.; Murray, M.J.; Toy-Choutka, S.; Conrad, P.A.; et al. Isolation and characterization of a novel marine *brucella* from a southern sea otter (*enhydra lutris nereis*), california, USA *J. Wildl. Dis.* **2017**, *53*, 215–227.
8. Richomme, C.; Gauthier, D.; Fromont, E. Contact rates and exposure to inter-species disease transmission in mountain ungulates. *Epidemiol. Infect.* **2006**, *134*, 21–30.
9. Singh, B.B.; Dhand, N.K.; Gill, J.P. Economic losses occurring due to brucellosis in indian livestock populations. *Prev. Vet. Med.* **2015**, *119*, 211–215.
10. Godfroid, J. Brucellosis in livestock and wildlife: Zoonotic diseases without pandemic potential in need of innovative one health approaches. *Arch. Public Health.* **2017**, *75*, 6.
11. Refai, M. Incidence and control of brucellosis in the near east region. *Vet. Microbiol.* **2002**, *90*, 81–110.
12. Menshawy, A.M.; Perez-Sancho, M.; Garcia-Seco, T.; Hosein, H.I.; Garcia, N.; Martinez, I.; Sayour, A.E.; Goyache, J.; Azzam, R.A.; Dominguez, L.; et al. Assessment of genetic diversity of zoonotic *brucella* spp. Recovered from livestock in egypt using multiple locus vntr analysis. *Biomed. Res. Int.* **2014**, *2014*, 7.
13. Wareth, G.; Hikal, A.; Refai, M.; Melzer, F.; Roesler, U.; Neubauer, H. Animal brucellosis in egypt. *J. Infect.*

Dev. Ctries. **2014**, *8*, 1365–1373.
14. Hegazy, Y.M.; Molina-Flores, B.; Shafik, H.; Ridler, A.L.; Guitian, F.J. Ruminant brucellosis in upper egypt (2005-2008). *Prev Vet. Med.* **2011**, *101*, 173–181.
15. Eltholth, M.M.; Hegazy, Y.M.; El-Tras, W.F.; Bruce, M.; Rushton, J. Temporal analysis and costs of ruminant brucellosis control programme in egypt between 1999 and 2011. *Transbound Emerg Dis.* **2017**, *64*, 1191–1199.
16. Dean, A.S.; Crump, L.; Greter, H.; Schelling, E.; Zinsstag, J. Global burden of human brucellosis: A systematic review of disease frequency. *PLoS Negl. Trop. Dis.* **2012**, *6*, e1865.
17. Pappas, G.; Papadimitriou, P.; Akritidis, N.; Christou, L.; Tsianos, E.V. The new global map of human brucellosis. *Lancet Infect. Dis.* **2006**, *6*, 91–99.
18. Godfroid, J.; Al Dahouk, S.; Pappas, G.; Roth, F.; Matope, G.; Muma, J.; Marcotty, T.; Pfeiffer, D.; Skjerve, E. A "one health" surveillance and control of brucellosis in developing countries: Moving away from improvisation. *Comp. Immunol. Microbiol. Infect. Dis.* **2013**, *36*, 241–248.
19. Tuon, F.F.; Gondolfo, R.B.; Cerchiari, N. Human to human transmission of *brucella* - a systematic review. *Trop Med. Int Health.* **2017**, *22*, 539–546.
20. El-Diasty, M.; Wareth, G.; Melzer, F.; Mustafa, S.; Sprague, L.D.; Neubauer, H. Isolation of *brucella abortus* and *brucella melitensis* from seronegative cows is a serious impediment in brucellosis control. *Vet. Sci.* **2018**, *5*, 4.
21. Godfroid, J.; Nielsen, K.; Saegerman, C. Diagnosis of brucellosis in livestock and wildlife. *Croat. Med. J.* **2010**, *51*, 296–305.
22. Mathew, C.; Stokstad, M.; Johansen, T.B.; Klevar, S.; Mdegela, R.H.; Mwamengele, G.; Michel, P.; Escobar, L.; Fretin, D.; Godfroid, J. First isolation, identification, phenotypic and genotypic characterization of *brucella abortus* biovar 3 from dairy cattle in tanzania. *BMC Vet. Res.* **2015**, *11*, 9.
23. Vicente, A.F.; Antunes, J.M.; Lara, G.H.; Mioni, M.S.; Allendorf, S.D.; Peres, M.G.; Appolinario, C.M.; Listoni, F.J.; Ribeiro, M.G.; Megid, J. Evaluation of three formulations of culture media for isolation of *brucella* spp. Regarding their ability to inhibit the growth of contaminating organisms. *Biomed. Res. Int.* **2014**, *2014*, 3.
24. Alton, G.G.; Jones, L.M.; Angus, R.D.; Verger, J.M. *Techniques for the brucellosis laboratory.: Paris: Institute national de la recherdie agrononique*. Institut National De La Recherche Agronomique, 147, rue de I,Universite, 75007: Paris, France, **1988**. https://www.cabi.org/isc/abstract/19892297928 (accessed on 19 November 2019).
25. Angeletti, S. Matrix assisted laser desorption time of flight mass spectrometry (maldi-tof ms) in clinical microbiology. *J. Microbiol. Method.* **2017**, *138*, 20–29.
26. Patel, R. Maldi-tof ms for the diagnosis of infectious diseases. *Clin. Chem.* **2015**, *61*, 100–111.
27. Sali, M.; De Maio, F.; Tarantino, M.; Garofolo, G.; Tittarelli, M.; Sacchini, L.; Zilli, K.; Pasquali, P.; Petrucci, P.; Marianelli, C., et al. Rapid and safe one-step extraction method for the identification of *brucella* strains at genus and species level by maldi-tof mass spectrometry. *PLoS ONE* **2018**, *13*, e0197864.
28. Hinic, V.; Brodard, I.; Thomann, A.; Cvetnic, Z.; Makaya, P.V.; Frey, J.; Abril, C. Novel identification and differentiation of *brucella melitensis, b. Abortus, b. Suis, b. Ovis, b. Canis,* and *b. Neotomae* suitable for both conventional and real-time pcr systems. *J. Microbiol Methods.* **2008**, *75*, 375–378.
29. Bricker, B.J.; Halling, S.M. Differentiation of *brucella abortus* bv. 1, 2, and 4, *brucella melitensis*, *brucella ovis*, and *brucella suis* bv. 1 by pcr. *J. Clin. Microbiol.* **1994**, *32*, 2660–2666.
30. Lopez-Goni, I.; Garcia-Yoldi, D.; Marin, C.M.; de Miguel, M.J.; Munoz, P.M.; Blasco, J.M.; Jacques, I.; Grayon, M.; Cloeckaert, A.; Ferreira, A.C.; et al. Evaluation of a multiplex pcr assay (bruce-ladder) for molecular typing of all *brucella* species, including the vaccine strains. *J. Clin. Microbiol.* **2008**, *46*, 3484–3487.
31. Ali, S.; Ali, Q.; Melzer, F.; Khan, I.; Akhter, S.; Neubauer, H.; Jamal, S.M. Isolation and identification of bovine *brucella* isolates from pakistan by biochemical tests and pcr. *Trop Anim Health Prod.* **2014**, *46*, 73–78.
32. Bricker, B.J.; Halling, S.M. Enhancement of the *brucella* amos pcr assay for differentiation of *brucella abortus* vaccine strains s19 and rb51. *J. Clin. Microbiol.* **1995**, *33*, 1640–1642.
33. Ariza, J.; Bosilkovski, M.; Cascio, A.; Colmenero, J.D.; Corbel, M.J.; Falagas, M.E.; Memish, Z.A.; Roushan, M.R.; Rubinstein, E.; Sipsas, N.V.; et al. Perspectives for the treatment of brucellosis in the 21st century: The ioannina recommendations. *PLoS Med.* **2007**, *4*, e317.
34. Del Pozo, J.S.G.; Solera, J. Treatment of human brucellosis-review of evidence from clinical trials. In *Updates on brucellosis*, InTech.Open: London, United Kingdom, **2015**.
35. Zakaa El-Din, M.; Samy, F.; Mohamed, A.; Hamdy, F.; Yasser, S.; Ehab, M. Egyptian community pharmacists' attitudes and practices towards antibiotic dispensing and antibiotic resistance; a cross-sectional survey in greater Cairo. *Curr Med. Res. Opin.* **2019**, *35*, 939–946.

36. Sabry, N.A.; Farid, S.F.; Dawoud, D.M. Antibiotic dispensing in Egyptian community pharmacies: An observational study. *Res. Soc. Adm Pharm.* **2014**, *10*, 168–184.
37. World Health Organization, Regional Office for the Eastern Mediterranean. Report on the consultative meeting on antimicrobial resistance for countries in the Eastern Mediterranean Region: from policies to action. Sharm el Sheikh, Egypt, 12–14 November 2013. https://apps.who.int/iris/handle/10665/116211, 2014; 8–9.
38. Abdulah, R. Antibiotic abuse in developing countries. *Pharm Regul Aff.* **2012**, *1*, 1000–1006.
39. Kasim, K.; Hassan, H. Self-medication problem in Egypt: A review of current and future perspective. *Int. J. Cur Res. Rev.* **2018**, *10*, 6.
40. Gebeyehu, E.; Bantie, L.; Azage, M. Inappropriate use of antibiotics and its associated factors among urban and rural communities of bahir dar city administration, northwest ethiopia. *PLoS ONE.* **2015**, *10*, e0138179.
41. Magiorakos, A.P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; Harbarth, S.; Hindler, J.F.; Kahlmeter, G.; Olsson-Liljequist, B.; et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol Infect.* **2012**, *18*, 268–281.
42. Landers, T.F.; Cohen, B.; Wittum, T.E.; Larson, E.L. A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Rep.* **2012**, *127*, 4–22.
43. Agunos, A.; Pierson, F.W.; Lungu, B.; Dunn, P.A.; Tablante, N. Review of nonfoodborne zoonotic and potentially zoonotic poultry diseases. *Avian Dis.* **2016**, *60*, 553–575.
44. Hoffman, S.J.; Outterson, K.; Rottingen, J.A.; Cars, O.; Clift, C.; Rizvi, Z.; Rotberg, F.; Tomson, G.; Zorzet, A. An international legal framework to address antimicrobial resistance. *Bull. World Health Organ.* **2015**, *93*, 1.
45. Johansen, T.B.; Scheffer, L.; Jensen, V.K.; Bohlin, J.; Feruglio, S.L. Whole-genome sequencing and antimicrobial resistance in *brucella melitensis* from a norwegian perspective. *Sci Rep.* **2018**, *8*, 9.
46. Liu, Z.G.; Di, D.D.; Wang, M.; Liu, R.H.; Zhao, H.Y.; Piao, D.R.; Zhao, Z.Z.; Hao, Y.Q.; Du, Y.N.; Jiang, H., et al. In vitro antimicrobial susceptibility testing of human *brucella melitensis* isolates from ulanqab of inner mongolia, china. *BMC Infect Dis.* **2018**, *18*, 6.
47. Marianelli, C.; Ciuchini, F.; Tarantino, M.; Pasquali, P.; Adone, R. Genetic bases of the rifampin resistance phenotype in *brucella* spp. *J. Clin. Microbiol.* **2004**, *42*, 5439–5443.
48. Deshmukh, A.; Hagen, F.; Sharabasi, O.A.; Abraham, M.; Wilson, G.; Doiphode, S.; Maslamani, M.A.; Meis, J.F. In vitro antimicrobial susceptibility testing of human *brucella melitensis* isolates from qatar between 2014 - 2015. *BMC Microbiol.* **2015**, *15*, 5.
49. Turkmani, A.; Psaroulaki, A.; Christidou, A.; Chochlakis, D.; Tabaa, D.; Tselentis, Y. In vitro-selected resistance to fluoroquinolones in two *brucella* strains associated with mutational changes in *gyr*a. *Int J Antimicrob Agents.* **2008**, *32*, 227–232.
50. Biswas, S.; Raoult, D.; Rolain, J.M. A bioinformatic approach to understanding antibiotic resistance in intracellular bacteria through whole genome analysis. *Int J. Antimicrob Agents.* **2008**, *32*, 207–220.
51. Valdezate, S.; Navarro, A.; Medina-Pascual, M.J.; Carrasco, G.; Saez-Nieto, J.A. Molecular screening for rifampicin and fluoroquinolone resistance in a clinical population of *brucella melitensis*. *J. Antimicrob Chemother.* **2010**, *65*, 51–53.
52. Ravanel, N.; Gestin, B.; Maurin, M. In vitro selection of fluoroquinolone resistance in *brucella melitensis*. *Int J Antimicrob Agents.* **2009**, *34*, 76–81.
53. OIE. Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*) (infection with *B. abortus, B. melitensis* and *B. suis*). In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019*, *OIE*; World Health Organization for Animal Health: Paris, France, 2019; pp. 355–398.
54. Karger, A.; Melzer, F.; Timke, M.; Bettin, B.; Kostrzewa, M.; Nockler, K.; Hohmann, A.; Tomaso, H.; Neubauer, H.; Al Dahouk, S. Interlaboratory comparison of intact-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry results for identification and differentiation of *brucella* spp. *J. Clin. Microbiol.* **2013**, *51*, 3123–3126.
55. Baily, G.G.; Krahn, J.B.; Drasar, B.S.; Stoker, N.G. Detection of *brucella melitensis* and *brucella abortus* by DNA amplification. *J. Trop Med. Hyg.* **1992**, *95*, 271–275.
56. Garcia-Yoldi, D.; Marin, C.M.; de Miguel, M.J.; Munoz, P.M.; Vizmanos, J.L.; Lopez-Goni, I. Multiplex pcr assay for the identification and differentiation of all *brucella* species and the vaccine strains *brucella abortus* s19 and rb51 and *brucella melitensis* rev1. *Clin. Chem.* **2006**, *52*, 779–781.

57. CLSI. Clinical and Laboratory Standard Institute: Performance standards for antimicrobial susceptibility testing, 29th edition. Wayne, Pennsylvania, **2019**, M100, 74-77.
58. EUCAST. The european committee on antimicrobial susceptibility testing. Breakpoint tables for interpretation of mics and zone diameters **2019**, *Version 9.0, 2019.* Available online: http://www.eucast.org (accessed on 19 November 2019).
59. Khazaei, Z.; Najafi, A.; Piranfar, V.; Mirnejad, R. Microarray-based long oligonucleotides probe designed for *brucella* spp. Detection and identification of antibiotic susceptibility pattern. *Electron. Physician.* **2016**, *8*, 2297–2303.
60. Mugizi, D.R.; Muradrasoli, S.; Boqvist, S.; Erume, J.; Nasinyama, G.W.; Waiswa, C.; Mboowa, G.; Klint, M.; Magnusson, U. Isolation and molecular characterization of *brucella* isolates in cattle milk in uganda. *Biomed. Res. Int.* **2015**, http://dx.doi.org/10.1155/2015/720413 (accessed on 19 November 2019).
61. Trott, D.J.; Abraham, S.; Adler, B. Antimicrobial resistance in *leptospira, brucella,* and other rarely investigated veterinary and zoonotic pathogens. *Microbiol Spectr.* **2018**, *6*, 13.
62. Barbosa Pauletti, R.; Reinato Stynen, A.P.; Pinto da Silva Mol, J.; Seles Dorneles, E.M.; Alves, T.M.; de Sousa Moura Souto, M.; Minharro, S.; Heinemann, M.B.; Lage, A.P. Reduced susceptibility to rifampicin and resistance to multiple antimicrobial agents among *brucella abortus* isolates from cattle in brazil. *PLoS ONE.* **2015**, *10*, e0132532.
63. Torkaman Asadi, F.; Hashemi, S.H.; Alikhani, M.Y.; Moghimbeigi, A.; Naseri, Z. Clinical and diagnostic aspects of brucellosis and antimicrobial susceptibility of *brucella* isolates in hamedan, iran. *Jpn J. Infect. Dis.* **2017**, *70*, 235–238.
64. Hashim, R.; Ahmad, N.; Mohamed Zahidi, J.; Tay, B.Y.; Mohd Noor, A.; Zainal, S.; Hamzah, H.; Hamzah, S.H.; Chow, T.S.; Wong, P.S.; et al. Identification and in vitro antimicrobial susceptibility of *brucella* species isolated from human brucellosis. *Int. J. Microbiol.* **2014**, *2014*, 5.
65. Abdel-Maksoud, M.; House, B.; Wasfy, M.; Abdel-Rahman, B.; Pimentel, G.; Roushdy, G.; Dueger, E. In vitro antibiotic susceptibility testing of *brucella* isolates from egypt between 1999 and 2007 and evidence of probable rifampin resistance. *Ann Clin Microbiol Antimicrob.* **2012**, *11*, 24.
66. Shevtsov, A.; Syzdykov, M.; Kuznetsov, A.; Shustov, A.; Shevtsova, E.; Berdimuratova, K.; Mukanov, K.; Ramankulov, Y. Antimicrobial susceptibility of *brucella melitensis* in kazakhstan. *Antimicrob Resist. Infect. Control.* **2017**, *6*, 5.
67. Khan, M.Z.; Zahoor, M. An overview of brucellosis in cattle and humans, and its serological and molecular diagnosis in control strategies. *Trop Med. Infect. Dis.* **2018**, *3*, 14.
68. Theuretzbacher, U. Global antimicrobial resistance in gram-negative pathogens and clinical need. *Curr. Opin. Microbiol.* **2017**, *39*, 106–112.
69. Asai, T.; Hiki, M.; Ozawa, M.; Koike, R.; Eguchi, K.; Kawanishi, M.; Kojima, A.; Endoh, Y.S.; Hamamoto, S.; Sakai, M., et al. Control of the development and prevalence of antimicrobial resistance in bacteria of food animal origin in japan: A new approach for risk management of antimicrobial veterinary medicinal products in japan. *Foodborne Pathog. Dis.* **2014**, *11*, 171–176.

C H A P T E R 5.

Genotyping of *Brucella* spp. in Egypt

Whole Genome sequencing for tracing the genetic diversity and virulence genes of *Brucella abortus* and *Brucella melitensis* isolated from livestock in Egypt

"Submitted and under revision"

Frontiers in Microbiology

Whole Genome Sequencing for tracing the genetic diversity and Virulence genes of *Brucella abortus* and *Brucella melitensis* isolated from livestock in Egypt

Aman Ullah Khan[1,2,3], Falk Melzer[1], Ashraf E. Sayour[4], Sherif Abdel Ghafar Elsayed El-Soally[5], Jörg Linde[1], Mostafa Abdel-Glil[1], Mandy C. Elschner[1], Waleed S. Shell[6], Eman Shawkat Ramadan[7], Shereen Aziz Mohamed[8], Uwe Roesler[2], Heinrich Neubauer[1] and Hosny El-Adawy[1,9]*

1 Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, 07743 Jena, Germany; AmanUllah.Khan@fli.de (A.U.K.); falk.melzer@fli.de (F.M.); mandy.elschner@fli.de (M.C.E.); Heinrich.neubauer@fli.de (H.N.); hosny.eladawy@fli.de (H. E-A)
2 Institut for Animal Hygiene and Environmental Health, Free University of Berlin, 14163, Berlin, Germany; uwe.roesler@fu-berlin.de (U.R);
3 Department of Pathobiology, College of Veterinary and Animal Sciences, Jhang 35200, Pakistan
4 Department of Brucellosis, Animal Health Research Institute, Agricultural Research Center, 12618 Dokki - Giza, Egypt; sayourashraf@gmail.com (A. E. S.)
5 Veterinary Service Department, Armed Forces Logistics Authority, Egyptian Armed Forces, 11765 Nasr City, Egypt; dr.sherifelsoaaly@gmail.com (S.A.G.E.E-S)
6 Central Laboratory for Evaluation of Veterinary Biologics, Agricultural Research Center, 11517 Abbasaia-Cairo , Egypt; tarikwaleedshell@hotmail.com (W. S. S.)
7 Animal Reproduction Research Institute, Agricultural Research Center, 12556 Al Ahram-Giza, Egypt; moc.liamg@1791131nadamarname
8 Veterinary Serum and Vaccine Research Institute, Agricultural Research Center, 11517 Abbasaia- Cairo, Egypt; shereenibraheem1968@gmail.com (S. A. M.)
9 Faculty of Veterinary Medicine, Kafrelsheikh University, 33516 Kafr El-Sheikh, Egypt
* Correspondence: Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, 07743 Jena, Germany; Hosny.eladawy@fli.de

Abstract

Background: Brucellosis is a highly contagious zoonosis worldwide with high economic and public health impacts. The use of whole-genome sequencing (WGS) using next-generation sequencing (NGS) technology has become a widely accepted molecular typing method for outbreak tracing and genomic epidemiology of brucellosis. This study aimed to genotype *Brucella (B.) abortus* and *B. melitensis* isolates from different animal species located in different governorates in Egypt.

Methodology: In total, 34 suspected *Brucella* isolates were recovered from lymph nodes, milk, and fetal abomasal contents of infected cattle, buffaloes, sheep, and goats originating 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. Antimicrobial susceptibility testing against ciprofloxacin, imipenem, and rifampicin were performed using E-Test. Illumina MiSeq® was used to sequence 29 *Brucella* isolates. Bioinformatics analysis was performed for in-depth genotyping, and resistome and virulome analysis. The antimicrobial resistance-mechanism caused by mutations in *rpo*B and *gyr*A and *gyr*B genes associated with rifampicin and ciprofloxacin resistance were identified.

Results: In total, 29 *Brucella* isolates (eight *B. abortus* biovar 1 and 21 *B. melitensis* biovar 3) were identified and typed. Using MLST typing, ST11 and ST1 were identified among *B. melitensis* and *B. abortus*, respectively. Egyptian *B. melitensis* strains showed MLST sequence types closer to the

Mediterranean lineage while *B. abortus* strains showed sequence types closer to strains isolated from the UK, American and European lineage. The 21 *B. melitensis* and 8 *B. abortus* isolates were shown to have genetic similarity coefficient by MLVA typing. *B. abortus* and *B. melitensis* strains clustered into 2 main clusters containing 2 and 10 distinct genotypes by MLVA-16 analysis, respectively. Similarly, *B. abortus* and *B. melitensis* strains were clustered into 1 and 2 main clusters containing 3 and 14 distinct genotypes by core-genome SNP analysis respectively. The genotypes were irregularly distributed over time and space in the study area. The phenotypic resistance of *B. melitensis* to ciprofloxacin, imipenem and rifampicin were 76.2% (n=16), 76.2% (n=16) and 66.7% (n=14), respectively. Whereas, 25.0% (n=2), 25.0% (n=2), and 37.5% (n=3) of *B. abortus* were resistant to ciprofloxacin, imipenem and rifampicin, respectively. One point mutation at position 297-GAT to GAA/Asp-Glu in *gyr*A and two-point mutations in the *rpo*B gene at position 2784 (CGC to CGT/Arg-Arg) and 2394 (ACG to ACT/Thr-Thr) were detected in 3 and 4 phenotypicaly resistant strains of *B. melitensis* against ciprofloxacin and rifampin, respectively. In total 43 virulent genes were identified in each *B. melitensis* and *B. abortus* isolate.

Conclusion: WGS demonstrated genetic heterogeneity of *Brucella* spp. in livestock in different localities in Egypt and highlighted the potential of whole-genome sequencing for improving routine surveillance of brucellosis by enhancing outbreak detection, source-tracing, and potentially prevention of infections. Strains with similar genotypes isolated from different governorates highlight the risk of spreading the pathogen by movement of animals among governorates. Hence, it may also reflect the long endemicity of brucellosis in Egypt with earlier dispersion of types and great local genetic diversity.

Key words: *B. abortus*, *B. melitensis*, WGS, genotyping, livestock, Egypt.

1. Introduction

Brucellosis is one of the most widespread zoonoses of public health significance and leading to huge economic losses in animal production systems globally [1,2]. Brucellosis is well controlled in developed countries but is still endemic in many others with high records in humans in the Middle East and Central Asian regions [3]. The disease is caused by bacteria of the genus *Brucella* (*B.*). These are highly pathogenic, intra-cellular living, Gram-negative, non-motile, non-spore forming coccobacilli bacteria. At present, twelve *Brucella* species have been recognized with apparent host preference, which includes six classical species *B. melitensis* (sheep and goats), *B. abortus* (cattle and buffaloes), *B. suis* (pigs, wild boar, hares, and rodents), *B. canis* (dogs), *B. ovis* (sheep) and *B. neotome* (rodents), and six newly recognized species, *B. microti* (voles), *B. ceti* (cetaceans), *B. pinipedialis* (seals), *B. inopinata* (from a human female breast implant infection), *B. papionis* (baboons) and *B. vulpis* (red foxes) [4-8]. However, the cross-infection of animal species with brucellae is also reported [9,10]. Among these, *B. melitensis, B. abortus, B. suis* (except bv 2) and *B. canis* are largely responsible for human brucellosis whereas *B. ceti, B. penipedialis* and *B. neotome* can be potentially zoonotic [6,11-14]. *Brucella melitensis* is highly pathogenic among other brucellae, primarily infecting sheep and goats and is the most frequent agent of human brucellosis leading to severe disease outcomes [2]. Brucellosis is usually transmitted in animals either by contact or through ingestion of contaminated feed and water, while in humans either by direct contact with infected animals or ingestion of contaminated animal products [15-19].

Infection with brucellae results in reproductive disease in domestic animals and chronic debilitating disease in humans [20]. Pathogenicity and virulence of brucellae mainly depend on escaping the innate

immune system, interfering with intracellular signaling mechanisms, resistance to respiratory burst, and adaptation to survive in intramacrophage oxygen-limited conditions [21]. *Brucella suis*, *B. melitensis*, and *B. abortus* have been reported in livestock in Egypt [22,23]. Brucellosis has likely been endemic in Egypt for thousands of years [24,25]. The disease has been detected with increasing prevalence in livestock species predominantly in ruminants [26,27]. Prevalences ranging from 2.47% to 26.66% were found in various animal populations [28]. *Brucella abortus* and *B. melitensis* were isolated from livestock and humans and *B. suis* was identified in cattle and pigs [25,29,30]. In Egypt, *B. melitensis* is the predominant *Brucella* spp. causing infection in animals as well as in humans [22,31].

The disease can be controlled by management and effective immunization in animals [32]. Vaccination with *B. abortus*-S19 and RB51 or *B. melitensis* REV1 vaccine strain are effective in controlling the disease in ruminants [20,33]. Treatment is not advised for animal brucellosis, however, human brucellosis is treatable. Antimicrobial regimens with doxycycline, rifampicin, streptomycin, quinolones, and aminoglycoside alone or in combination are used to treat brucellosis [34]. Especially the combination of doxycycline with either aminoglycosides or rifampicin is a good choice for the treatment [35]. But also quinolones are promising alternative antibioticsas they have good bioavailability and affinity for bone and soft tissues [36]. Contraindications of tetracyclines (e.g. in pregnant women and children) may limit the treatment of acute brucellosis [37].

Diverse analysis methods are used to identify pathogens, specifically *Brucella* species or biovars. Both traditional and modern typing methods are used in the epidemiology of *Brucella*.

Classical bio-typing methods recommended by World Organization for Animal Health (OIE) are based on culture characteristics and biochemical tests including phage typing and agglutination with monospecific sera divide brucellae into different species and their biovars [38]. This bio-typing divides *B. melitensis* into three biovars (biovar 1-3), while *B. abortus* is divided into seven biovars (biovar 1-6 and 9) [38]. The method is laborious and requires living bacteria handling and the results are often insufficient for epidemiological purposes as they do not provide sufficient resolution between the isolates [2,39]. Moreover, an individual biotype often predominates in particular areas, as seen in Egypt, where *B. melitensis* biovar 3 and *B. abortus* biovar 1 is almost exclusively isolated from the local animal populations [22,31,40,41]. Owing to its highly clonal features like monomorphism brucellae are difficult to differentiate at the strain level [42]. Techniques such as pulsed-field gel electrophoresis and amplified fragment length polymorphism have been applied in the past, but these techniques were not able to differentiate *Brucella* at the subspecies level, which correlated with low intra- and interlaboratory reproducibility [43]. Therefore, PCR-based typing like Multilocus Sequence Typing (MLST), Multiple Loci VNTR (Variable Number of Tandem Repeats) Analysis (MLVA), is now commonly used as an alternative to the culture-dependent typing methods [2,44,45]. MLVA has been considered the most efficient and standard typing method possessing a high discriminating resolution, in congruence with MLST, and is sufficient for in-depth study of either genome evolution or outbreak epidemiology of *Brucella* species [46]. However, this typing method has several weaknesses, related both to the nature of variable-number tandem repeats (VNTRs) as well as to laboratory demands of the technique itself [2].

Whole-Genome-Sequencing (WGS) sequencing is considered as the ideal tool to study detailed genomic variations in organisms. With the advancement and development of next-generation sequencing technologies, now complete bacterial genome sequencing has become easy, economical, and highly

accessible [47]. The organisms in genus *Brucella* particularly *B. suis*, *B. melitensis*, and *B. abortus* share >90% homology in their DNA [48]. The genome of Brucella is very stable and contains two circular chromosomes of approximately 2.1 and 1.2 Mb and both share similar GC contents, equivalent housekeeping genes, and a similar proportion of encoding regions [49]. Molecular epidemiology and typing of Brucella could be challenging owing to low genetic variations in its genome [50]. However, WGS provides the most comprehensive collection of an individual's genetic variation and could provide unprecedented resolution in discriminating even highly genetically related bacteria like *Brucella* [2,47,51,52]. WGS is much superior to conventional genotyping tools for studying outbreaks, geographical distribution, and newly emerging infections [53]. Comparatively, new methods of *Brucella* typing, including gene-by-gene comparison using core genome multilocus sequence typing (cgMLST), as well as single-nucleotide polymorphism (SNP) calling based on a reference sequence analysis are considered to be a suitable and more informative replacement of the gold standard typing schemes [2,47,54]. Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variations in closely related microbial species, strains, or isolates. SNPs are powerful genetic markers for distinguishing closely related strains [55]. Janowicz *et. al*., recommends that WGS based typing approaches can be used as standalone tools in establishing phylogenetic relationships and provide insights into the epidemiology of *B. melitensis*, and it is a candidate to be a benchmark tool for outbreak investigation and assessment of antimicrobial resistance in human and animal brucellosis [2,39,56]. Recently, MLVA based study explains the epidemiological situation of brucellae in Egypt [22]. Fewer studies reported the emergence of antimicrobial resistance in *Brucella* spp. from Egypt [31,57]. The high prevalence of *B. melitensis* bv-3 throughout the country as a predominating *Brucella* species compromises the whole epidemiological situation necessitates a high discriminatory tool to assess the genetic diversity and relatedness among the local genotypes [40].

This study was aimed to assess the genetic diversity and relationships among the *B. melitensis* and *B. abortus* circulated different animal species in different regions in Egypt through whole-genome sequencing analysis.

2. Materials and Methods

2.1. Brucella strains isolation and identification

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

Brucella spp. was isolated by following the OIE standard protocol [38]. Briefly, isolates were inoculated on calf blood agar, Brucella medium, and Brucella selective medium plates (Oxoid GmbH, Wesel, Germany) at 37ºC in the absence and presence of 5-10% CO_2 for up to 2 weeks. Typically, round, glistening, pinpoint and honey drop-like cultures were picked and stained with Gram and modified Ziehl-Nelsen stain (MZN) initially. Subsequent biochemical tests, motility tests, hemolysis on blood agar, and agglutination with monospecific sera were performed. Bacterial identification was additionally carried out using MALDI-TOF MS as described previously [31,58].

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). The AMOS-PCR was performed to differentiate *Brucella* species [59,60] followed by multiplex Bruce-ladder PCR assay for strain and biovar typing [61,62].

Table 1. Microbiological and molecular identification of *Brucella* spp. isolated from animals species in Egypt

Sample ID	Year of isolation	Governorate	District	Host	Type of sample	Biochemical identification	MALDI-TOF-MS	Molecular identification
18RB17227	2017	Giza	Al Badrashin	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. abortus)*	*B. melitensis*
18RB17228	2017	Giza	Al Badrashin	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. abortus)*	*B. melitensis*
18RB17229	2017	Giza	Al Badrashin	Cattle	Lymph node	*B. melitensis* 3	*Brucella melitensis*	*B. melitensis*
18RB17230	2017	Giza	Al Badrashin	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. melitensis)*	*B. melitensis*
18RB17231	2017	Giza	Al Badrashin	Cattle	Lymph node	*NA	*Achromobacter* spp.	-ve
18RB17232	2017	Giza	Al Badrashin	Cattle	Lymph node	NA	*Achromobacter* spp.	-ve
18RB17233	2017	Giza	Al Badrashin	Cattle	Lymph node	*B. abortus* 1	*Brucella abortus*	*B. abortus*
18RB17234	2017	Giza	El-Hawamdeyya	Cattle	Lymph node	NA	*Achromobacter* spp.	-ve
18RB17235	2017	Giza	El-Hawamdeyya	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. microti)*	*B. melitensis*
18RB17236	2017	Giza	El-Hawamdeyya	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. melitensis)*	*B. melitensis*
18RB17237	2017	Giza	El-Hawamdeyya	Cattle	Lymph node	NA	*Achromobacter* spp.	-ve
18RB17238	2017	Giza	El-Hawamdeyya	Cattle	Lymph node	*B. abortus* 1	*Brucella* spp (*B. microti)*	*B. melitensis*
18RB17239	2017	Giza	Al Ayat	Cattle	Lymph node	NA	*Achromobacter* spp.	-ve
18RB17240	2016	Beheira	Damanhour	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. microti)*	*B. melitensis*
18RB17241	2016	Beheira	Damanhour	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. microti)*	*B. melitensis*
18RB17242	2016	Beheira	Damanhour	Cattle	Lymph node	*B. abortus* 1	*Brucella abortus*	*B. abortus*
18RB17243	2016	Beheira	Damanhour	Cattle	Lymph node	*B. abortus* 1	*Brucella abortus*	*B. abortus*
18RB17244	2017	Asyut	Asyut	Buffalo	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. abortus)*	*B. melitensis*
18RB17245	2017	Asyut	Asyut	Buffalo	Lymph node	*B. abortus* 1	*Brucella abortus*	*B. abortus*
18RB17246	2015	Beni Suef	Al Wasta	Goat	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. microti)*	*B. melitensis*
18RB17247	2017	Asyut	Asyut	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. melitensis)*	*B. melitensis*
18RB17248	2017	Qalyubia	Tukh	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. microti)*	*B. melitensis*
18RB17249	2017	Qalyubia	Qaha	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. melitensis)*	*B. melitensis*
18RB17250	2015	Beni Suef	Al Wasta	Sheep	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. melitensis)*	*B. melitensis*
18RB17251	2015	Beni Suef	Al Wasta	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. microti)*	*B. melitensis*
18RB17252	2017	Ismailia	Ismailia	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. melitensis)*	*B. melitensis*
18RB17253	2017	Ismailia	Ismailia	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. abortus)*	*B. melitensis*
18RB17254	2017	Ismailia	Ismailia	Cattle	Lymph node	*B. melitensis* 3	*Brucella* spp	*B. melitensis*
18RB17255	2017	Beheira	Ad Dilinjat	Cattle	Fetal stomach content	*B. abortus* 1	*Brucella abortus*	*B. abortus*
18RB17256	2017	Dakahlia	Mansoura	Cattle	Lymph node	*B. abortus* 1	*Brucella abortus*	*B. abortus*
18RB17257	2017	Monufia	Sirs Al Layyanah	Cattle	Lymph node	*B. abortus* 1	*Brucella abortus*	*B. abortus*
18RB17258	2017	Monufia	Sirs Al Layyanah	Cattle	Milk	*B. melitensis* 3	*Brucella* spp (*B. abortus)*	*B. melitensis*
18RB17259	2017	Qalyubia	Tukh	Cattle	Lymph node	*B. abortus* 1	*Brucella abortus*	*B. abortus*
18RB17260	2017	Qalyubia	Tukh	Buffalo	Lymph node	*B. melitensis* 3	*Brucella* spp (*B. microti)*	*B. melitensis*

* NA: not applied

2.2. Whole-genome sequencing

Total genomic DNA was quantified with the Qubit fluorometer (QubitTM DNA HS assay; Life Technologies, Thermo Fisher Scientific, Inc.), and library preparation was performed using the Nextera XT library preparation kit (Illumina Inc., San Diego, CA).

Quality assessment of the paired-end Illumina sequence data was performed using the qc_pipeline, a bioinformatics workflow that was developed for quick assessment of Illumina reads. The source code is available here https://gitlab.com/FLI_Bioinfo/qc_pipeline. Briefly, FastQC (Babraham Bioinformatics, Babraham Institute, Cambridge) was used to check the quality metrics of Illumina sequence data. Mash was used to estimating genome size from k-mer content. Theoretical coverage was calculated by dividing the total number of sequenced bases over the estimated genome size. The results of the qc_pipeline are summarized in a multiqc report.

Bioinformatics analysis for the characterization and analysis of genomic data was performed using WGSBAC (v 2.0.0), available at https://gitlab.com/FLI_Bioinfo/WGSBAC.

Briefly, Illumina reads were processed and assembled using the software shovill (v 1.0.4). This software includes steps for adapters trimming using trimmomatic, overlapping paired-end reads using FLASH2, *de novo* assembly using SPAdes (run without error correction in mode –only assembler), assembly improvement using pilon and filtering contigs which are below 3x k-mer coverage and 500 bp contig length. Quality control of assembled contigs was performed using QUAST (v 5.0.2)[63]. Check for contamination was performed at read level and contig level using kraken2(v2.0.7_beta) [64]. Assembled contigs were searched for antimicrobial resistance and virulence determinant using abricate (v 0.8.10) and the databases NCBI AMRFinderPlus (Accession: PRJNA313047) and the virulence factor database (DOI: 10.1093/nar/gkv1239), respectively. MLST profiles were identified for the *Brucella* isolates using mlst (https://github.com/tseemann/mlst) and mlst scheme "brucella" from pubmlst. *In silico* MLVA baed on assembled contigs was performed as previously described [39] by adapting the tool MISTReSS. Core genome SNPs were identified using snippy (v4.3.6) and the core genome tree was built using RAxML (v8.2.12). As a reference for *B. melitensis* strain 16M (accessions NC_003317 and NC_003318) and for *B. abortus* strain BDW (accessions NZ_CP007680 and NZ_CP007681) was used. snp-dists (0.6.3) was used to calculate the SNP distance between each pair of genomes. Tree visualization using iTOL v4. All generated data were submitted to the National Center for Biotechnology Information (NCBI).

2.3. Antimicrobial susceptibility testing

The antimicrobial susceptibility of *B. melitensis* and *B. abortus* isolates was performed against ciprofloxacin, imipenem, and rifampicin using gradient strip method (E-test, bioMerieux, Marcy L'Etoile, France) as described previously [65]. The breakpoints guidelines for slow-growing bacteria (*Haemophilus influenzae*) were used as an alternative [66]. Quality control assays were performed with *E. coli* (161008BR3642, DSM 1103, ATCC 25922). The interpretations were performed using CLSI [66] and EUCAST [67] susceptibility criteria for slow-growing bacteria. Based on these criteria the strains were classified as resistant or sensitive.

3. Results

3.1. Identification and differentiation of Brucella isolates

Microbiological, biochemical methods and MALDI-TOF MS confirmed 29 isolates as *Brucella* species while the remaining five isolates as *Achromobacter* species (**Table 1**). Molecular assays (AMOS-PCR and Bruce-Ladder PCR) differentiated 21 isolates as *B. melitensis* (17 from cattle, 2 from buffaloes, 1 from sheep and 1 from a goat) and 8 isolates as *B. abortus* (7 from cattle and 1 from a buffalo). PCR assays confirmed these isolates as field strains. Biochemical methods revealed biovar 3 in B. melitensis and biovar 1 in B. abortus. (**Table 1**).

3.2. Brucella genome

In this study, 21 genomes of *B. melitensis* and 8 genomes of *B. abortus* were sequenced. The average number of reads was 962993, 9 (min 358990, max 2068092) and 1194287, 25 (min 449286, max 1967264) for *B. melitensis* and *B. abortus*, respectively (Table S1) which yields an average genome coverage of 69, 8 fold (min 26, max167) and 87,1fold (range min max31 - 143). Genome assembly yielded an average genome size of 3289627,4 bp (min 3278036 bp, max 3300052 bp) and 326493 bp (min 3263728 bp, max 3265874 bp) for *B. melitensis* and *B. abortus*, respectively, with average N50 average values of 295160,05 bp and 208398,9 bp (Table S1).

3.3. Classical MLST

By MLST typing, ST11 and ST1 were identified among *B. melitensis* and *B. abortus*, respectively (Table S2). ST11 comprises 21 *B. melitensis* strains that are found to be predominant identified from humans and clustered into Mediterranean lineage, indicating a close phylogenetic relationship of Egyptian *B. melitensis* strains to those from the Mediterranean region. All B. *abortus* were typed into ST1 which predominantly detected in bovines and humans from UK, Ireland, New Zealand, Mexico, USA, India, Portugal, Bolivia, and Zimbabwe.

3.4. In silico MLVA

All *B. abortus* strains clustered into 1 main cluster producing 2 distinct genotypes (GT-1 and GT-2) after MLVA-16 analysis. The dendrogram of genetic relatedness of *B. abortus* strains depicted in Figure 1 and detailed description of *B. abortus* strains metadata given in Table 1. All loci from panel 1, panel 2A, and panel 2B were monomorphic except from one locus *Bruce16* from panel 2B (Table S3). As shown in figure 1, *B. abortus* bv1 strain 18RB17256 isolated from cattle in 2017 from Mansoura-Dakahlia Governorate has the same genotype (GT-1) of bv1 strain 18RB17259 recovered from cattle in 2017 from Tukh-Qalyubia Governorate. The other six *B.abortus* bv1 strains 18RB17257isolated from cattle in 2017 from Sirs Al-Layyanah, Monufia Governorate ,the strain18RB17233 isolated from cattle from Al-Badrashin, Giza Governorate in 2017, the strains 18RB17242 and 18RB17243 isolated from Beheira (Damanhour) in 2016 from cattle, the strain 18RB17255 recovered from cattle from Beheira (Ad Dilinjat) Governorate in 2017, and the strain 18RB17245 that was obtained from buffalo in 2017 from Asyut Governorate share the same genotype (GT-2) (Figure 1.).

All 21 *B. melitensis* strains were 2 main clusters (1 and 2) containing 10 genotypes (GT-1 to GT-10) by MLVA-16 analysis. All loci of the panel 1 and panel 2A except *Bruce18* and *Bruce19* were homogenous. In contrast, the most discriminatory loci were Bruce18 (Panel 2A) and Bruce4, Bruce7, Bruce9, and Bruce16 from panel-2B (Table S4). The dendrogram of genetic relatedness of *B. melitensis* strains depicted in Figure 2 and detailed description of *B. melitensis* strains metadata given in Table 1. As shown in figure 2, *B. melitensis* strains isolated from cattle in 2017 from Al-Badrashin (18RB17227, 18RB17228,

18RB17229, 18RB17230) and El-Hawamdeyya (18RB17235, 18RB17236, and 18RB17238) Giza Governorate shared same genotypes (GT-1) with strains (18RB17240 and 18RB17241) of *B. melitensis* bv3 recovered from cattle in 2016 from Damanhour-Beheira Governorate. The *B. melitensis* strain isolated from goat in 2015 (18RB17246, GT-2) from Al Wasta-Beni Suef Governorate was very similar to strain (18RB17244, GT-3) isolated from buffalo in 2017 from Asyut Governorate with very low genetic diversity (Bruce09: 6 to 7). The identical genotypes (GT-4) were found in circulating strains (18RB17252, 18RB17253, and 18RB17254) in Ismailia Governorate isolated from cattle in 2017. Beni Suef Governorate had the same genotype (GT-5) of *B. melitensis* bv1 isolated from sheep (18RB17250) in 2015 with the strain at Qalyubia Governorate (18RB17249) isolated from cattle in 2017. *Brucella melitensis* strains recovered from cattle (18RB17247, GT-6 isolated in 2017 from Asyut, and 18RB17248, GT-7 isolated in 2017 from Tukh, Qalyubia) had very low genetic similarity (Bruce04: 6 to 7 and Bruce09:9 to 10). A *B. melitensis* strain isolated from cattle (18RB17251, GT-8) in 2015 from Al-Wasta, Beni Suef has a distinct genotype. A genotype strain 18RB17258 (GT-9) isolated from cattle in 2017 from Sirs Al-Layyanah, Monufia have a distinct genotype from *B. melitensis* bv3 strain 18RB17260 (GT-10) isolated from buffalo in 2017 from Tukh, Qalyubia (Figure 2).

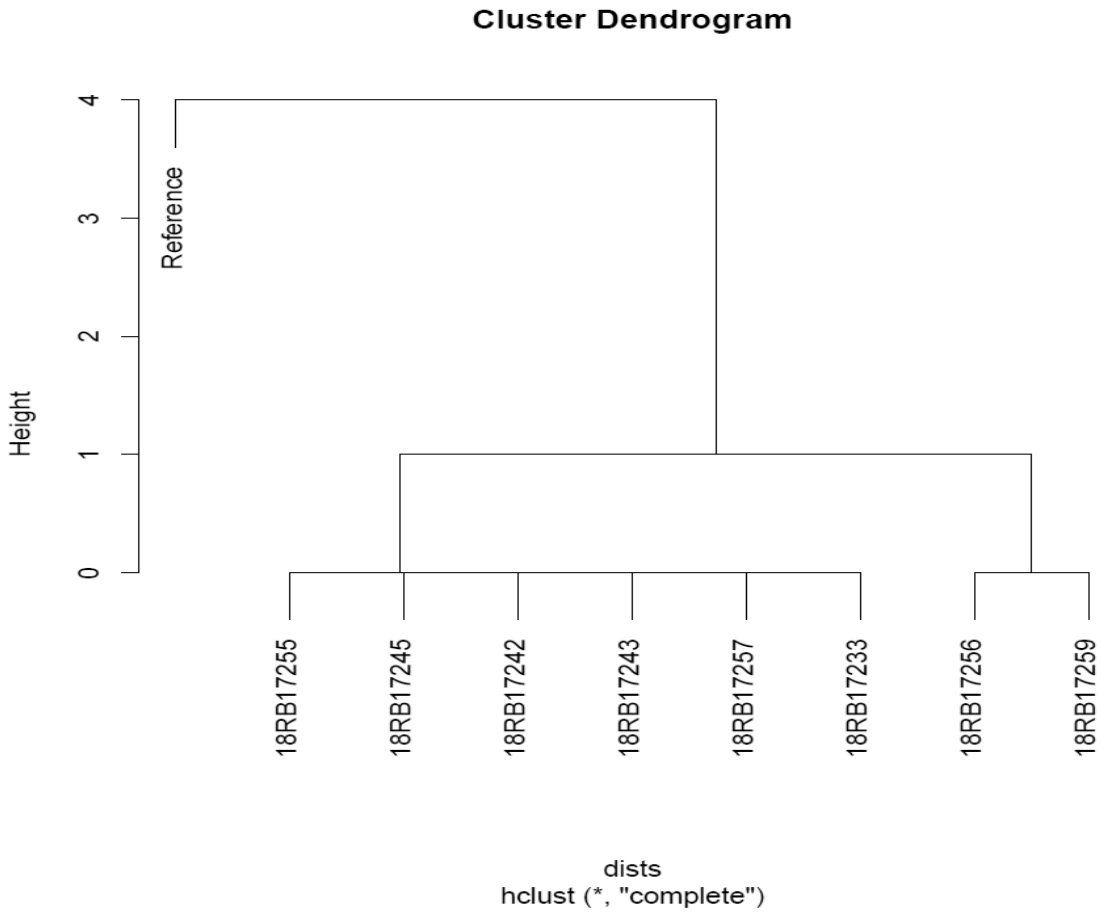

Figure 1. **Dendrogram based on the MLVA-16 genotyping assay (in silico), showing relationships between the 8 *B. abortus* (B) isolates**.

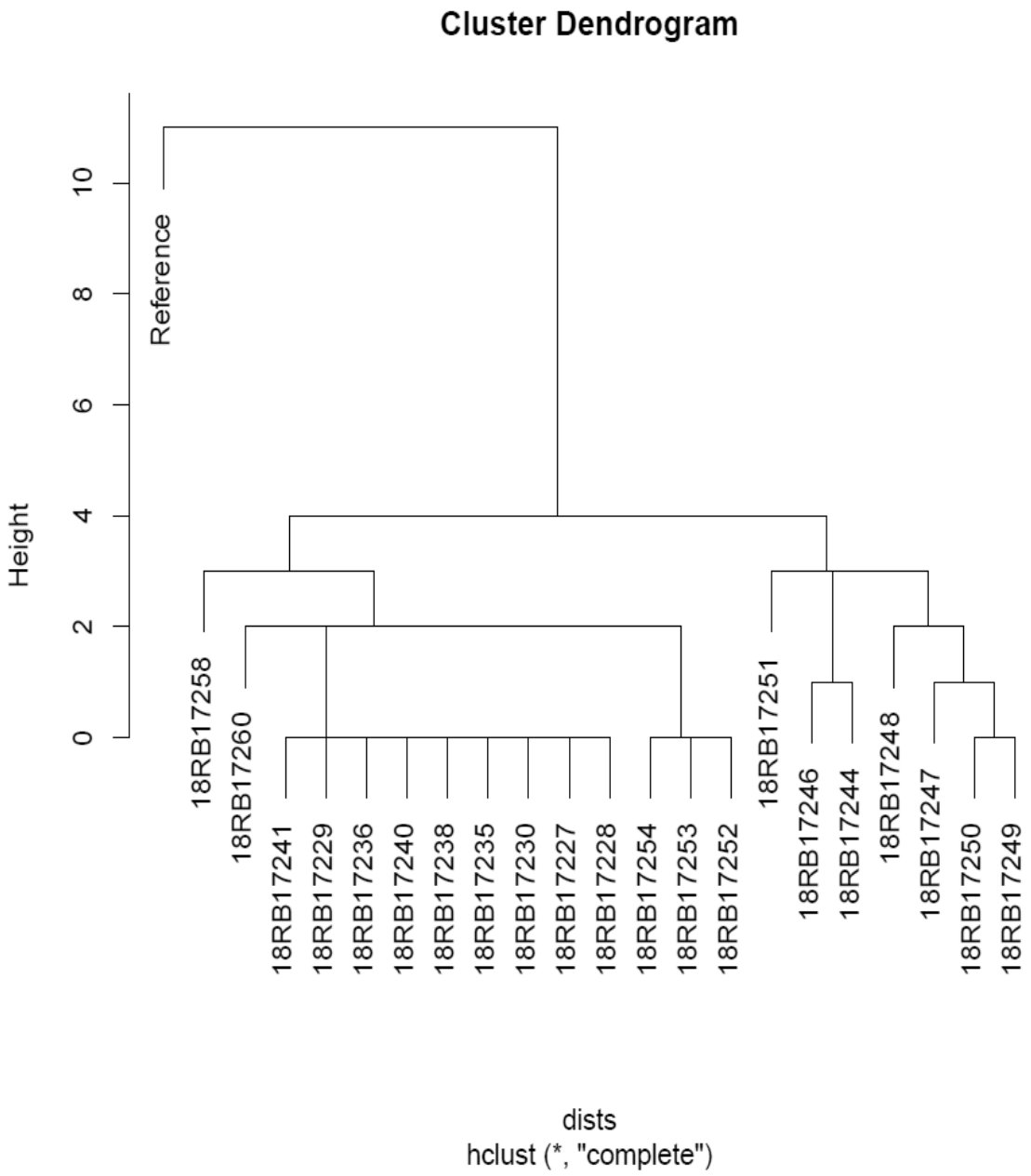

Figure 2. **Dendrogram based on the MLVA-16 genotyping assay (in silico), showing relationships between the 21 *B. melitensis*.**

3.5. SNP typing

Core genome Single Nucleotide Polymorphism analysis (cgSNPs) revealed 1 major cluster/clade divided the *B. abortus* strains into 3 genotypes (GT-1 to GT-3). As shown in figure 3, the 5 *B. abortus* bv1 strains (18RB17233, 18RB17242, 18RB17243, 18RB17245, and 18RB17255) were depicting identical genotypes GT-1 (0 SNPs distance) isolated from cattle and buffaloes from different Governorates in Egypt in different years. The strain 18RB17233 was isolated from cattle from Al-Badrashin, Giza Governorate in 2017. While the strains 18RB17242 and 18RB17243 were isolated from Beheira (Damanhour) in 2016 from cattle. Similarly, the strain 18RB17255 was obtained from cattle from Beheira

Governorate but different districts (Ad Dilinjat) in 2017. The strain 18RB17245 was obtained from buffalo in 2017 from Asyut Governorate. The second genotype (GT-2) contains 2 identical (0 SNPs distance) strains of *B. abortus* (18RB17256 and 18RB17259) isolated from cattle in 2017 but from different governorates (18RB17256 from Mansoura, Dakahlia, and 18RB17259 from Tukh, Qalyubia). The *B. abortus* strain 18RB17257 was singleton genotype (GT-3) isolated from cattle in 2017 from Sirs Al-Layyanah, Monufia Governorate (Table 1, Table S5, and Figure 3).

Similarly, cgSNPs analysis divided *B. melitensis* strains into 2 main clusters/clades containing 14 genotypes (GT-1 to GT-14). Cluster 1 contains 5 strains (18RB17252, 18RB17253, 18RB17254, 18RB17258, and 18RB17260) out of 21 *B. melitensis* strains. As shown in figure 4, the *B. melitensis* strains (18RB17252, 18RB17253, and 18RB17254) were shown identical (0 SNPs distance) genotypes (GT-1) recovered from cattle in 2017 from Ismailia Governorate. A *B. melitensis* strain (18RB17258) in cluster 1 isolated from cattle in 2017 from Sirs Al-Layyanah District of Monufia Governorate was singleton genotypes (GT-2) and it varies with 42 SNPs from a strain (18RB17260, GT-3) recovered from buffalo in 2017 from Tukh-Qalyubia. While the cluster 2 contained 16 strains (18RB17227, 18RB17228, 18RB17229, 18RB17230, 18RB17235, 18RB17236, 18RB17238, 18RB17240, 18RB17241, 18RB17244, 18RB17246, 18RB17247, 18RB17248, 18RB17249, 18RB17250 and 18RB17251) out of 21 *B. melitensis* strains (Figure 4). Six strains from cluster 2 (18Rb17227, 18RB17228, 18RB17230, 18RB1717235, 18RB17236, and 18RB17241) were identical genotypes (GT-4) (0 SNPs distance) isolated from cattle in 2017 from Giza Governorate with exception of 1 strain (18RB17241) that was isolated in 2016 from Beheira Governorate. Three *Brucella melitensis* strain (18RB17229, 18RB17238, and 18RB17240) were singleton genotypes showing very low genetic diversity (3-4 SNPs distance), strain 18RB17229 (GT-5) and 18RB17238 (GT-6) were isolated from cattle in 2017 from Al-Badrashin and El-Hawamdeyya Districts of Giza Governorate respectively. However, strain (18RB17240, GT-7) recovered from cattle in 2016 from Danhour-Beheira Governorate. Another strain (18RB17251, GT-8) fro the same cluster (cluster 2) was singleton genotype and isolated from cattle in 2015 from Al Wasta-Beni Suef Governorate.

Two *B. melitensis* genotypes (18RB17244, GT-9, and 18RB17246, GT-10) that were highly related and have an only difference of 6 SNPs were isolated from buffalo and goat in 2017 and 2015 from Asyut and Beni Suef Governorates respectively. Furthermore, two *B. melitensis* genotypes (18RB17248, GT-11, and 18RB17249, GT-12) were showing low genetic diversity by an only difference of 6-12 SNPs were isolated from cattle in 2017 from Tukh and Qaha Districts of Qalyubia Governorate, respectively. The *B. melitensis* genotype (18RB17247, GT-13) isolated from cattle varies by 25 SNPs from a different genotype (18RB17244) recovered from buffalo in 2017 from same (Asyut) Governorate. The *B. melitensis* genotype (18RB17250, GT-14) isolated from sheep varies by 26 SNPs from another genotype (18RB17246) recovered from goat in 2015 from Al-Wasta District of Beni Suef Governorate (Table 1, Table S6 and Figure 4).

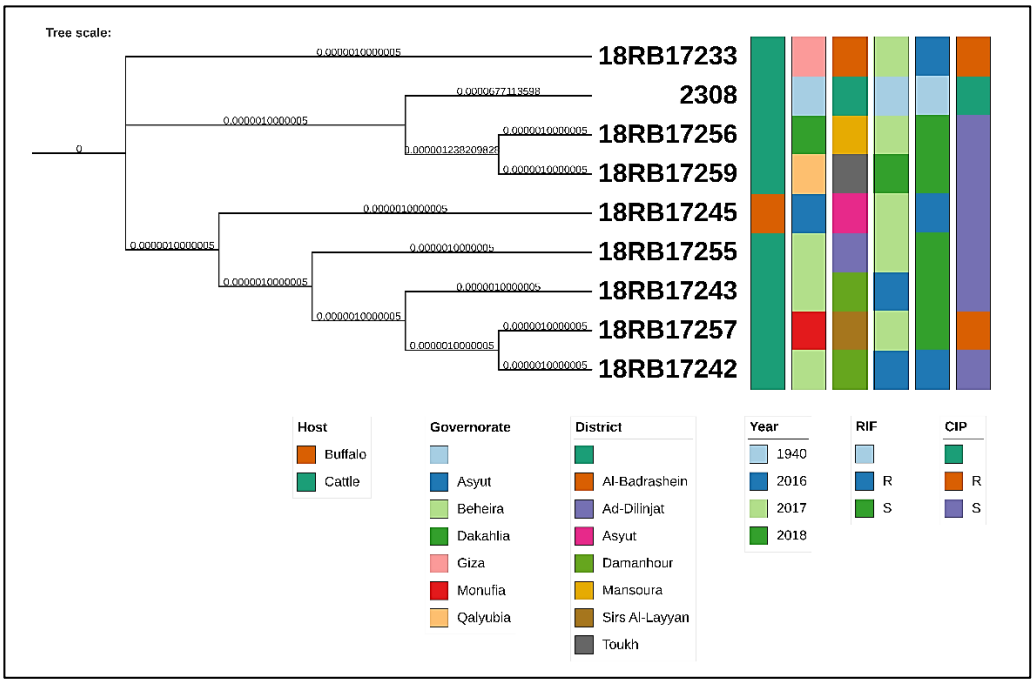

Figure 3. Dendrogram based on of cgSNP analysis of 8 B. abortus bv1 isolates recovered from different animal species in Egypt. The columns show strain identification numbers, host, geographic origin (governorate and distrcit), year, specimen/source of isolation and resistance to rifampicin and ciprofloxacin.

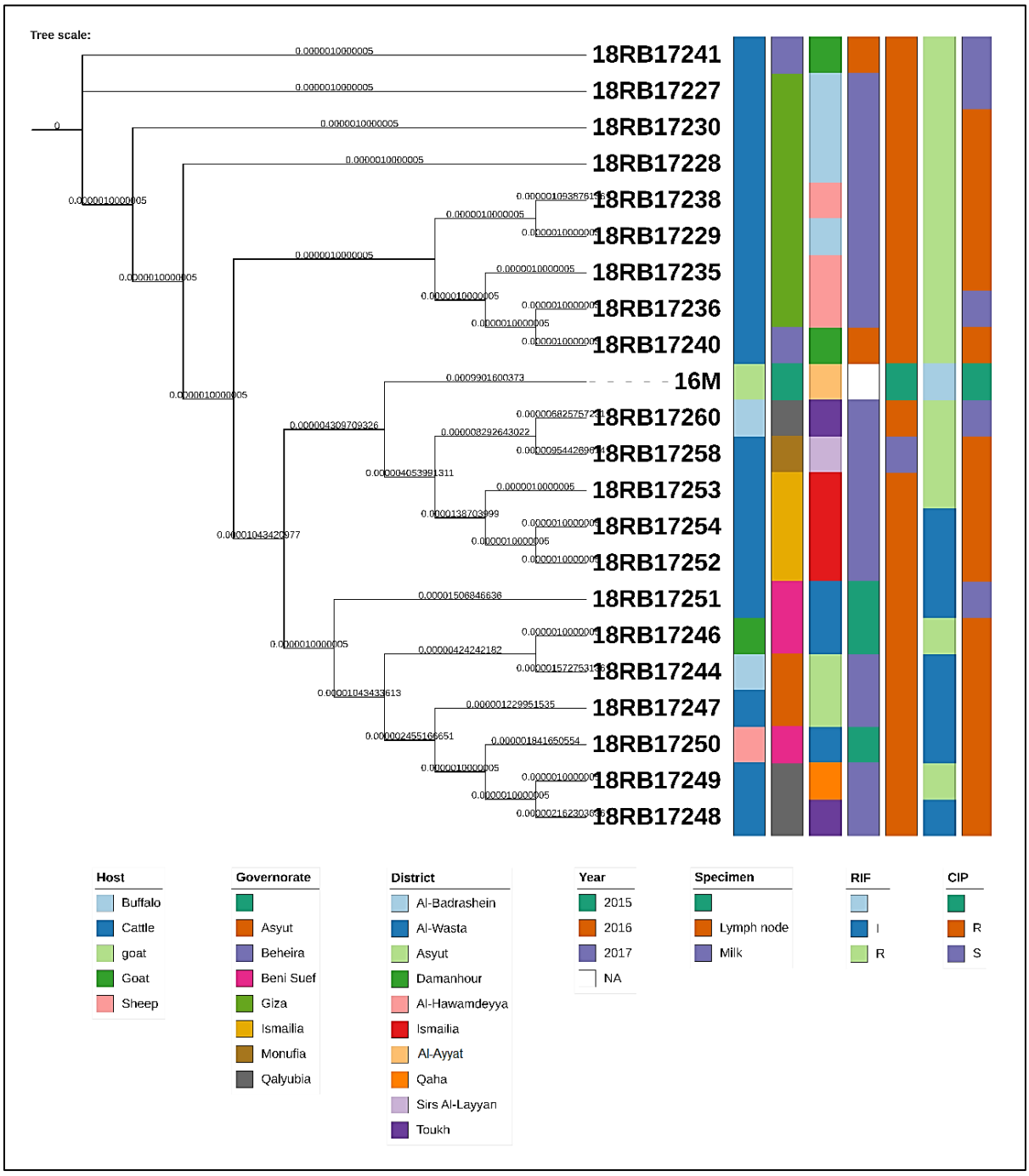

Figure 4. Dendrogram based on cgSNP analysis of 21 B. melitensis bv3 isolates recovered from different animal species in Egypt. The columns show strain identification numbers, host, geographic origin (governorate and distrcits), year, specimen/source of isolation and resistance to rifampicin and ciprofloxacin.

3.6. Antimicrobial susceptibility and Resistome analysis

In this study, 76.19%, 76.19%, and 66.66% of the *B. melitensis* isolates were resistant against ciprofloxacin, imipenem, and rifampicin/rifampin, respectively. While, 25%, 25%, and 37.5% of *B. abortus* isolates were phenotypically resistant to ciprofloxacin, imipenem, and rifampicin/rifampin, respectively.

Resistance Gene Identifier (RDI) available at the Comprehensive Antibiotic Resistance Database (CARD) identified two genes (*Brucella suis*-mprF and TriC) with 100% identity with available database involved in antimicrobial resistance mechanisms. Further, the tool also identifies Bifidobacterium adolescentis *rpo*B conferring resistance to rifampicin. However, AMRFinderPlus (NCBI) and RDI (CARD) did not identify any of the proposed genes responsible for antimicrobial resistance against macrolides (*erm*, *mef*, *msr*), tetracyclines (*tet* genes), beta-lactams (*mec*A) or trimethoprim (*fol*A).

A point mutation was identified in 3 isolates of *B. melitensis* (18RB17252, 18RB17253, and 18RB17254) in *rpo*B gene at position 2784-CGC to CGT/Arg to Arg. Another mutation was identified in *rpo*B of *B. melitensis* strain (18RB17260) at position 2394-ACG to ACT/Thr to Thr (table 2a, figure 5a) One point mutation was identified in *gyr*A gene of *B. melitensis* strains (18RB17252, 18RB17253, and 18RB17254) at 297-GAT to GAA/Asp to Glu (table 2b, figure 5b).

Table 2a. Mutation analysis of *rpoB* gene in phenotypically resistant isolates of *B. melitensis*.

ID	*Brucella* spp.	RIF	Mutation site	Mutation	Amino acid change
18RB17252	*B. melitensis*	3	2784	CGC to CGT	Arg to Arg
18RB17253	*B. melitensis*	4	2784	CGC to CGT	Arg to Arg
18RB17254	*B. melitensis*	3	2784	CGC to CGT	Arg to Arg
18RB17260	*B. melitensis*	4	2394	ACG to ACT	Thr to Thr

MIC for Rifampin: sensitive: ≤1, intermediate resistant: 2, resistant: ≥ 4 (CLSI, EUCAST)

Table 2b. Mutation analysis of *gyrA* gene in phenotypically resistant isolates of *B. melitensis*.

ID	*Brucella* spp.	CIP	Mutation site	Mutation	Amino acid change
18RB17252	*B. melitensis*	0.12	297	GAT to GAA	Asp to Glu
18RB17253	*B. melitensis*	0.12	297	GAT to GAA	Asp to Glu
18RB17254	*B. melitensis*	0.12	297	GAT to GAA	Asp to Glu

MIC for Ciprofloxacin: sensitive: ≤0.06, resistant: > 0.06 (CLSI, EUCAST)

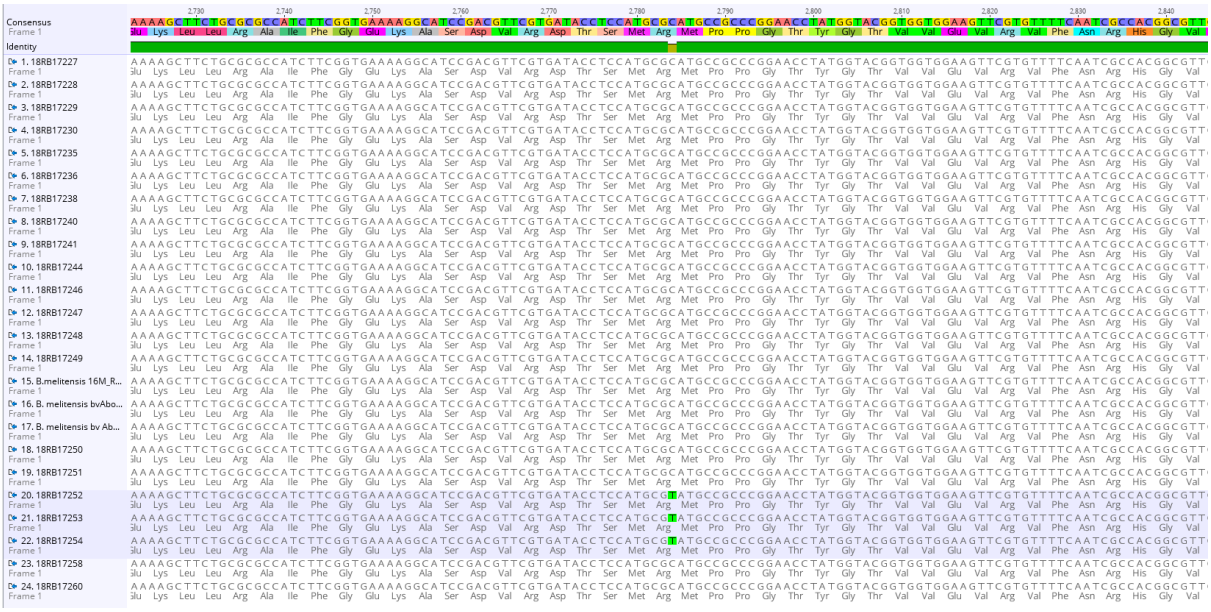

Figure 5a. Mutations in rpoB gene of *B. melitensis* isolates.

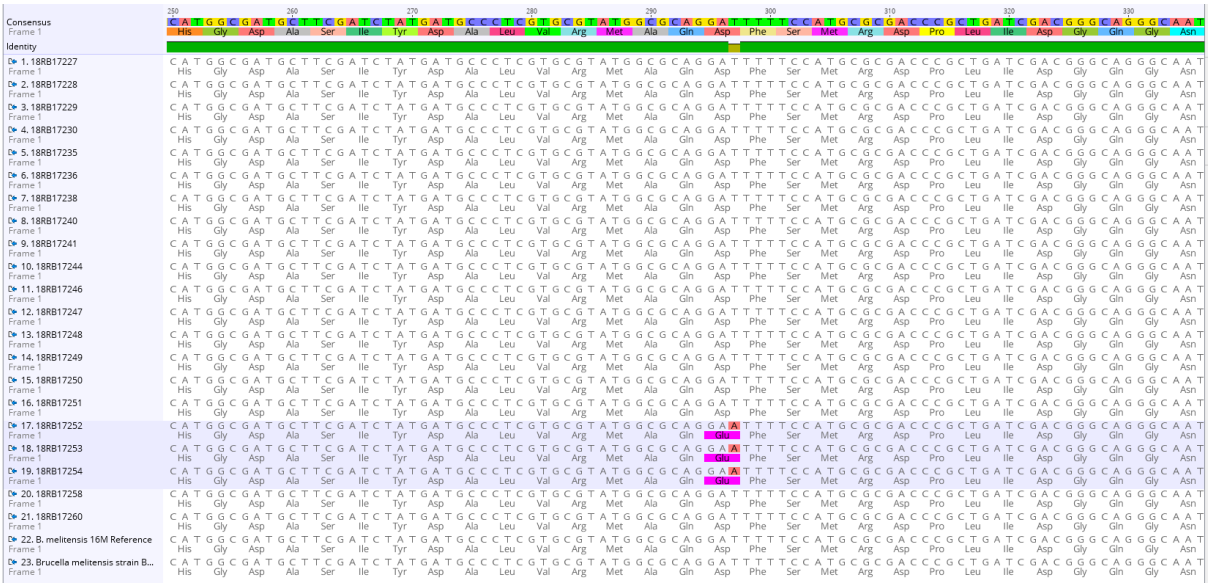

Figure 5b. Mutations in gyrA gene of *B. melitensis* isolates

3.7. Virulence factors

In total 43 virulent genes were identified in each *B. melitensis* and *B. abortus* isolates (Table. S7). These genes are mainly responsible for host immune evasion system, intracellular survival, regulation, and expression of the Type IV secretion system in brucellae. Genes responsible for lipopolysaccharide (LPS) synthesis, maturation, and functioning were identified in *B. melitensis* and *B. abortus* isolates. They include acpXL, fabZ, gmd, htrB, kdsA, kdsB, lpsA, lpsB/lpcC, lpxA, lpxB, lpxC, lpxD, lpxE, manAoAg, manCoAg, per, pgm, pmm, wbdA, wbkA, wbkB, wbkC, wboA, wbpL, wbpZ, wzm and wzt. LPS of brucellae facilitate pathogenesis by encountering the host innate immune system. Type IV secretory system (T4SS) plays an important role in pathogenesis by adhering bacterium to the host cell and cell entry, and intracellular trafficking and survival of brucellae. Several genes (virB1-virB12) were identified in *B. melitensis* and *B. abortus* clinical isolates. *cgs* gene responsible for Cyclic ß-1,2-glucans (CßGs) synthesis was also identified. CßGs also interfere with intracellular trafficking and prevent the phagosome-lysosome fusion cycle. Furthermore, genes for TIR domain-containing proteins (btpA and btpB) were also identified. These proteins interfere with Toll-like receptor (TLR) signaling and hinder the primary inflammatory response. ricA is another important gene and its expression believed to affect the maturation of the brucellae containing vesicle and help in immune evasion of brucellae. The detailed genes and their product/protein description were given in the table. S7.

4. Discussion

Brucellosis is a zoonotic disease of public health importance characterized by reproductive losses in animals. It is a debilitating disease in humans and is still endemic in many countries including Egypt [68]. In this study, molecular characterization and genotyping of *Brucella* isolates from cattle, buffaloes, sheep, and goats obtained from different geographical locations of Egypt was performed. Additionally, the assessment of antimicrobial resistance genes and virulence factors based on whole-genome sequencing in *Brucella* isolates from Egypt is reported. These results contribute to a better understanding of geographic transmission and the spread of brucellae in livestock in Egypt and pave a way for specific treatment and control of the disease in animals and humans also.

These brucellae (*B. abortus* and *B. melitensis*) are circulating in the animal population and recovered from cattle and buffaloes, sheep and goats, and dogs and cats previously [40,41,69,70]. The isolation of these brucellae particularly *B. melitensis* from cattle, buffaloes, sheep, and goats confirms its ability to establish permanent animal reservoirs in the country and the region [22,41]. Cross-species transmission of the small ruminant pathogen *B. melitensis* to other animal species were reported from Egypt previously [26,41,71]. The majority of animals are owned by individual households and are kept in small, mixed herds and moved daily between house yards and grazing lands. Circulation of brucellae particularly *B. melitensis*, the most virulent pathogen for human brucellosis, in cattle and buffaloes increases the risk for human infection. The isolation of *B. abortus* from cat and dogs also depicts the biological role of carrier hosts in the dissemination and re-emergence of *Brucella* spp. Identification of brucellae particularly *B. suis* and *B. melitensis* from pigs highlighted the endemicity of the disease in the country [19]. This complex epidemiological situation may result in difficulties for effective surveillance and control of brucellosis in Egypt.

Brucellosis control largely relies on efficient diagnosis and epidemiological assessment of prevailing *Brucella* species in a particular region. Routine culture isolation, serological and molecular assays help in disease diagnosis but unable to differentiate and trace back the circulating genotypes [40,43]. The genus *Brucella* is a highly homogenous and highly monomorphic species of bacteria with minimal genetic variations. The classification and biological typing mainly rely on isolation, identification based on biochemical characteristics, or immunological characteristics as agglutination with monospecific sera or phage typing[38]. However, these criteria are unable to trace back the origin of *Brucella* and discriminate among strains effectively [72]. Molecular advancements led the development of such assays that can differentiate, identify, and genotype brucellae at strain levels. Several studies have proved the usefulness of MLVA in genotyping and identification of *Brucella* strains along with their epidemiological monitoring and tracing back the source of brucellae [40,46]. However, these methods showed some limitations notably hyper-variability of some VNTR loci and homoplasy (convergent evolution) and currently suggested to be replaced by WGS based molecular tools providing detailed and better resolution in discriminating the genotypes of brucellae [2,73].

Brucella melitensis was identified in lymph nodes and milk samples. These isolates were recovered from preference host (1 from sheep and 1 from goats) in addition to occasional host (17 from cattle and 2 from buffaloes). The bovine infection with *B. melitensis* represents a serious public health problem as most of the milk is produced by cattle and buffaloes in the country and can be a potential source of human infection. Additionally, these animals may lead to environmental contamination as a result of abortions and the birth of infected calves [40]. *B. melitensis* also consider the most virulent type among other brucellae [74]. *Brucella melitensis* is the predominant species in Egypt and almost identified from every animal species as well as from humans [22,40,41]. The high prevalence of *B. melitensis* bv3 in large ruminants (secondary host) in the country represents a complex epidemiological situation that necessitates the detailed epidemiological investigations and genotyping of prevalent strains. *Brucella abortus* was identified from the lymph node and fetal stomach contents. These isolates were recovered from preference hosts (7 from cattle and 1 from buffaloes).

In silico MLVA-16, whole genome classical MLST and core genome SNPs analysis were applied to evaluate the epidemiological situation for *B. melitensis* and *B. abortus* strains recovered from cattle, buffaloes, sheep, and goats from several Governorate of Egypt. The findings suggest that different genotypes of brucellae are heterogeneously circulating in the country and brucellae may be endemic in

the country for a long time. Uncontrolled movement of animals among various governorates particularly during the religious festivals might be spreading brucellae from one region to another. Open and mixed animal market countrywide results in the spread of various diseases including brucellosis [41].

According to WGS classical MLST, all *B. melitensis* strains belongs to sequence type 11 (ST-11) while all *B. abortus* strains were sequence type 1 (ST-1). All 21 *B. melitensis* strains clustered into Mediterranean lineage, indicating a close phylogenetic relationship of Egyptian *B. melitensis* strains to those from Mediterranean region while the *B. abortus* strains showed a relationship to bovines and humans from UK, Ireland, New Zealand, Mexico, USA, India, Portugal, Bolivia, and Zimbabwe. According to *in silico* MLVA-16 profiles, all tested *B. melitensis* and *B. abortus* strains showed complete homogeneity in the panel 1 markers. These markers are sued for species assignment of strains. No difference was observed in strains collected from different animals from different governorates. Thus exhibiting typical Egyptian clusters [41]. Loci from Bruce16-Panel 2B in *B. abortus* profiles found to be discriminatory. Similarly, the loci (Bruc04, Bruc09, and Bruce16) of panel 2B discriminating *B. melitensis* strains into different genotypes.

Although, MLVA-16 typing is easy and cheap, however it poses some limitations in in-silico MLVA-16 analysis. Two loci, *Bruce21* (Panel 2A) and *Bruce07* (Panel 2B) were missing in 2 and 7 strains of *B. melitensis*, respectively. These missing values may attributed to the incomplete genomes of *B. melitensis* strains. However, for the closed genomes (reference genomes) in-silico MLVA-16 analysis worked properly [39,41,52].

Genotyping analysis of *B. abortus* and *B. melitensis* strains collected from cattle, buffaloes, sheep, and goats revealed 2 and 10 genotypes respectively. The *B. abortus* genotype 1 (GT-1) circulating in cattle from Mansoura and Qalyubia Governorate highlighted the animal movement and spread of the brucellae. Similarly, other genotypes (GT-2) of *B. abortus* were identified in cattle and buffaloes from Asyut, Beheira, Giza, and Monufia Governorates. MLVA-16 analysis identified 10 genotypes of *B. melitensis* recovered from cattle, buffaloes, sheep, and goats. GT-1 of *B. melitensis* exclusively circulating in Giza and Beheira governorate. Genotype 2, 3, 5, 6, 7, 8, 9, and 10 are singleton and recovered from cattle and buffaloes from Beni Suef, Asyut, Qalyubia, Asyut, Qalyubia, Beni Suef, Monufia and Qalyubia governorates respectively. Qalyubia and Monufia governorates have more heterogenous *B. melitensis* strains circulating in animals. Genotype 2 and 5 were recovered from goats and sheep respectively from different governorates. MLVA profile highlighted the genetic diversity of *B. melitensis* strains in Egypt. Genotyping analysis using MLVA-15 and MLVA-16 were performed in *B. abortus* and *B. melitensis* strain recovered from human and animals in Egypt previously [22,40,41,69,75]. Six strains of *B. abortus* in this study belonging to GT-2 shared the same MLVA-16 profile with Egypt-66 strain isolated from buffaloe at Ismailia Governorate [41]. Two genotypes (GT-7 and GT-8) of *B. melitensis* strains isolated from cattle at Qalyubia and Beni Suef Governorates in this study are very closely related to strain 23-Bm3-Suef and 36-Bm3-Suef isolated from cattle at Beni Suef Governorates [22].

Core genome single nucleotide polymorphism (cgSNPs) analysis revealed 3 and 14 genotypes of *B. abortus* and *B. melitensis* strains recovered from cattle, buffaloes, sheep, and goats in Egypt from various governorates in different years. Although, MLVA-16 and cgSNPs profiles share almost the same genotypes of *B. abortus* strains with the difference of only 1 strain (18RB17257) that was included in GT-2 in MLVA-16 profile while it is singleton genotype revealed by cgSNPs. However, cgSNPs showed

better genetic variation and discriminate *B. melitensis* strains into 14 genotypes as compared to MLVA-16 that revealed 10 genotypes. To the best of our knowledge, this is the first study highlighting the genotypes and epidemiology of brucellosis in Egypt using WGS based cgSNPs analysis. WGS based genotyping particularly cgSNPS provided high discriminatory power and identified several genotypes as compared to MLST and MLVA-16 profiling [2,39,52].

In this study, we have also analyzed phenotypical resistance patterns and compared the results with WGS data, both to evaluate phenotypic test results and to investigate possible genetic markers that could predict resistance. Notably, high phenotypic antimicrobial resistance was observed in *B. melitensis* isolates against clinically used antimicrobials (ciprofloxacin, imipenem, and rifampicin). Previous reports from Egypt [57], Norway [76], and Kazakhstan [77] describe comparatively low phenotypic resistance. However, the increased use of these antimicrobials in Egypt in veterinary and public health settings may be the cause of the emergence of higher phenotypic resistance in brucellae [78]. One SNP variant was detected in *gyr*A, a gene associated with ciprofloxacin resistance, while two SNP variants were identified in *rpo*B, a gene associated with rifampicin resistance [79] in 3 and 4 strains of *B. melitensis* respectively. The point mutation at position 297 (GAT to GAA) in *gyr*A gene leads to an amino acid change from Aspartic acid (Asp) to Glutamic acid (Glu). While the point mutations in *rpo*B gene reveal silent mutations at position 2784 (CGC to CGT/Arg-Arg) and 2394 (ACG to ACT/Thr-Thr). These alterations are different from mutations previously described as a cause of rifampicin resistance in *Brucella* [80]. Similarly, the mutations in *gyr*A gene also different from the mutations described previously [81]. However, mutations in rpoB and gyrA genes of *B. melitensis* described previously were observed in mutant strains. The SNP changes, therefore, does not seem associated with phenotypic rifampicin resistance. However, our findings question if the recommended broth microdilution method by the CLSI or EUCAST [66,67], and to some extent the gradient strip method, might overestimate in vitro rifampicin resistance in *B. melitensis*. This topic needs to be further addressed in larger multicentre studies. None of the proposed antimicrobial resistance genes responsible for antimicrobial resistance development in *Brucella* were detected against macrolides (*erm, mef, msr*), tetracyclines (*tet* genes), beta-lactams (*mec*A) or trimethoprim (*fol*A) [79]. The enhanced phenotypic resistance against *Brucella* isolates in this study may result due to the efflux of antimicrobial or other unknown mechanisms.

However, *Brucella suis*-mprF and TriC genes were identified in all *Brucella* isolates. Many bacterial pathogens achieve resistance to defensin-like cationic antimicrobial peptides (CAMPs) by the multiple peptide resistance factor (MprF) protein. MprF plays a crucial role in *Staphylococcus aureus* virulence and it is involved in resistance to the CAMP-like antibiotic daptomycin [82]. MprF is responsible for the modification of anionic phosphatidylglycerol with positively charged L-lysine which results in the repulsion of CAMPs. This protein also is known to affect susceptibility to antimicrobials (methicillin, oxacillin, bacitracin, gentamicin, beta-lactams) and other cationic peptides. It also resistance and susceptibility to moenomycin and vancomycin, resistance to human defensins (HNP1-3), and evasion of oxygen-independent neutrophil killing [83]. The identification of this protein may suggest the involvement especially intracellular survival and repulsion of cationic antimicrobials in *Brucella*. This protein identified in *B. suis* genome, however, not much is known about its virulence and antimicrobial resistance in *Brucella* [84].

TriC is a resistance nodulation cell division (RND) transporter that is a part of TriABC-OpmH a triclosan-specific efflux protein. It has been identified in *Pseudomonas aeruginosa* which contains two membrane fusion proteins, TriA and TriB [85]. Its function in *Brucella* is not known, however, increased

phenotypic resistance against ciprofloxacin and imipenem may suggest the role of efflux pump as described earlier in *Brucella melitensis* resistance against quinolones [86].

Brucellae are intracellular, facultative bacteria that can avoid killing mechanisms and proliferate within the cells of the reticuloendothelial system of the host. The pathogenesis of brucellae involves adhesion, invasion, establishment, and dissemination within the host. Several studies focused on the virulence factors in brucellae directed to the involvement of the outer membrane. The outer membrane contains Lipopolysaccharide (LPS), which is the major virulence factor of *Brucella*. It possesses a peculiar non-classical LPS as compared to the classical LPS from Enterobacteria, such as *Escherichia coli* [87]. In this study, we identified set of genes (lpsB/lpcC, lpxC, lpxD, fabZ, lpxA, lpxB, kdsA, kdsB, pgm, gmd, per, wzm, wzt, wbkB, wbkC, wbpL, acpXL, lpxE, lpsA, htrB, acpXL, wboA, wbdA, wbpZ, manAoAg, manCoAg, pmm, wbkA) regulating the LPS synthesis and functions. These genes are reported previously in brucellae [88]. LPS facilitates brucellae pathogenesis by countering the innate immune defense correlating with poor myeloid differentiation-2 (MD2) binding and low endotoxicity. Further, limiting the complement deposition and activation and killing of several neutrophils [89]. The TIR domain-containing proteins BtpA/Btp1/TcpB and BtpB interfere with Toll-like receptor (TLR) signaling to temper the host inflammatory response [90]. In addition to LPS, Type IV secretory system (T4SS) plays an important role in adherence of the bacterium to the host cell and cell entry, and intracellular trafficking and survival [87,91]. Cyclic ß-1,2-glucans (CßGs) also interferes with cellular trafficking and prevent the phagosome-lysosome fusion cycle. The brucellae containing vesicle (BCV) does not fuse with the lysosomes, instead, it interacts with the endoplasmic reticulum (ER), leading to the creation of a specialized vacuole in which the bacteria multiply [21]. The fusion between the endoplasmic reticulum and the BVC depended on the *Brucella* virB encoded T4SS. Genes responsible for TIR domain-containing proteins (btpA and btpB), T4SS (virB1-virB12), and CßGs (cgs) were identified in this study as reported previously [88]. Besides, RicA (Rab2 interacting conserved protein A) also identified in this study. The RicA-Rab2 interaction may affect the maturation of the BCV in a way that slows intracellular replication, thereby contributing to the evasion of the innate immune system [92].

Thus, higher resolution molecular tools based on next-generation sequencing (NGS) technology are now to be preferred and required for epidemiological studies and identification of the outbreaks of *Brucella* [39]. The application of core-genome multilocus sequence typing (cgMLST) and SNP analysis provided a higher phylogenetic distance resolution than MLVA for *B. melitensis* isolates belonging to the same lineage. This helped in the accurate typing, identification, clustering, and distinguishing of diverse and unrelated genotypes [2,52].

5. Conclusion

In summary, this study corroborates WGS to be a suitable tool for trace-back analysis of *B. melitensis* and *B. abortus* suggesting the potential geographic relation of a given strain. The WGS achieves a higher resolution compared to common typing approaches. Core genome SNPs analysis revealed 14 genotypes as compared to *in-silico* MLVA-16 analysis. Classical MLST showed limited variation and discriminated *B. abortus* and *B. melitensis* into sequence types 1 and 11 respectively. *Brucella melitensis* sequence types predominately infection animal population in Egypt and clustered closely to Mediterranean lineage however B. abortus sequence types showed closer affiliation with a strain isolated from Asian, American, and European lineages. Strains with similar genotypes isolated from different Governorates highlight the movement of the pathogen among governorates. Hence, it may also reflect the long endemicity of brucellosis in Egypt with earlier dispersion of types and great local

genetic diversity. Antimicrobial resistance development highlights the importance of control the use of antibiotics for the treatment of brucellosis and other diseases in the country. The irregular use of antibiotics may result in the development of antimicrobial resistance in brucellae in Egypt. The identification of similar types of virulence factors may highlight the pathogenicity of both brucellae strains among animals and humans.

Author Contributions: Data curation, S. A. G. E. E-S. and H. E-A.; Investigation, A. U. K., F. M., S. Abdel G. E. E-S., W. S. S. and H. E-A.; Methodology, A. U. K., A. E. S., F. M., A. H., H. N. and H. E-A.; Supervision, F. M., M. C. E., U. R., H. N. and H. E-A.; Writing – original draft, Aman U. K. and H. E-A.; Writing – review & editing, A. U. K., A. E. S., F. M., S. A. G. E. E-S., M. C. E., W. S. S., S. A. M., A. H., U. R., H. N. and H. E-A.

Funding: This research received no external funding.

Acknowledgments: The authors thank Michaela Ganss and Katja Fischer at the Institute of Bacterial Infections and Zoonoses, Friedrich–Loeffler Institut (FLI) for their cooperation and technical assistance. This research work was supported by the International Research Project as part of the "German Biosecurity Program" funded by the Federal Foreign Office, Germany. The authors thank Islamic Development Bank (IDB), Jeddah, Saudi Arabia for PhD grant.

Conflicts of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper

References

1. Seleem, M.N.; Boyle, S.M.; Sriranganathan, N. Brucellosis: a re-emerging zoonosis. *Vet Microbiol.* **2010**, *140*, 392-398, doi:10.1016/j.vetmic.2009.06.021.
2. Janowicz, A.; De Massis, F.; Ancora, M.; Camma, C.; Patavino, C.; Battisti, A.; Prior, K.; Harmsen, D.; Scholz, H.; Zilli, K., et al. Core genome multilocus sequence typing and single nucleotide polymorphism analysis in the epidemiology of *Brucella melitensis* infections. *J Clin Microbiol.* **2018**, *56*, doi:10.1128/jcm.00517-18.
3. Kirk, M.D.; Pires, S.M.; Black, R.E.; Caipo, M.; Crump, J.A.; Devleesschauwer, B.; Dopfer, D.; Fazil, A.; Fischer-Walker, C.L.; Hald, T., et al. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: A data synthesis. *PLoS Med.* **2015**, *12*, e1001921, doi:10.1371/journal.pmed.1001921.
4. Foster, G.; Osterman, B.S.; Godfroid, J.; Jacques, I.; Cloeckaert, A. *Brucella ceti* sp nov and *Brucella pinnipedialis* sp nov for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int J Syst Evol Micr.* **2007**, *57*, 2688-2693, doi:10.1099/ijs.0.65269-0.
5. Scholz, H.C.; Hubalek, Z.; Sedlacek, I.; Vergnaud, G.; Tomaso, H.; Al Dahouk, S.; Melzer, F.; Kampfer, P.; Neubauer, H.; Cloeckaert, A., et al. *Brucella microti* sp nov., isolated from the common vole Microtus arvalis. *Int J Syst Evol Micr.* **2008**, *58*, 375-382, doi:10.1099/ijs.0.65356-0.
6. Scholz, H.C.; Nockler, K.; Gollner, C.; Bahn, P.; Vergnaud, G.; Tomaso, H.; Al Dahouk, S.; Kampfer, P.; Cloeckaert, A.; Maquart, M., et al. *Brucella inopinata* sp nov., isolated from a breast implant infection. *Int J Syst Evol Micr.* **2010**, *60*, 801-808, doi:10.1099/ijs.0.011148-0.
7. Whatmore, A.M.; Davison, N.; Cloeckaert, A.; Al Dahouk, S.; Zygmunt, M.S.; Brew, S.D.; Perrett, L.L.; Koylass, M.S.; Vergnaud, G.; Quance, C., et al. *Brucella* papionis sp nova, isolated from baboons (*Papio* spp.). *Int J Syst Evol Micr.* **2014**, *64*, 4120-4128, doi:10.1099/ijs.0.065482-0.
8. Scholz, H.C.; Revilla-Fernandez, S.; Al Dahouk, S.; Hammerl, J.A.; Zygmunt, M.S.; Cloeckaert, A.; Koylass, M.; Whatmore, A.M.; Blom, J.; Vergnaud, G., et al. *Brucella vulpis* sp nov., isolated from mandibular lymph nodes of red foxes (Vulpes vulpes). *Int J Syst Evol Micr.* **2016**, *66*, 2090-2098, doi:10.1099/ijsem.0.000998.
9. Richomme, C.; Gauthier, D.; Fromont, E. Contact rates and exposure to inter-species disease transmission in mountain ungulates. *Epidemiol Infect.* **2006**, *134*, 21-30, doi:10.1017/s0950268805004693.
10. Saeed, U.; Ali, S.; Khan, T.M.; El-Adawy, H.; Melzer, F.; Khan, A.U.; Iftikhar, A.; Neubauer, H. Seroepidemiology and the molecular detection of animal brucellosis in Punjab, Pakistan. *Microorganisms.* **2019**, *7*, doi:10.3390/microorganisms7100449.
11. Villalobos-Vindas, J.M.; Amuy, E.; Barquero-Calvo, E.; Rojas, N.; Chacón-Díaz, C.; Chaves-Olarte, E.; Guzman-Verri, C.; Moreno, E. Brucellosis caused by the wood rat pathogen *Brucella neotomae*: two case reports. *J Med Case Rep.* **2017**, *11*, 352-352, doi:10.1186/s13256-017-1496-8.

12. Hensel, M.E.; Negron, M.; Arenas-Gamboa, A.M. Brucellosis in dogs and public health risk. *Emerg Infect Dis.* **2018**, *24*, 1401-1406, doi:10.3201/eid2408.171171.
13. Hull, N.C.; Schumaker, B.A. Comparisons of brucellosis between human and veterinary medicine. *Infect Ecol Epidemiol.* **2018**, *8*, 1500846, doi:10.1080/20008686.2018.1500846.
14. Saddique, A.; Ali, S.; Akhter, S.; Khan, I.; Neubauer, H.; Melzer, F.; Khan, A.U.; Azam, A.; El-Adawy, H. Acute febrile illness caused by *Brucella abortus* infection in humans in Pakistan. *Int J Environ Res Public Health.* **2019**, *16*, doi:10.3390/ijerph16214071.
15. Jamil, T.; Melzer, F.; Saqib, M.; Shahzad, A.; Khan Kasi, K.; Hammad Hussain, M.; Rashid, I.; Tahir, U.; Khan, I.; Haleem Tayyab, M., et al. Serological and molecular detection of bovine brucellosis at institutional livestock farms in Punjab, Pakistan. *Int J Environ Res Public Health.* **2020**, *17*, doi:10.3390/ijerph17041412.
16. Hassan, H.; Salami, A.; Nehme, N.; Hakeem, R.A.; Hage, J.E.; Awada, R. Prevalence and prevention of brucellosis in cattle in Lebanon. *Vet World.* **2020**, *13*, 364-371, doi:10.14202/vetworld.2020.364-371.
17. Liu, Z.; Shen, T.; Wei, D.; Yu, Y.; Huang, D.; Guan, P. Analysis of the epidemiological, clinical characteristics, treatment and prognosis of human brucellosis during 2014-2018 in Huludao, China. *Infect Drug Resist.* **2020**, *13*, 435-445, doi:10.2147/idr.S236326.
18. Njenga, M.K.; Ogolla, E.; Thumbi, S.M.; Ngere, I.; Omulo, S.; Muturi, M.; Marwanga, D.; Bitek, A.; Bett, B.; Widdowson, M.A., et al. Comparison of knowledge, attitude, and practices of animal and human brucellosis between nomadic pastoralists and non-pastoralists in Kenya. *BMC Public Health.* **2020**, *20*, 269, doi:10.1186/s12889-020-8362-0.
19. Khan, A.U.; Melzer, F.; El-Soally, S.; Elschner, M.C.; Mohamed, S.A.; Sayed Ahmed, M.A.; Roesler, U.; Neubauer, H.; El-Adawy, H. Serological and molecular identification of *Brucella* spp. in pigs from Cairo and Giza governorates, Egypt. *Pathogens.* **2019**, *8*, doi:10.3390/pathogens8040248.
20. Byndloss, M.X.; Tsolis, R.M. *Brucella* spp. Virulence Factors and Immunity. *Annu Rev Anim Biosci.* **2016**, *4*, 111-127, doi:10.1146/annurev-animal-021815-111326.
21. Seleem, M.N.; Boyle, S.M.; Sriranganathan, N. *Brucella*: a pathogen without classic virulence genes. *Vet Microbiol.* **2008**, *129*, 1-14, doi:10.1016/j.vetmic.2007.11.023.
22. Sayour, A.E.; Elbauomy, E.; Abdel-Hamid, N.H.; Mahrous, A.; Carychao, D.; Cooley, M.B.; Elhadidy, M. MLVA fingerprinting of Brucella melitensis circulating among livestock and cases of sporadic human illness in Egypt. *Transbound Emerg Dis.* **2020**.
23. Sayour, A.E.; Sayour, H.E. Binomial identification of Brucella isolates by MALDI-TOF mass spectrometry. In Proceedings of Second Scientific Conference of Food Safety and Technology, Cairo, Egypt; pp. 28-49.
24. Refai, M. Incidence and control of brucellosis in the Near East region. *Vet Microbiol.* **2002**, *90*, 81-110.
25. Menshawy, A.M.; Perez-Sancho, M.; Garcia-Seco, T.; Hosein, H.I.; Garcia, N.; Martinez, I.; Sayour, A.E.; Goyache, J.; Azzam, R.A.; Dominguez, L., et al. Assessment of genetic diversity of zoonotic *Brucella* spp. recovered from livestock in Egypt using multiple locus VNTR analysis. *Biomed Res Int.* **2014**, *2014*, 353876, doi:10.1155/2014/353876.
26. Wareth, G.; Hikal, A.; Refai, M.; Melzer, F.; Roesler, U.; Neubauer, H. Animal brucellosis in Egypt. *J Infect Dev Ctries.* **2014**, *8*, 1365-1373, doi:10.3855/jidc.4872.
27. Hegazy, Y.M.; Molina-Flores, B.; Shafik, H.; Ridler, A.L.; Guitian, F.J. Ruminant brucellosis in Upper Egypt (2005-2008). *Prev Vet Med.* **2011**, *101*, 173-181, doi:10.1016/j.prevetmed.2011.05.007.
28. Eltholth, M.M.; Hegazy, Y.M.; El-Tras, W.F.; Bruce, M.; Rushton, J. Temporal analysis and costs of ruminant brucellosis control programme in Egypt between 1999 and 2011. *Transbound Emerg Dis.* **2017**, *64*, 1191-1199, doi:10.1111/tbed.12491.
29. Samaha, H.; Al-Rowaily, M.; Khoudair, R.M.; Ashour, H.M. Multicenter study of brucellosis in Egypt. *Emerg Infect Dis.* **2008**, *14*, 1916-1918, doi:10.3201/eid1412.071452.
30. Khan, A.U.; Melzer, F.; El-Soally, S.; Elschner, M.C.; Mohamed, S.A.; Sayed Ahmed, M.A.; Roesler, U.; Neubauer, H.; El-Adawy, H. Serological and Molecular Identification of *Brucella* spp. in Pigs from Cairo and Giza Governorates, Egypt. *Pathogens* **2019**, *8*, doi:10.3390/pathogens8040248, doi:10.3390/pathogens8040248.
31. Khan, A.U.; Shell, W.S.; Melzer, F.; Sayour, A.E.; Ramadan, E.S.; Elschner, M.C.; Moawad, A.A.; Roesler, U.; Neubauer, H.; El-Adawy, H. Identification, genotyping and antimicrobial susceptibility testing of *Brucella* spp. isolated from livestock in Egypt. *Microorganisms.* **2019**, *7*, doi:10.3390/microorganisms7120603.
32. Moreno, E. Retrospective and prospective perspectives on zoonotic brucellosis. *Front Microbiol.* **2014**, *5*, 213, doi:10.3389/fmicb.2014.00213.

33. Siadat, S.D.; Salmani, A.S.; Aghasadeghi, M.R. Brucellosis vaccines: an overview. In *Zoonosis.* , Intech: Croatia, 2012; pp. 143-166.
34. Ariza, J.; Bosilkovski, M.; Cascio, A.; Colmenero, J.D.; Corbel, M.J.; Falagas, M.E.; Memish, Z.A.; Roushan, M.R.; Rubinstein, E.; Sipsas, N.V., et al. Perspectives for the treatment of brucellosis in the 21st century: the Ioannina recommendations. *PLoS Med.* **2007**, *4*, doi:10.1371/journal.pmed.0040317.
35. Solera, J.; Beato, J.L.; Martinez-Alfaro, E.; Segura, J.C.; de Tomas, E. Azithromycin and gentamicin therapy for the treatment of humans with brucellosis. *Clin Infect Dis.* **2001**, *32*, 506-509, doi:10.1086/318503.
36. Valdezate, S.; Navarro, A.; Medina-Pascual, M.J.; Carrasco, G.; Saez-Nieto, J.A. Molecular screening for rifampicin and fluoroquinolone resistance in a clinical population of *Brucella melitensis*. *J Antimicrob Chemother.* **2010**, *65*, 51-53, doi:10.1093/jac/dkp389.
37. Solera, J.; Martinez-Alfaro, E.; Espinosa, A. Recognition and optimum treatment of brucellosis. *Drugs.* **1997**, *53*, 245-256, doi:10.2165/00003495-199753020-00005.
38. OIE. Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*) (infection with *B. abortus, B. melitensis* and *B. suis*). In *In Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* 7th ed.; OIE; World Health Organization for Animal Health: Paris, France, 2018; Vol. 1, pp. 355-398.
39. Sacchini, L.; Wahab, T.; Di Giannatale, E.; Zilli, K.; Abass, A.; Garofolo, G.; Janowicz, A. Whole genome sequencing for tracing geographical origin of imported cases of human brucellosis in Sweden. *Microorganisms.* **2019**, *7*, doi:10.3390/microorganisms7100398.
40. Abdel-Hamid, N.H.; El-Bauomy, E.M.; Ghobashy, H.M.; Shehata, A.A. Genetic variation of Brucella isolates at strain level in Egypt. *Veterinary medicine and science* **2020**, 10.1002/vms3.260, 1-12, doi:10.1002/vms3.260.
41. Wareth, G.; El-Diasty, M.; Melzer, F.; Schmoock, G.; Moustafa, S.A.; El-Beskawy, M.; Khater, D.F.; Hamdy, M.E.R.; Zaki, H.M.; Ferreira, A.C., et al. MLVA-16 Genotyping of Brucella abortus and Brucella melitensis Isolates from Different Animal Species in Egypt: Geographical Relatedness and the Mediterranean Lineage. *Pathogens (Basel, Switzerland)* **2020**, *9*, doi:10.3390/pathogens9060498.
42. Wattam, A.R.; Foster, J.T.; Mane, S.P.; Beckstrom-Sternberg, S.M.; Beckstrom-Sternberg, J.M.; Dickerman, A.W.; Keim, P.; Pearson, T.; Shukla, M.; Ward, D.V., et al. Comparative phylogenomics and evolution of the *Brucellae* reveal a path to virulence. *J Bacteriol.* **2014**, *196*, 920-930, doi:10.1128/jb.01091-13.
43. Whatmore, A.M. Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. *Infect Genet Evol.* **2009**, *9*, 1168-1184, doi:10.1016/j.meegid.2009.07.001.
44. Le Flèche, P.; Jacques, I.; Grayon, M.; Al Dahouk, S.; Bouchon, P.; Denoeud, F.; Nöckler, K.; Neubauer, H.; Guilloteau, L.A.; Vergnaud, G. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiology.* **2006**, *6*, 9, doi:10.1186/1471-2180-6-9.
45. Whatmore, A.M.; Perrett, L.L.; MacMillan, A.P. Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiol.* **2007**, *7*, 34, doi:10.1186/1471-2180-7-34.
46. Vergnaud, G.; Hauck, Y.; Christiany, D.; Daoud, B.; Pourcel, C.; Jacques, I.; Cloeckaert, A.; Zygmunt, M.S. Genotypic expansion within the population structure of classical *Brucella* species revealed by MLVA16 Typing of 1404 *Brucella* isolates from different animal and geographic origins, 1974-2006. *Front Microbiol.* **2018**, *9*, 1545-1545, doi:10.3389/fmicb.2018.01545.
47. Tan, K.K.; Tan, Y.C.; Chang, L.Y.; Lee, K.W.; Nore, S.S.; Yee, W.Y.; Mat Isa, M.N.; Jafar, F.L.; Hoh, C.C.; AbuBakar, S. Full genome SNP-based phylogenetic analysis reveals the origin and global spread of *Brucella melitensis*. *BMC Genomics.* **2015**, *16*, 93, doi:10.1186/s12864-015-1294-x.
48. Ratushna, V.G.; Sturgill, D.M.; Ramamoorthy, S.; Reichow, S.A.; He, Y.; Lathigra, R.; Sriranganathan, N.; Halling, S.M.; Boyle, S.M.; Gibas, C.J. Molecular targets for rapid identification of *Brucella* spp. *BMC Microbiol.* **2006**, *6*, 13, doi:10.1186/1471-2180-6-13.
49. Pelerito, A.; Nunes, A.; Nuncio, M.S.; Gomes, J.P. Genome-scale approach to study the genetic relatedness among *Brucella melitensis* strains. *PLoS One.* **2020**, *15*, e0229863, doi:10.1371/journal.pone.0229863.
50. Foster, J.T.; Beckstrom-Sternberg, S.M.; Pearson, T.; Beckstrom-Sternberg, J.S.; Chain, P.S.; Roberto, F.F.; Hnath, J.; Brettin, T.; Keim, P. Whole-genome-based phylogeny and divergence of the genus *Brucella*. *J Bacteriol.* **2009**, *191*, 2864-2870, doi:10.1128/jb.01581-08.
51. Ng, P.; Kirkness, E.F. Whole genome sequencing. In *Genetic variation*, Springer: 2010; pp. 215-226.
52. Georgi, E.; Walter, M.C.; Pfalzgraf, M.T.; Northoff, B.H.; Holdt, L.M.; Scholz, H.C.; Zoeller, L.; Zange, S.; Antwerpen, M.H. Whole genome sequencing of *Brucella melitensis* isolated from 57 patients in Germany reveals high diversity in strains from Middle East. *PloS one.* **2017**, *12*, e0175425-e0175425, doi:10.1371/journal.pone.0175425.

53. Roetzer, A.; Diel, R.; Kohl, T.; Rückert, C.; Nübel, U.; Blom, J.; Wirth, T.; Jaenicke, S.; Schuback, S.; Rüsch-Gerdes, S. Whole genome sequencing versus traditional genotyping for investigation of a *Mycobacterium tuberculosis* outbreak: a longitudinal molecular epidemiological study. *PLoS medicine.* **2013**, *10*, e1001387.
54. Georgi, E.; Walter, M.C.; Pfalzgraf, M.T.; Northoff, B.H.; Holdt, L.M.; Scholz, H.C.; Zoeller, L.; Zange, S.; Antwerpen, M.H. Whole genome sequencing of *Brucella melitensis* isolated from 57 patients in Germany reveals high diversity in strains from Middle East. *PLoS One.* **2017**, *12*, e0175425, doi:10.1371/journal.pone.0175425.
55. Song, J.; Xu, Y.; White, S.; Miller, K.W.; Wolinsky, M. SNPsFinder--a web-based application for genome-wide discovery of single nucleotide polymorphisms in microbial genomes. *Bioinformatics.* **2005**, *21*, 2083-2084, doi:10.1093/bioinformatics/bti176.
56. Johansen, T.B.; Scheffer, L.; Jensen, V.K.; Bohlin, J.; Feruglio, S.L. Whole-genome sequencing and antimicrobial resistance in *Brucella melitensis* from a Norwegian perspective. *Sci Rep.* **2018**, *8*, 8538, doi:10.1038/s41598-018-26906-3.
57. Abdel-Maksoud, M.; House, B.; Wasfy, M.; Abdel-Rahman, B.; Pimentel, G.; Roushdy, G.; Dueger, E. In vitro antibiotic susceptibility testing of *Brucella* isolates from Egypt between 1999 and 2007 and evidence of probable rifampin resistance. *Ann Clin Microbiol Antimicrob.* **2012**, *11*, 24, doi:10.1186/1476-0711-11-24.
58. Sali, M.; De Maio, F.; Tarantino, M.; Garofolo, G.; Tittarelli, M.; Sacchini, L.; Zilli, K.; Pasquali, P.; Petrucci, P.; Marianelli, C., et al. Rapid and safe one-step extraction method for the identification of *Brucella* strains at genus and species level by MALDI-TOF mass spectrometry. *PLoS One.* **2018**, *13*, e0197864, doi:10.1371/journal.pone.0197864.
59. Bricker, B.J.; Halling, S.M. Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J Clin Microbiol.* **1995**, *33*, 1640-1642.
60. Bricker, B.J.; Halling, S.M. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *J Clin Microbiol.* **1994**, *32*, 2660-2666.
61. Garcia-Yoldi, D.; Marin, C.M.; de Miguel, M.J.; Munoz, P.M.; Vizmanos, J.L.; Lopez-Goni, I. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clin Chem.* **2006**, *52*, 779-781, doi:10.1373/clinchem.2005.062596.
62. Lopez-Goni, I.; Garcia-Yoldi, D.; Marin, C.M.; de Miguel, M.J.; Munoz, P.M.; Blasco, J.M.; Jacques, I.; Grayon, M.; Cloeckaert, A.; Ferreira, A.C., et al. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. *J Clin Microbiol.* **2008**, *46*, 3484-3487, doi:10.1128/jcm.00837-08.
63. Mikheenko, A.; Prjibelski, A.; Saveliev, V.; Antipov, D.; Gurevich, A. Versatile genome assembly evaluation with QUAST-LG. *Bioinformatics (Oxford, England)* **2018**, *34*, i142-i150, doi:10.1093/bioinformatics/bty266.
64. Wood, D.E.; Lu, J.; Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome biology* **2019**, *20*, 257, doi:10.1186/s13059-019-1891-0.
65. Deshmukh, A.; Hagen, F.; Sharabasi, O.A.; Abraham, M.; Wilson, G.; Doiphode, S.; Maslamani, M.A.; Meis, J.F. In vitro antimicrobial susceptibility testing of human *Brucella melitensis* isolates from Qatar between 2014 - 2015. *BMC Microbiol.* **2015**, *15*, 5, doi:10.1186/s12866-015-0458-9.
66. CLSI. Guideline M100-ED29. Performance standards for antimicrobial susceptibility testing, 29th edition. . 29th ed.; Wayne, PA: Clinical and Laboratory Standard Institute, USA.: 2019.
67. EUCAST. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. EUCAST: 2019; Vol. 9.
68. El-Diasty, M.; Wareth, G.; Melzer, F.; Mustafa, S.; Sprague, L.D.; Neubauer, H. Isolation of *Brucella abortus* and *Brucella melitensis* from seronegative cows is a serious impediment in brucellosis control. *Vet Sci.* **2018**, *5*, 4, doi:10.3390/vetsci5010028.
69. Menshawy, A.M.; Perez-Sancho, M.; Garcia-Seco, T.; Hosein, H.I.; García, N.; Martinez, I.; Sayour, A.E.; Goyache, J.; Azzam, R.A.; Dominguez, L., et al. Assessment of genetic diversity of zoonotic Brucella spp. recovered from livestock in Egypt using multiple locus VNTR analysis. *BioMed research international* **2014**, *2014*, 353876, doi:10.1155/2014/353876.
70. Wareth, G.; Melzer, F.; Tomaso, H.; Roesler, U.; Neubauer, H. Detection of Brucella abortus DNA in aborted goats and sheep in Egypt by real-time PCR. *BMC research notes* **2015**, *8*, 212, doi:10.1186/s13104-015-1173-1.

71. El-Tras, W.F.; Tayel, A.A.; Eltholth, M.M.; Guitian, J. Brucella infection in fresh water fish: Evidence for natural infection of Nile catfish, Clarias gariepinus, with Brucella melitensis. *Veterinary microbiology* **2010**, *141*, 321-325, doi:10.1016/j.vetmic.2009.09.017.
72. Al Dahouk, S.; Flèche, P.L.; Nöckler, K.; Jacques, I.; Grayon, M.; Scholz, H.C.; Tomaso, H.; Vergnaud, G.; Neubauer, H. Evaluation of Brucella MLVA typing for human brucellosis. *Journal of microbiological methods* **2007**, *69*, 137-145, doi:10.1016/j.mimet.2006.12.015.
73. Allen, A.R.; Milne, G.; Drees, K.; Presho, E.; Graham, J.; McAdam, P.; Jones, K.; Wright, L.; Skuce, R.; Whatmore, A.M., et al. Genomic epizootiology of a *Brucella abortus* outbreak in Northern Ireland (1997-2012). *Infect Genet Evol.* **2020**, *81*, 104235, doi:10.1016/j.meegid.2020.104235.
74. Corbel, M.J. *Brucellosis in humans and animals. The World Health Organization, in collaboration with the Food and Agriculture Organization of the United Nations and the World Organization for Animal Health*; WHO Press: Geneva, 2006; pp. 89.
75. Tiller, R.V.; De, B.K.; Boshra, M.; Huynh, L.Y.; Van Ert, M.N.; Wagner, D.M.; Klena, J.; Mohsen, T.S.; El-Shafie, S.S.; Keim, P., et al. Comparison of two multiple-locus variable-number tandem-repeat analysis methods for molecular strain typing of human Brucella melitensis isolates from the Middle East. *Journal of clinical microbiology* **2009**, *47*, 2226-2231, doi:10.1128/jcm.02362-08.
76. Johansen, T.B.; Scheffer, L.; Jensen, V.K.; Bohlin, J.; Feruglio, S.L. Whole-genome sequencing and antimicrobial resistance in *Brucella melitensis* from a Norwegian perspective. *Sci Rep.* **2018**, *8*, 9, doi:10.1038/s41598-018-26906-3.
77. Shevtsov, A.; Syzdykov, M.; Kuznetsov, A.; Shustov, A.; Shevtsova, E.; Berdimuratova, K.; Mukanov, K.; Ramankulov, Y. Antimicrobial susceptibility of *Brucella melitensis* in Kazakhstan. *Antimicrob Resist Infect Control.* **2017**, *6*, 5, doi:10.1186/s13756-017-0293-x.
78. S., S.E. *Report on the consultative meeting on antimicrobial resistance for countries in the Eastern Mediterranean Region:from policies to action, Egypt.* ; Regional Office for the Eastern Mediterranean, Berlin, 2014; pp 8-9.
79. Biswas, S.; Raoult, D.; Rolain, J.M. A bioinformatic approach to understanding antibiotic resistance in intracellular bacteria through whole genome analysis. *International journal of antimicrobial agents* **2008**, *32*, 207-220, doi:10.1016/j.ijantimicag.2008.03.017.
80. Marianelli, C.; Ciuchini, F.; Tarantino, M.; Pasquali, P.; Adone, R. Genetic bases of the rifampin resistance phenotype in Brucella spp. *Journal of clinical microbiology* **2004**, *42*, 5439-5443, doi:10.1128/jcm.42.12.5439-5443.2004.
81. Turkmani, A.; Psaroulaki, A.; Christidou, A.; Chochlakis, D.; Tabaa, D.; Tselentis, Y. In vitro-selected resistance to fluoroquinolones in two Brucella strains associated with mutational changes in gyrA. *International journal of antimicrobial agents* **2008**, *32*, 227-232, doi:10.1016/j.ijantimicag.2008.03.012.
82. Ernst, C.M.; Staubitz, P.; Mishra, N.N.; Yang, S.J.; Hornig, G.; Kalbacher, H.; Bayer, A.S.; Kraus, D.; Peschel, A. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. *PLoS Pathog.* **2009**, *5*, e1000660, doi:10.1371/journal.ppat.1000660.
83. Andrä, J.; Goldmann, T.; Ernst, C.M.; Peschel, A.; Gutsmann, T. Multiple peptide resistance factor (MprF)-mediated Resistance of *Staphylococcus aureus* against antimicrobial peptides coincides with a modulated peptide interaction with artificial membranes comprising lysyl-phosphatidylglycerol. *J Biol Chem.* **2011**, *286*, 18692-18700, doi:10.1074/jbc.M111.226886.
84. Paulsen, I.T.; Seshadri, R.; Nelson, K.E.; Eisen, J.A.; Heidelberg, J.F.; Read, T.D.; Dodson, R.J.; Umayam, L.; Brinkac, L.M.; Beanan, M.J., et al. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proceedings of the National Academy of Sciences of the United States of America* **2002**, *99*, 13148-13153, doi:10.1073/pnas.192319099.
85. Mima, T.; Joshi, S.; Gomez-Escalada, M.; Schweizer, H.P. Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. *J Bacteriol.* **2007**, *189*, 7600-7609, doi:10.1128/jb.00850-07.
86. Ravanel, N.; Gestin, B.; Maurin, M. In vitro selection of fluoroquinolone resistance in *Brucella melitensis*. *Int J Antimicrob Agents.* **2009**, *34*, 76-81, doi:10.1016/j.ijantimicag.2009.01.002.
87. Christopher, S.; Umapathy, B.L.; Ravikumar, K.L. Brucellosis: review on the recent trends in pathogenicity and laboratory diagnosis. *J Lab Physicians.* **2010**, *2*, 55-60, doi:10.4103/0974-2727.72149.
88. DelVecchio, V.G.; Kapatral, V.; Redkar, R.J.; Patra, G.; Mujer, C.; Los, T.; Ivanova, N.; Anderson, I.; Bhattacharyya, A.; Lykidis, A., et al. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. *Proceedings of the National Academy of Sciences of the United States of America* **2002**, *99*, 443-448, doi:10.1073/pnas.221575398.

89. Smith, J.A. *Brucella* lipopolysaccharide and pathogenicity: The core of the matter. *Virulence.* **2018**, *9*, 379-382, doi:10.1080/21505594.2017.1395544.
90. Felix, C.; Kaplan Türköz, B.; Ranaldi, S.; Koelblen, T.; Terradot, L.; O'Callaghan, D.; Vergunst, A.C. The *Brucella* TIR domain containing proteins BtpA and BtpB have a structural WxxxE motif important for protection against microtubule depolymerisation. *Cell Commun Signal.* **2014**, *12*, 53-53, doi:10.1186/s12964-014-0053-y.
91. Ke, Y.; Wang, Y.; Li, W.; Chen, Z. Type IV secretion system of *Brucella* spp. and its effectors. *Front Cell Infect Microbiol.* **2015**, *5*, 72-72, doi:10.3389/fcimb.2015.00072.
92. de Barsy, M.; Jamet, A.; Filopon, D.; Nicolas, C.; Laloux, G.; Rual, J.F.; Muller, A.; Twizere, J.C.; Nkengfac, B.; Vandenhaute, J., et al. Identification of a *Brucella* spp. secreted effector specifically interacting with human small GTPase Rab2. *Cell Microbiol.* **2011**, *13*, 1044-1058, doi:10.1111/j.1462-5822.2011.01601.x.

General discussion

General discussion

Brucellosis is an important worldwide zoonosis caused by bacteria of the genus *Brucella* [1]. Brucellosis is classified by WHO among the top seven world´s neglected zoonotic diseases [2]. It is a disease causing extensive worldwide economic losses in animal production and a debilitating disease in human health. Since its discovery, the brucellosis is constantly emerging zoonosis and is re-emerging at many geographical areas [3]. The epidemiology of human brucellosis has drastically changed due to globalization, socio-economic situation, and increased international travel. New foci of human brucellosis have emerged and the situation is rapidly worsening in some countries of the Middle East [4]. In most countries, it is a nationally notifiable disease and international notifiable to OIE. However, it is still under-reported and official notifications show only fractions of the true incidence. Although the disease has been eradicated and well-controlled in many developed countries, it is still present in the Mediterranean countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico, and Central and South America [3]. Diagnosis of brucellosis is currently based on serological assays and it is well established within the Egyptian national surveillance program. However, isolation and identification are limited to few laboratories due to biosafety reasons. Brucellae are classified S3 agents posing a great risk of infection to laboratory personal.

The current work is divided into 4 studies, the first study discussed the epidemiology of brucellae in pigs in Cairo and Giza using serological and molecular tools. This is the first study comprehensively highlighting the circulating brucellae in pigs from two governorates which have a considerable number of pigs. These pigs were raised in slums, rural, and peri-urban areas likely having close contact with other livestock (cattle, sheep, and goats) which may lead to the sharing of pathogens with each other [5]. In this study, 331 samples of pigs were collected at slaughterhouses. A higher number of seropositive pigs was recorded by c-ELISA (10.8%) when compared to the i-ELISA (4.83%). Swine brucellosis is widely prevalent in pig rearing countries. However, this is the first report from Egypt that may help in better understanding the epidemiology of the disease in the country. *Brucella* DNA was detected in 3.02% of pig samples. Qualitative multiplex real-time PCR confirmed seven *B. melitensis* and three *B. suis* DNAs. *Brucella suis* and *B. melitensis* DNA detected in pig sera highlighted the endemicity of these brucellae in the country and region [6]. The detection of *B. melitensis* DNA highlights the fact that it is the predominant species infecting farm animals in Egypt [7,8]. The detection of a higher number of *B. melitensis* DNA positive samples was expected as these pigs were in close contact with free-grazing sheep and goat flocks. It is common in extensive livestock farming to share pastures and water supplies. Such type of mixing of animals is an important risk factor to spread the disease from infected to healthy animals or other livestock

species [9]. Open animal markets and the uncontrolled movement of animals among different governorates in Egypt may spread the brucellae from one region to another [7]. Most of the sheep and goat flocks are mobile in Egypt and movements of infected animals can contaminate feeding and grazing areas and may also indirectly spread the infection to other animals (e.g., cattle, buffalo, or camel). Thus, the prevalence of *B. melitensis* and *B. suis* in swine can be attributed to cross-contamination or co-rearing of pigs with other animals.

In the second study, camel brucellosis was investigated in three Egyptian governorates with the highest number of camels, viz. Giza, Aswan and Al-Bahr Al-Ahmar (The Red Sea).The latter two governorates are the main entry portals for camels imported from Sudan. Giza receives imported Sudanese camels from Aswan as well as Somali camels shipped to the port of Suez. Apart from camels smuggled through the desert, Egypt has been importing camels officially from east Africa where brucellosis is enzootic in ruminants including camels [10-12]. High seroprevalences of 15.5%, 22.8%, 20.2%, and 31.0% were observed using RBPT, i-ELISA, c-ELISA, and CFT, respectively. Although few reports on camel brucellosis from Egypt are available, they show lower prevalences as compared to these findings [13]. A previous report from camel-keeping countries has revealed that seroprevalence of camel brucellosis can range from 1.0 to 23.3% [14]. Detection of *B. abortus* DNA in the three target governorates in addition to *B. melitensis* in camels reared in Aswan and Al-Bahr Al-Ahmar (The Red Sea) governorates was expected as previous reports showed the endemicity of *B. melitensis* and *B. abortus* in these regions already [6]. Previously, *B. melitensis* was isolated from camel stomach contents of an aborted fetus [13] as well as from whole citrated blood samples from Al-Bahr Al-Ahmar (The Red Sea) governorate [15]. Similar studies reported the identification of *B. melitensis* DNA from camel milk from Giza and Aswan [16,17]. The source of *B. melitensis* in camels might be attributed to small ruminants as camels are usually reared in herds with sheep and goats in mobile flocks [18,19]. However, the detection of *B. suis* DNA is a new finding of this study probably attributable to the first use of recently developed highly sensitive and specific primer for *B. suis* biovars 1 to 4 to test camel sera [20]. *Brucella suis* has previously been isolated from cattle [6] and *B. suis* or its DNA was identified in pigs in Egypt [21,22]. The source of *B. suis* in camels could be traceable to either domestic or wild pigs e.g. the wild boars (*Sus scrofa*) of the adjacent Eastern Desert. Being a border governorate with Sudan, Al-Bahr Al-Ahmar (The Red Sea) is also likely to have *B. suis* imported from Sudan, where some pig farms in Khartoum state to the west of Kassala state exist. The uncontrolled transboundary movement of Sudanese cattle to adjacent African countries, i.e. South Sudan and Central African Republic may contribute to the spread of *B. suis* as both states have domestic and wild pigs [23].

In the third part of the thesis, 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. 21 *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, Egypt. In developing countries including Egypt, antimicrobials particularly quinolones, tetracycline, beta-lactams, aminoglycosides, and imipenem are still overused non-therapeutically to treat various human infections [24-26]. This improper use of antimicrobials/antibiotics may result in the emergence of multidrug-resistant bacteria [27-29]. The development of antimicrobial resistance in *Brucella* has been reported from various parts of the world i.e. Egypt, Qatar, Iran, Malaysia, and China [30]. In the present work, massive phenotypic antimicrobial resistance was observed against ciprofloxacin, erythromycin, imipenem, rifampicin, and streptomycin in *Brucella* isolates. The 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 [31,32]. An alarming high rifampicin resistance (66.66%) of *B. melitensis* isolates was detected in this study. Reduced rifampicin susceptibility in *B. melitensis* strains was also reported from Iran, Malaysia, China, and Kazakhstan [33-36]. The emergence of phenotypic antimicrobial resistance against erythromycin (19.04%), imipenem (76.19%) and streptomycin (4.76%) in *B. melitensis* isolates is shown. However, the increased use of these antimicrobials in Egypt in veterinary and human practices may be the cause of the emergence 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 proven previously. Multidrug resistant strains of *B. abortus* isolated from cattle were reported previously from Brazil [38]. Resistance to commonly used antimicrobials is mediated by mutations of *rpo*B gene (rifampicin), *gyr*A, *gyr*B, *par*C, *par*E genes (quinolones), *erm, mef, msr* (macrolides) or *tet* genes (tetracyclines), *mec*A (beta-lactams) and *fol*A (trimethoprim) [39]. Genetic mutations were observed in *rpo*B, *gyr*A, and *gyr*B genes associated with phenotypic resistance in rifampicin and quinolones, respectively. In this study, mutations were identified in the *rpo*B gene associated with phenotypic rifampicin resistant *Brucella* strains isolated from clinical specimens of animals. Mutations were detected in all phenotypically resistant brucellae. 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 *rpo*B gene of phenotypically resistant *B. melitensis* were detected.

The mutations in the *gyr*B 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 *B. melitensis* are considered as novel findings of this study. None of these mutations was detected in *gyr*A or *gyr*B genes of *B. abortus* strains. Genes responsible for resistance against chloramphenicol (*cat*B), gentamicin (*Aac*) and tetracycline (*tet*A, *tet*B, *tet*M and *tet*O) were not detected in the investigated *Brucella* isolates of this study, which is in accordance with their phenotypic antimicrobial susceptibility results. Phenotypic and genotypic antimicrobial resistance in *B. melitensis* was also reported previously [40-42]. In contrast to previously reported antimicrobial resistance against rifampicin and quinolones, the phenotypic resistance against imipenem, streptomycin, and erythromycin was observed for the first time. These findings may again endorse the statement of the development of resistance due to misuse/overuse of antimicrobials in the Egypt [24-29]. Another interesting finding of this study is that, all resistant *Brucella* strains were isolated from animals and they showed resistance to antimicrobials clinically used in humans to treat brucellosis suggesting that the source of these *Brucella* strains maybe humans. These findings point to the fact that inter-species and intra-host species *Brucella* transmission is common but spillback may occur also when chronic human brucellosis is mistreated and resistant strains are shedded [43]. A likely scenario would be the animal keeper interface.

In the fourth part, the genetic diversity of 29 *Brucella* spp. (21 *B. melitensis* and 8 *B. abortus*) recovered from cattle, buffaloes, sheep, and goats from various governorates in different years in Egypt was evaluated. Advancement in molecular methodologies has enabled to sequence the whole genome and map the genes available for virulence and development of antimicrobial resistance in *Brucella*. The recently implemented whole genome sequencing (WGS) typing methods provide higher resolution genetic clustering and can overcome the drawbacks of missing VNTR calls. Thus, higher resolution molecular tools based on next-generation sequencing (NGS) technology are now to be preferred and required for epidemiological studies and identification of the outbreaks of brucellosis [44]. In this study, whole-genome sequencing-based next-generation sequencing technology i.e. Illumina technology was used to sequence the complete genomes of these *B. melitensis* and *B. abortus* isolated from Egypt. *In-silico* MLST revealed two different sequence types for *B. abortus* and *B. melitensis* i.e sequence types 1 and 11, respectively. These sequence types showed a close relationship with Mediterranean or Asian, American, and European lineage of *B. melitensis* and *B. abortus* respectively. Furthermore, MLVA-16 profiles of *B. abortus* clustered into 2 main clusters and *B. melitensis* profiles were also found in 2 main clusters consisting of 2 and 10 distinct genotypes. Moreover, core genome SNP analysis demonstrated high discriminatory power and divided *B. abortus* and *B. melitensis* into 2 main clusters each consisting of 3 and 14

distinct genotypes. These findings suggest that the brucellae may be endemic for a long time in Egypt and circulate in different farm animals in different governorates [7]. Additionally, the mechanism of antimicrobial resistance in brucellae against rifampicin, quinolones, and imipenem was investigated. The findings of this study revealed one SNP variant in *gyr*A, a gene associated with ciprofloxacin resistance while two SNP variants were identified in *rpo*B, a gene associated with rifampicin resistance [45] in 3 and 4 strains of *B. melitensis*, respectively. No mutation was observed in *B. abortus* strains. The point mutation at position 297 (GAT to GAA) in *gyr*A gene leads to an amino acid change from Aspartic acid (Asp) to Glutamic acid (Glu). The point mutations at position 2784 (CGC to CGT/Arg-Arg) and 2394 (ACG to ACT/Thr-Thr) in the *rpo*B gene are silent mutations. These alterations are different from mutations previously described as a cause of rifampicin and quinolone resistance in *Brucella* [32,42]. The high phenotypic antimicrobial resistance observed in this study may be attributed to other factors i.e. efflux of antimicrobials or other unknown mechanisms till now as none of the proposed genes responsible for antimicrobial resistance in brucellae were detected using WGS tools [45]. No difference was observed in virulence factors identified in *B. melitensis* and *B. abortus* strains from Egypt suggesting the pathogenicity and cross-infection of brucellae in human and farm animal species.

In conclusion, *B. melitensis* bv3 and *B. abortus* bv1 are the prevalent strains in Egypt [46]. Both, *B. abortus* and *B. melitensis* were found associated with infection in cattle, buffaloes, sheep, goats, pigs, camels, and humans. Effectiveness of control and eradication programs largely rely on accurate and timely identification of brucellosis cases. Conventional diagnostic methods have limitations in the efficient identification and typing of brucellae from animals and humans. Thus, the use of WGS based typing tools helped in accurate typing, identification, clustering, and distinguishing of diverse and unrelated genotypes. The emergence of antimicrobial resistance in brucellae necessitates the need for further research on the epidemiology and spread of antimicrobial-resistant *Brucella* strains in Egypt. Furthermore, the existing WHO regimes have to be reevaluated and awareness among physicians about AMR needs to be raised. It is also advocated that further investigations are necessary to assess the prevalence of *Brucella* species particularly *B. suis* in swine and other domestic ruminants as well as in humans using adequate diagnostic techniques.

References

1. Cutler, S.J.; Whatmore, A.M.; Commander, N.J. Brucellosis--new aspects of an old disease. *J Appl Microbiol* **2005**, *98*, 1270-1281, doi:10.1111/j.1365-2672.2005.02622.x.
2. Gorvel, J.-P. "If you bring an alarm, we will destroy it," said *Brucella* to the host cell. *Virulence* **2014**, *5*, 460-462, doi:10.4161/viru.29092.
3. Seleem, M.N.; Boyle, S.M.; Sriranganathan, N. Brucellosis: a re-emerging zoonosis. *Veterinary microbiology* **2010**, *140*, 392-398, doi:10.1016/j.vetmic.2009.06.021.

4. Pappas, G.; Papadimitriou, P.; Akritidis, N.; Christou, L.; Tsianos, E.V. The new global map of human brucellosis. *The Lancet. Infectious diseases* **2006**, *6*, 91-99, doi:10.1016/s1473-3099(06)70382-6.
5. Ashraf, M.; Hassan, A.; Raafat, M.; Fathia, A. Occupational health hazard of egyptian employees in contact with wastage nourished swine. *Journal of American Science* **2011**, *7*.
6. Menshawy, A.; Perez-Sancho, M.; Garcia-Seco, T.; Hosein, H.; Garcia, N.; Martinez, I.; Sayour, A.; Goyache, J.; Azzam, R.; Dominguez, L., et al. Assessment of genetic diversity of zoonotic *Brucella* spp. recovered from livestock in Egypt using multiple locus VNTR analysis. *Biomed Res Int* **2014**, *2014*, 353876, doi:10.1155/2014/353876.
7. Wareth, G.; El-Diasty, M.; Melzer, F.; Schmoock, G.; Moustafa, S.A.; El-Beskawy, M.; Khater, D.F.; Hamdy, M.E.R.; Zaki, H.M.; Ferreira, A.C., et al. MLVA-16 Genotyping of *Brucella abortus* and *Brucella melitensis* Isolates from Different Animal Species in Egypt: Geographical Relatedness and the Mediterranean Lineage. *Pathogens (Basel, Switzerland)* **2020**, *9*, doi:10.3390/pathogens9060498.
8. Sayour, A.E.; Elbauomy, E.; Abdel-Hamid, N.H.; Mahrous, A.; Carychao, D.; Cooley, M.B.; Elhadidy, M. MLVA fingerprinting of *Brucella melitensis* circulating among livestock and cases of sporadic human illness in Egypt. *Transboundary and emerging diseases* **2020**, 10.1111/tbed.13581, doi:10.1111/tbed.13581.
9. Díaz Aparicio, E. Epidemiology of brucellosis in domestic animals caused by *Brucella melitensis*, *Brucella suis* and *Brucella abortus*. *Revue scientifique et technique (International Office of Epizootics)* **2013**, *32*, 43-51, 53-60.
10. Chisholm, K.; Dueger, E.; Fahmy, N.T.; Samaha, H.A.T.; Zayed, A.; Abdel-Dayem, M.; Villinski, J.T. Crimean-congo hemorrhagic fever virus in ticks from imported livestock, Egypt. *Emerg Infect Dis* **2012**, *18*, 181-182, doi:10.3201/eid1801.111071.
11. Wakene, W.Z.; Mamo, G. Review on epidemiology of camel and human brucellosis in East Africa, Igad Member Countries. *J. Clin. Med.* **2017**, *6*, 109-115.
12. Gwida, M.M.; El-Gohary, A.H.; Melzer, F.; Tomaso, H.; Rosler, U.; Wernery, U.; Wernery, R.; Elschner, M.C.; Khan, I.; Eickhoff, M., et al. Comparison of diagnostic tests for the detection of *Brucella* spp. in camel sera. *BMC Res Notes* **2011**, *4*, 525, doi:10.1186/1756-0500-4-525.
13. El-Sayed, A.M.; Sayed-Ahmed, M.Z.; El-Diasty, M.M.; El-Beskawy, M.A.; Shoieb, S.M.; Younis, E.E. Sero-prevalence of camel brucellosis (*Camelus dromedarius*) and phenotypic characteristics of *Brucella melitensis* biovar 3 in Shalateen City, Red Sea Governorate, Egypt. *Afr. J. Microbiol. Res.* **2017**, *11*, 1259-1266.
14. Gwida, M.; El-Gohary, A.; Melzer, F.; Khan, I.; Rosler, U.; Neubauer, H. Brucellosis in camels. *Res Vet Sci* **2012**, *92*, 351-355, doi:10.1016/j.rvsc.2011.05.002.
15. Sayour, A.E.; Elbauomy, E.; Abdel-Hamid, N.H.; Mahrous, A.; Carychao, D.; Cooley, M.B.; Elhadidy, M. MLVA fingerprinting of Brucella melitensis circulating among livestock and cases of sporadic human illness in Egypt. *Transbound Emerg Dis.* **2020**.
16. Hosein, H.I.; Rouby, S.; Menshawy, A.; Ghazy, N. Seroprevalence of camel brucellosis and molecular characterization of *Brucella melitensis* recovered from dromedary camels in Egypt. *Res. J. Vet. Pract* **2016**, *4*, 17-24.
17. Hamdy, M.E.R.; Amin, A.S. Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR. *Vet. J.* **2002**, *163*, 299-305.
18. Musa, M.T.; Eisa, M.Z.; El Sanousi, E.M.; Abdel Wahab, M.B.; Perrett, L. Brucellosis in camels (*Camelus dromedarius*) in Darfur, Western Sudan. *J Comp Pathol* **2008**, *138*, 151-155, doi:10.1016/j.jcpa.2007.10.005.
19. Wareth, G.; Melzer, F.; El-Diasty, M.; Schmoock, G.; Elbauomy, E.; Abdel-Hamid, N.; Sayour, A.; Neubauer, H. Isolation of *Brucella abortus* from a Dog and a Cat Confirms their Biological Role in Re-emergence and Dissemination of Bovine Brucellosis on Dairy Farms. *Transboundary and emerging diseases* **2017**, *64*, e27-e30, doi:10.1111/tbed.12535.
20. Hansel, C.; Mertens, K.; Elschner, M.C.; Melzer, F. Novel real-time PCR detection assay for *Brucella suis*. *Vet Rec Open* **2015**, *2*, e000084, doi:10.1136/vetreco-2014-000084.
21. Khan, A.U.; Melzer, F.; El-Soally, S.; Elschner, M.C.; Mohamed, S.A.; Sayed Ahmed, M.A.; Roesler, U.; Neubauer, H.; El-Adawy, H. Serological and Molecular Identification of *Brucella* spp. in Pigs from Cairo and Giza Governorates, Egypt. *Pathogens (Basel, Switzerland)* **2019**, *8*, doi:10.3390/pathogens8040248, doi:10.3390/pathogens8040248.
22. Ibrahim, S.I. Studies on swine brucellosis in Egypt. *J Egypt Vet Med Ass.* **1996**, *56*, 1-12.
23. Gilbert, M.; Nicolas, G.; Cinardi, G.; Van Boeckel, T.P.; Vanwambeke, S.O.; Wint, G.R.W.; Robinson, T.P. Global distribution data for cattle, buffaloes, horses, sheep, goats, pigs, chickens and ducks in 2010. *Sci Data.* **2018**, *5*, 180227, doi:10.1038/sdata.2018.227.

24. Zakaa El-Din, M.; Samy, F.; Mohamed, A.; Hamdy, F.; Yasser, S.; Ehab, M. Egyptian community pharmacists' attitudes and practices towards antibiotic dispensing and antibiotic resistance; a cross-sectional survey in Greater Cairo. *Curr Med Res Opin.* **2019**, *35*, 939-946, doi:10.1080/03007995.2018.1544119.
25. Sabry, N.A.; Farid, S.F.; Dawoud, D.M. Antibiotic dispensing in Egyptian community pharmacies: an observational study. *Res Social Adm Pharm.* **2014**, *10*, 168-184, doi:10.1016/j.sapharm.2013.03.004.
26. S., S.E. *Report on the consultative meeting on antimicrobial resistance for countries in the Eastern Mediterranean Region:from policies to action, Egypt.* ; Regional Office for the Eastern Mediterranean, Berlin, 2014; pp 8-9.
27. Abdulah, R. Antibiotic abuse in developing countries. *Pharm Regul Aff.* **2012**, *1*, 1000-1006.
28. Kasim, K.; Hassan, H. Self medication problem in Egypt: A review of current and future perspective. *Int J Cur Res Rev.* **2018**, *10*, 6.
29. Gebeyehu, E.; Bantie, L.; Azage, M. Inappropriate use of antibiotics and its associated factors among urban and rural communities of Bahir Dar City administration, Northwest Ethiopia. *PLoS One.* **2015**, *10*, e0138179, doi:10.1371/journal.pone.0138179.
30. del Pozo, J.S.G.; Solera, J. Treatment of human brucellosis-Review of evidence from clinical trials. In *Updates on Brucellosis*, InTech.: 2015.
31. Ravanel, N.; Gestin, B.; Maurin, M. In vitro selection of fluoroquinolone resistance in *Brucella melitensis*. *International journal of antimicrobial agents* **2009**, *34*, 76-81, doi:10.1016/j.ijantimicag.2009.01.002.
32. Turkmani, A.; Psaroulaki, A.; Christidou, A.; Chochlakis, D.; Tabaa, D.; Tselentis, Y. In vitro-selected resistance to fluoroquinolones in two *Brucella* strains associated with mutational changes in gyrA. *International journal of antimicrobial agents* **2008**, *32*, 227-232, doi:10.1016/j.ijantimicag.2008.03.012.
33. Liu, Z.G.; Di, D.D.; Wang, M.; Liu, R.H.; Zhao, H.Y.; Piao, D.R.; Zhao, Z.Z.; Hao, Y.Q.; Du, Y.N.; Jiang, H., et al. In vitro antimicrobial susceptibility testing of human *Brucella melitensis* isolates from Ulanqab of Inner Mongolia, China. *BMC infectious diseases* **2018**, *18*, 43, doi:10.1186/s12879-018-2947-6.
34. Deshmukh, A.; Hagen, F.; Sharabasi, O.A.; Abraham, M.; Wilson, G.; Doiphode, S.; Maslamani, M.A.; Meis, J.F. In vitro antimicrobial susceptibility testing of human *Brucella melitensis* isolates from Qatar between 2014 - 2015. *BMC microbiology* **2015**, *15*, 121, doi:10.1186/s12866-015-0458-9.
35. Torkaman Asadi, F.; Hashemi, S.H.; Alikhani, M.Y.; Moghimbeigi, A.; Naseri, Z. Clinical and Diagnostic Aspects of Brucellosis and Antimicrobial Susceptibility of *Brucella* Isolates in Hamedan, Iran. *Japanese journal of infectious diseases* **2017**, *70*, 235-238, doi:10.7883/yoken.JJID.2016.133.
36. Hashim, R.; Ahmad, N.; Mohamed Zahidi, J.; Tay, B.Y.; Mohd Noor, A.; Zainal, S.; Hamzah, H.; Hamzah, S.H.; Chow, T.S.; Wong, P.S., et al. Identification and in vitro antimicrobial susceptibility of *Brucella* species isolated from human brucellosis. *International journal of microbiology* **2014**, *2014*, 596245, doi:10.1155/2014/596245.
37. World Health Organization, R.O.f.t.E.M. *Report on the consultative meeting on antimicrobial resistance for countries in the Eastern Mediterranean Region: From policies to action. Sharm el Sheikh, Egypt, 12–14 November 2013*; 2014.
38. Barbosa Pauletti, R.; Reinato Stynen, A.P.; Pinto da Silva Mol, J.; Seles Dorneles, E.M.; Alves, T.M.; de Sousa Moura Souto, M.; Minharro, S.; Heinemann, M.B.; Lage, A.P. Reduced Susceptibility to Rifampicin and Resistance to Multiple Antimicrobial Agents among *Brucella abortus* Isolates from Cattle in Brazil. *PloS one* **2015**, *10*, e0132532, doi:10.1371/journal.pone.0132532.
39. Biswas, S.; Raoult, D.; Rolain, J.M. A bioinformatic approach to understanding antibiotic resistance in intracellular bacteria through whole genome analysis. *Int J Antimicrob Agents.* **2008**, *32*, 207-220, doi:10.1016/j.ijantimicag.2008.03.017.
40. Johansen, T.B.; Scheffer, L.; Jensen, V.K.; Bohlin, J.; Feruglio, S.L. Whole-genome sequencing and antimicrobial resistance in *Brucella melitensis* from a Norwegian perspective. *Sci Rep.* **2018**, *8*, 9, doi:10.1038/s41598-018-26906-3.
41. Shevtsov, A.; Syzdykov, M.; Kuznetsov, A.; Shustov, A.; Shevtsova, E.; Berdimuratova, K.; Mukanov, K.; Ramankulov, Y. Antimicrobial susceptibility of *Brucella melitensis* in Kazakhstan. *Antimicrob Resist Infect Control.* **2017**, *6*, 5, doi:10.1186/s13756-017-0293-x.

42. Marianelli, C.; Ciuchini, F.; Tarantino, M.; Pasquali, P.; Adone, R. Genetic bases of the rifampin resistance phenotype in *Brucella* spp. *J Clin Microbiol.* **2004**, *42*, 5439-5443, doi:10.1128/jcm.42.12.5439-5443.2004.
43. Khan, M.Z.; Zahoor, M. An overview of brucellosis in cattle and humans, and its serological and molecular diagnosis in control strategies. *Trop Med Infect Dis.* **2018**, *3*, 14, doi:10.3390/tropicalmed3020065.
44. Sacchini, L.; Wahab, T.; Di Giannatale, E.; Zilli, K.; Abass, A.; Garofolo, G.; Janowicz, A. Whole Genome Sequencing for Tracing Geographical Origin of Imported Cases of Human Brucellosis in Sweden. *Microorganisms* **2019**, *7*, doi:10.3390/microorganisms7100398.
45. Biswas, S.; Raoult, D.; Rolain, J.M. A bioinformatic approach to understanding antibiotic resistance in intracellular bacteria through whole genome analysis. *International journal of antimicrobial agents* **2008**, *32*, 207-220, doi:10.1016/j.ijantimicag.2008.03.017.
46. Wareth, G.; Hikal, A.; Refai, M.; Melzer, F.; Roesler, U.; Neubauer, H. Animal brucellosis in Egypt. *J Infect Dev Ctries* **2014**, *8*, 1365-1373, doi:10.3855/jidc.4872.

Summary

Summary

Molecular Epidemiology, Genotyping and Antimicrobial Susceptibility Studies on *Brucella* spp. Isolated from Livestock

Brucellosis is a notifiable zoonotic disease of public health importance affecting animals and humans globally. Although the disease is controlled and eradicated from the developed world, it is still an endemic and often neglected zoonosis in developing countries including Egypt. The disease is emerging and remains endemic at high prevalences in ruminants particularly at established intensive breeding farms. The disease is also continuously introduced by the import of animals especially camels from neighboring brucellosis endemic countries like Sudan. Serological surveillance programs in the country have given indirect proof of brucellosis in cattle, buffaloes, sheep, goats, camels, equines, dogs, cats, and pigs. Published scientific literature has highlighted the dominance of *B. melitensis* bv3 and *B. abortus* bv1 infecting animals and humans in the country. The disease can be transmitted to other animals via direct or indirect contact. Clinically healthy animals may be a carrier of infection and a source of disease spread. Humans get infected via the consumption of unpasteurized milk and milk products and contact with infected animals. Movement of infected animals can also spread the infection to atypical hosts that become carriers.

Although brucellosis is endemic in Egypt, reports were scarce regarding its epidemiology in pigs. Thus, seroepidemiological surveillance was performed in pigs using serological and molecular assays. To achieve this objective, 331 serum samples were collected from two governorates (Cairo and Giza) having pig production. Samples were analyzed using competitive and indirect *Brucella* ELISAs. Anti-*Brucella* antibodies were detected in 4.83% and 10.8% pig sera by i-ELISA and c-ELISA, respectively. Molecular assays identified *Brucella* DNA in 3.02% of tested serum samples and identified as *B. melitensis* (7/10) and *B. suis* (3/10). The identification of both, *B. melitensis* and *B. suis* DNA, in serum samples of pigs was never reported till now. The results of this study provide help to develop effective control strategies for animal and human brucellosis in Egypt (Chapter 2).

The transmission of host-specific *Brucella* spp. to non-preferred hosts may occur due to the mixed rearing of farm animals. Data regarding *Brucella* spp. in the camel population in Egypt are scarce. Thus, this thesis focused on the seroepidemiology of camel brucellosis in three governorates (Giza, Aswan, and Al-Bahr Al-Ahmar (The Red Sea)) with the highest camel population and the largest camel markets in Egypt to determine the seroprevalence and to identify the *Brucella* spp. of local camel holdings. Serologic assays conducted on 381 serum

samples of camels showed that anti-*Brucella* antibodies were detected in 59 (15.5%), 87 (22.8%), 77 (20.2%) and 118 (31.0%) of sera by RBPT, i-ELISA, c-ELISA and CFT, respectively. Using real-time PCR, *Brucella* DNA was amplified from 32 (8.4%) seropositive samples and DNA of all species supposingly prevalent in Egypt was found: *Brucella abortus* (25/32), *Brucella suis* (5/32) and *Brucella melitensis* (2/32). Identification of *B. suis* DNA in camel sera reflects the complex epidemiological situation of brucellosis in Egypt. A high prevalence of brucellosis was observed in male animals. No significant association of brucellosis was found with age, sex, breed, and regions. The role of open markets in cross-species transmission and dissemination of brucellae nationwide and regionally is highlighted (Chapter 3).

The complex epidemiological situation of brucellosis in livestock poses risk to public health also. In this study, 29 *B. abortus* and *B. melitensis* strains were isolated from lymph nodes, milk, and fetal abomasal contents of infected cattle, buffaloes, sheep, and goats from nine districts. The phenotypic and genotypic patterns of antimicrobial resistance in local *Brucella* isolates were investigated. Notably, massive phenotypic resistance was observed in *B. melitensis* strains against ciprofloxacin, erythromycin, imipenem, rifampicin, and streptomycin, 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* strains were resistant to ciprofloxacin, erythromycin, imipenem, and rifampicin, respectively. Molecular analysis identified mutations in the *rpo*B gene associated with rifampicin resistance. These mutations were identified in all phenotypically resistant isolates. Mutations in *gyr*A and *gyr*B genes associated with ciprofloxacin resistance were also identified in *B. melitensis*. Repeated mutations were detected at positions 676, 677 (TAC to CTC/tyrosine to leucine) and 1435 (AAG to CAG/lysine to glutamine) in the *rpo*B gene of phenotypic resistant *B. melitensis* isolates while the same was recorded at position 2890 (CGT to GGT/arginine to glycine) in the *rpo*B gene for *B. abortus* isolates. Similarly, mutations in the *gyr*A gene were detected at positions 167 (ATG to AGG/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* strains. Mutations detected in genes associated with antimicrobial resistance unravel the molecular mechanisms of resistance in *Brucella* isolates from Egypt. The mutations in the *rpo*B gene in phenotypically resistant *B. abortus* isolates in this study were reported for the first time for Egypt. The results of this study advocate for more research on the epidemiology and spread of antimicrobial resistance in *Brucella* strains (Chapter 4).

Summary

Several studies from Egypt highlight the endemicity of brucellosis in the country. However, there are limited data available on the genetic diversity of brucellae circulating in Egypt. In this study, WGS was used to discriminate the genotypes along with virulence-associated factors and genetic markers responsible for antimicrobial resistance in 29 *Brucella* isolates (21 *B. melitensis* and 8 *B. abortus*) recovered from livestock in Egypt. *In silico* classical MLST identified sequence type-11 and sequence type-1 of *B. melitensis* and *B. abortus* strains, respectively in animal species in Egypt. These sequence types (ST-11 and ST-1) share a close relationship with Mediterranean and Asian, American, or European lineages respectively. *In silico* MLVA-16 analysis divided *B. abortus* and *B. melitensis* strains into 2 and 10 distinct genotypes, respectively. These strains types circulate in different governorates. However, cgSNPs analysis provided higher resolution and discriminated *B. abortus* and *B. melitensis* strains into 3 and 14 genotypes. Additionally, SNP variants were identified in *rpo*B and *gyr*A genes responsible for antimicrobial resistance in brucellae against rifampin and ciprofloxacin, respectively. These mutations are present at position 297-GAT to GAA/Asp-Glu in *gyr*A and two-point mutations were found in the *rpo*B gene at position 2784 (CGC to CGT/Arg-Arg) and 2394 (ACG to ACT/Thr-Thr) in 3 and 4 strains of *B. melitensis*, respectively (Chapter 5).

Zusammenfassung

Studien zur molekularen Epidemiologie, Genotypisierung und Antibiotikaresistenz an Brucellaisolaten von landwirtschaftlichen Nutztieren

Brucellose ist eine anzeigepflichtige weltweit auftretende Zoonose, die in vielen Ländern von großer Bedeutung für den öffentlichen Gesundheitssektor ist. Die Krankheit ist Bekämpfungs- und Überwachungsprogramme in vielen Industrieländern nur noch von geringer Bedeutung. In vielen Entwicklungsländern, einschließlich Ägypten, ist sie immer noch endemisch und wird in ihrer Bedeutung oft vernachlässigt. Deshalb ist die Brucellose insbesondere in den etablierten Zuchtbetrieben weit verbreitet. Die Krankheit wird außerdem kontinuierlich durch die Einfuhr von Tieren, vor allem von Kamelen, aus brucellose-endemischen Nachbarländern wie dem Sudan importiert. Bei serologische Überwachungsuntersuchungen in Ägypten wurde Brucellose bei Rindern, Büffeln, Schafen, Ziegen, Kamelen, Pferden, Hunden, Katzen und Schweinen nachgewiesen. In der wissenschaftlichen Literatur dominieren dabei *B. melitensis* bv3 und *B. abortus* bv1 bei Tieren und Menschen. Die Infektion wird zwischen den Tieren durch direkten oder indirekten Kontakt übertragen. Auch Tiere ohne Krankheitssymptome können als Infektionsquelle fungieren. Der Mensch infiziert sich meist durch den Verzehr von unpasteurisierter Milch und Milchprodukten sowie durch den Kontakt mit infizierten Tieren.

Obwohl die Brucellose in Ägypten endemisch ist, gab es kaum Berichte über die Situation bei Schweinen. Deshalb wurden im Rahmen dieser Arbeit seroepidemiologische Untersuchungen bei Schweinen mittels immunologischer und molekularer Assays durchgeführt. Dafür wurden 331 Serumproben aus zwei Gouvernoraten (Kairo und Gizeh) mit hohen Schweinebeständen entnommen und mittels kompetitivem und indirektem *Brucella*-ELISA analysiert. Anti-*Brucella*-Antikörper wurden in 16 (4,83%) und 36 (10,8%) Schweineseren mittels i-ELISA bzw. c-ELISA nachgewiesen. Molekulare Assays identifizierten *Brucella*-DNA in 3,02% (10) der untersuchten Serumproben. Über die Identifizierung sowohl von *B. melitensis*-DNA (7/10) als auch von *B. suis*-DNA (3/10) in Schweineserumproben wurde bisher noch nie berichtet. Die Ergebnisse dieser Studie tragen dazu bei, wirksame Bekämpfungsstrategien für Brucellose in Ägypten zu entwickeln (Kapitel 2).

Die Übertragung von *Brucella* spp. von bevorzugten auf nicht bevorzugte Wirte kann aufgrund der gemischten Aufzucht von Nutztieren auftreten. Über *Brucella* spp. in der Kamelpopulation in Ägypten liegen nur wenige Daten vor. Diese Arbeit ist auf Untersuchung der Kamelbrucellose in drei Gouvernoraten (Gizeh, Assuan und Al-Bahr Al-Ahmar (Rotes Meer)) mit der höchsten Kamelpopulation und den größten Kamelmärkten in Ägypten fokusiert, um hier die Seroprävalenz zu bestimmen und *Brucella* spp.-DNA in Kamelbeständen zu identifizieren. Serologische Untersuchungen an 381 Seren von Kamelen zeigen, dass bei 59

(15,5%), 87 (22,8%), 77 (20,2%) und 118 (31,0%) der Proben Anti-Brucella-Antikörper durch RBPT, i-ELISA, c-ELISA bzw. KBR nachgewiesen wurden. Mittels Real-time-PCR wurde *Brucella*-DNA in 32 (8,4%) seropositiven Proben amplifiziert, darunter *Brucella abortus* (25/32), *Brucella suis* (5/32) und *Brucella melitensis* (2/32). Es wurde eine hohe Prävalenz von Brucellose bei männlichen Tieren im Vergleich zu weiblichen Tieren beobachtet. Es konnte kein signifikanter Zusammenhang zwischen dem Auftreten von Brucellose und dem Alter, dem Geschlecht, der Rasse und den Regionen nachgewiesen werden. Das Vorkommen von *B. melitensis*-, *B. suis*- und *B. abortus*-Infektionen bei Kamelen weist auf eine komplexe epidemiologische Situation der Kamelbrucellose in Ägypten hin. Dies kann vermutlich auch auf die Rolle wenig kontrollierter Märkte bei der artübergreifenden Übertragung und Verbreitung von Brucellosen auf nationaler und regionaler Ebene (Kapitel 3).

Die weite Verbreitung von Brucellose bei Nutztieren in Ägypten, stellt auch ein Risiko für die öffentliche Gesundheit dar. In dieser Studie wurden 29 *B. abortus*- und *B. melitensis*-Stämme aus Lymphknoten, Milch und fetalen Labmageninhalten von infizierten Rindern, Büffeln, Schafen und Ziegen aus neun Distrikten in Ägypten isoliert. Diese Isolate wurden hinsichtlich ihrer phänotypischen und genotypischen antimikrobiellen Resistenzeigenschaften untersucht. Dabei wurde Resistenzen bei *B. melitensis* gegen Ciprofloxacin (76,2%), Erythromycin (19,0%), Imipenem (76,2%), Rifampicin (66,7%) und Streptomycin (4,8%) festgestellt. Bei *B. abortus* waren jeweils 25,0%, 87,5%, 25,0% und 37,5% der Isolate resistent gegen Ciprofloxacin, Erythromycin, Imipenem und Rifampicin. Durch die Genotypisierung wurden Mutationen im *rpo*B-Gen, die mit der Rifampicin-Resistenz assoziiert sind, in allen phänotypisch resistenten Isolaten identifiziert. Mutationen in den *gyr*A- und *gyr*B-Genen, die mit Ciprofloxacin-Resistenz assoziiert sind, wurden auch bei *B. melitensis* gefunden. Wiederholte Mutationen wurden an den Positionen 676, 677 (TAC zu CTC/Tyrosin zu Leucin) und 1435 (AAG zu CAG/Lysin zu Glutamin) im *rpo*B-Gen von phänotypisch resistenten *B. melitensis*-Isolaten nachgewiesen, während Mutationen an Position 2890 (CGT zu GGT/Arginin zu Glycin) im *rpo*B-Gen von *B. abortus*-Isolaten festgestellt wurde. In ähnlicher Weise wurden Mutationen im *gyr*A-Gen an den Positionen 167 (ATG zu AGG/Methionin zu Arginin), 197 (CCC zu CGC/Prolin zu Arginin), 202 (CGC zu AGC/Arginin zu Serin), 235 (GGT zu CGT/Glycin zu Arginin), 941 (GCC zu GAC/Alanin zu Asparaginsäure), 944 (GTG zu GAG/Valin zu Glutaminsäure), 944-945 (GTG zu GGA/Valin zu Glycin), 946 (GCC zu TCC/Alanin zu Serin) und 962 (AAC zu ACC/Asparagin zu Threonin) in *B. melitensis* nachgewiesen.

Mutationen, die in Genen entdeckt wurden, die mit antimikrobieller Resistenz assoziiert sind, entschlüsseln die molekularen Mechanismen der Resistenz in Brucella-Isolaten aus Ägypten.

Über die Mutationen im rpoB-Gen in phänotypisch resistenten *B. abortus*-Isolaten wurde in dieser Studie zum ersten Mal in Ägypten berichtet. Die Ergebnisse dieser Studie geben einen Einblick in die Epidemiologie und Verbreitung antimikrobiell resistenter Brucella-Stämme im Land (Kapitel 4).

Es gibt nur wenige Daten über die genetische Vielfalt der in Ägypten zirkulierenden Brucellen. Mittels Gesamtgenomsequenzierung wurden 29 ägyptische Brucella-Isolate (21 *B. melitensis* und 8 *B. abortus*) genotypisiert und virulenzassoziierte Faktoren und Marker bestimmt, die für die antimikrobielle Resistenz verantwortlich sind. Mittels *in-silico* MLST wurden die bei Nutztieren in Ägypten vorherrschenden Sequenztypen-11 und -1 von *B. melitensis* und *B. abortus* gefunden. Diese Sequenztypen sind auch bei anderen mediterranen, asiatischen, amerikanischen und europäischen Linien gefunden worden. Die *in-silico* MLVA-16-Analyse ergab 2 *B. abortus*- und 10 *B. melitensis*-Genotypen, die in verschiedenen Gouvernoraten zirkulieren. Die cgSNP-Analyse lieferte eine höhere Auflösung und diskriminierte *B. abortus*- und *B. melitensis*-Stämme in 3 bzw. 14 Genotypen. Zusätzlich wurden SNP-Varianten in den *rpo*B- und *gyr*A-Genen identifiziert, die für die antimikrobielle Resistenz von Brucellen gegen Rifampin bzw. Ciprofloxacin verantwortlich sind. Diese Mutationen betreffen bei 3 *B. melitensis* Stämmen die Position 297-GAT zu GAA/Asp-Glu in *gyr*A. Zwei-Punkt-Mutationen im *rpo*B-Gen an Position 2784 (CGC zu CGT/Arg-Arg) und 2394 (ACG zu ACT/Thr-Thr) wurden 4 Stämmen von *B. melitensis* nachgewiesen (Kapitel 5).

List of published articles

1. **Khan AU**, Sayour AE, Melzer F, El-Soally SAGE, Elschner MC, Shell WS, Moawad AA, Mohamed SA, Hendam A, Roesler U, Neubauer H, El-Adawy H. **(2020)**. Seroprevalence and molecular identification of *Brucella* spp. in camels in Egypt. *Microorganisms*. 8(7):E1035. doi: 10.3390/microorganisms8071035.
2. **Khan AU**, Shell WS, Melzer F, Sayour AE, Ramadan ES, Elschner MC, Moawad AA, Roesler U, Neubauer H, El-Adawy H. **(2019)**. Identification, genotyping and antimicrobial susceptibility testing of *Brucella* spp. isolated from livestock in Egypt. *Microorganisms*. 7(12):603. doi: 10.3390/microorganisms7120603.
3. **Khan AU**, Melzer F, El-Soally SAGE, Elschner MC, Mohamed SA, Sayed Ahmed MA, Roesler U, Neubauer H, El-Adawy H. **(2019)**. Serological and molecular identification of *Brucella* spp. in pigs from Cairo and Giza governorates, Egypt. *Pathogens*. 8(4):248. doi: 10.3390/pathogens8040248.
4. **Khan AU**, Melzer F, Elschner M C, Roesler U, Neubauer H and El-Adawy H. **(2020)**. Whole Genome sequencing for tracing the genetic diversity and virulence genes of *Brucella abortus* and *Brucella melitensis* isolated from livestock in Egypt. (*Under review*).
5. **Khan AU**, Melzer F, Hendam A, Sayour A E, Khan I, Elschner M C, Younus M, Ehtisham-ul-Haque S, Waheed U, Farooq M, Ali S, Neubauer H and El-Adawy H. **(2020)**. Seroprevalence and molecular identification of *Brucella* spp. in bovines in Pakistan – association investigation with risk factors using machine learning. (*Submitted to MDPI: Pathogens*)
6. Usama Saeed, Shahzad Ali, Tooba Latif, Muhammad Rizwan, Attaullah Saif, Anam Iftikhar, Syed Ghulam Mohayud Din Hashmi, **Khan AU**, Iahtisham Khan, Falk Melzer, Hosny El-Adawy, Heinrich Neubauer (**2020**). Prevalence and Spatial Distribution of Animal Brucellosis in Central Punjab, Pakistan. *Int. J. Environ. Res. Public Health. 17*(18), 6903; https://doi.org/10.3390/ijerph17186903.
7. Saddique A, Ali S, Akhter S, Khan I, Neubauer H, Melzer F, **Khan AU**, Azam A, El-Adawy H (**2019**). Acute Febrile Illness Caused by *Brucella abortus* Infection in Humans in Pakistan. *Int. J. Environ. Res. Public Health.* doi: 10.3390/ijerph16214071.
8. Saeed U, Ali S, Khan TM, El-Adawy H, Melzer F, **Khan AU**, Iftikhar A, Neubauer H (**2019**). Seroepidemiology and the Molecular Detection of Animal Brucellosis in Punjab, Pakistan. *Microorganisms*. doi: 10.3390/microorganisms7100449.
9. Mehmood K, Zhang H, Sabir AJ, Abbas RZ, Ijaz M, Durrani AZ, Saleem MH, Ur Rehman M, Iqbal MK, Wang Y, Ahmad HI, Abbas T, Hussain R, Ghori MT, Ali S, **Khan AU**, Li J (**2017**). A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. *Microb Pathog*. doi: 10.1016/j.micpath.2017.06.006.
10. Jaleel S, Younus M, Idrees A, Arshad M, **Khan AU**, Ehtisham-Ul-Haque S, Zaheer MI, Tanweer M, Towakal F, Munibullah, Tipu MY, Sohail ML, Umar S (**2017**). Pathological Alterations in Respiratory System During Co-infection with Low Pathogenic Avian Influenza Virus (H9N2) and *Escherichia Coli* in Broiler Chickens. *J Vet Res*. doi: 10.1515/jvetres-2017-0035.
11. Urva, Shafique H, Jamil Y, Haq ZU, Mujahid T, **Khan AU**, Iqbal M, Abbas M (**2017**). Low power continuous wave-laser seed irradiation effect on Moringa oleifera germination, seedling growth and biochemical attributes. *J Photochem Photobiol B*. doi: 10.1016/j.jphotobiol.2017.04.001.

Acknowledgment

In the beginning, I would like to thank **ALLAH** the Merciful for his tenderness and satisfaction.

First of all, I am deeply indebted to express my deep thanks and warmest gratitude to my supervisor, **Prof. Dr. habil. Heinrich Neubauer**, the head of the Institute of Bacterial Infections and Zoonoses [IBIZ] at Friedrich-Loeffler-Institut [FLI], Jena, Germany for hosting me to complete my study. I would like to thank him for his continuous guidance, orientation, support, and help during my work. Also, I would like to thank him for his inspiration and careful reading and correction of my manuscripts. Without his help and persistent guidance, this dissertation would not have been possible. I felt great honor to work under his kind supervision and I have gained during working with him superb and valuable experiences either in research or in life.

I would like to convey my deep thanks and sincere gratitude also to **Prof. Dr. habil. Uwe Rösler**, the head of the Institute of Animal Hygiene and Environmental Health at the Free University of Berlin, Berlin, Germany for his kind supervision, unforgettable help and precious time given to me.

Also, I wish to express my sincere gratitude for the kindness and encouragement of **Dr. Falk Melzer**, the head of the National Reference Laboratory for animal brucellosis at IBIZ, FLI, Jena, Germany for his continuous support, valuable advice, careful guidance, and patience during my thesis. His motivation and kindness always inspired me.

My particular thanks to **Ass. Prof. Dr. Hosny El-Adawy**, the head of NRL Campylobacteriosis at IBIZ, FLI, Jena, Germany for his generous help and advice during my research, writing my manuscripts and thesis. Also, he arranged my study, research, and above all, he treated me like his brother. I learned from him a lot of experiences either in brucellosis or in communication. I was proud to work with him.

I am deeply grateful to **Dr. Mandy Elschner**, the head of L-3 pathogens workgroup at IBIZ, FLI, Jena, Germany for her guidance and friendly cooperation and important support throughout this work.

I would like to appreciate the great effort from **Mrs. P. Flemmeng**, the institute secertery for her assistance.

I also want to pay my deep thanks to **Prof. Ashraf E. Sayour** and **Prof. Waleed S. Shell**, scientists at Agricultural Research Centre (ARC), Egypt for help in my research and manuscript preparations.

I wish to present a particular thanks to all staff members of AGr 130 at IBIZ, FLI, Jena, Germany (**Dr. A. Rassbach**, **M. Ganß**, **K. Fischer**, **P. Marten**, **L-M. Karnbach**, **T. Jamil** and **A. Moawad**) for their assistance and support in all personality subjects throughout my stay.

From the side of the family, I would like to present this work to my father, who always was the source of support and encouragement for me. My special gratitude is to my mother for her prayers and support. I would like to express my deep and warmest thanks to my wife **Safina Aman** and my son **Muhammad Ahmed Khan** for their love, encouragement, patience and support during my research time. Without their encouragement, support and understanding, it would have been impossible for me to finish this work.

I would like to thank the Islamic Development Bank Jeddah, Saudi Arabia (IDB) for financial support (grant no. 36/14203) and the “German biosecurity program” funded by Federal Foreign Office, Germany.

Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 29.09.2020 **Aman Ullah Khan**

mbvberlin mensch und buch verlag

49,90 Euro | ISBN: 978-3-96729-072-1