



Molecular characterization and antimicrobial susceptibility testing of clinical and non-clinical *Brucella melitensis* and *Brucella abortus* isolates from Egypt

Gamal Wareth^{a,b,c,*}, Mohamed El-Diasty^d, Nour H. Abdel-Hamid^d, Katharina Holzer^e, Mahmoud E.R. Hamdy, Prof.^d, Shawky Moustafa, Prof.^c, Momtaz A. Shahein, Prof.^d, Falk Melzer^a, Wolfgang Beyer^e, Mathias W. Pletz^b, Heinrich Neubauer^a

^a Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, Naumburger Str. 96a, 07743 Jena, Germany

^b Institute for Infectious Diseases and Infection Control, Jena University Hospital, Am Klinikum 1, 07747 Jena, Germany

^c Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh 13736, Egypt

^d Animal Health Research Institute, Agricultural Research Center, P.O. Box 264-Giza, Cairo 12618, Egypt

^e Institute of Animal Science, Department of Livestock Infectiology and Environmental Hygiene, University of Hohenheim, 70599 Stuttgart, Germany

ARTICLE INFO

Keywords:

Brucella abortus

Brucella melitensis

Antimicrobial susceptibility

MALDI-TOF

WGS, Egypt

ABSTRACT

Brucellosis is a highly contagious and incapacitating disease of humans, livestock and wildlife species globally. Treatment of brucellosis in animals is not recommended, and in humans, combinations of antibiotics recommended by the World Health Organization are used. However, sporadic antimicrobial-resistant (AMR) isolates and relapse cases have been reported from different endemic regions. In the current study, molecular characterization and antibiotic susceptibility testing using the microdilution method for 35 *B. abortus* and *B. melitensis* strains isolated from humans, milk and animal were carried out. Additionally, Next-Generation-Sequencing (NGS) technology was applied to confirm *Brucella* at the species level and investigate AMR and pathogenicity-associated determinants. MALDI-TOF seemed to be a rapid and reliable tool for routine identification of brucellae to the genus level; however, DNA-based identification is indispensable for accurate species identification. *Brucella abortus* strains were isolated from two human cases and a sheep. Such infections are uncommon in Egypt. Egyptian *Brucella* strains are still in-vitro susceptible to doxycycline, tetracyclines, gentamicin, ciprofloxacin, levofloxacin, chloramphenicol, streptomycin, trimethoprim/sulfamethoxazole and tigecycline. Probable (no CLSI/EUCAST breakpoints have been defined yet) in-vitro resistance to rifampicin and azithromycin was observed. WGS failed to determine classical AMR genes, and no difference in the distribution of virulence-associated genes in all isolates was found. Isolates of human and non-human origins were still susceptible to the majority of antibiotics used for treatment in humans. The absence of classical AMR genes in genomes of “resistant” *Brucella* strains may reflect a lack of information in databases, or resistance might not be encoded by single resistance genes. The One Health approach is necessary for tackling brucellosis. Continuous susceptibility testing, updating of breakpoints, assessing mutations that lead to resistance are needed.

1. Introduction

Brucellosis is a common infectious disease in animals causing substantial economic losses in the livestock industry and posing a serious threat to veterinary and public health worldwide [1]. Brucellae are

debilitating bacteria that can infect a wide range of domesticated vertebrate animals, e.g. livestock and companion animals, wildlife, and marine mammal [1–3]. The disease is transmitted to humans mainly by consuming unpasteurized milk/dairy products or undercooked meat/meat products and by direct contact with infected animal's discharges

* Corresponding author at: Institute of Bacterial Infections and Zoonoses, Naumburger Str. 96a, 07743 Jena, Germany.

E-mail addresses: gamal.wareth@fli.de (G. Wareth), dr_mesbah_m@yahoo.com (M. El-Diasty), nour78_78@yahoo.com (N.H. Abdel-Hamid), katharina.holzer@uni-hohenheim.de (K. Holzer), merhamdy@hotmail.com (M.E.R. Hamdy), dr.shawky.gesriha@gmail.com (S. Moustafa), montaz.shahein@yahoo.com (M.A. Shahein), falk.melzer@fli.de (F. Melzer), wolfgang.beyer@uni-hohenheim.de (W. Beyer), mathias.pletz@med.uni-jena.de (M.W. Pletz), heinrich.neubauer@fli.de (H. Neubauer).

<https://doi.org/10.1016/j.onehlt.2021.100255>

Received 9 March 2021; Received in revised form 23 April 2021; Accepted 25 April 2021

Available online 27 April 2021

2352-7714/© 2021 The Author(s).

Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

[4]. *Brucella* (*B.*) can also be transmitted to people via skin abrasion, conjunctival inoculation and inhalation of contaminated aerosol [5]. Human-to-human transmission is rare. However, transmission via blood transfusion and bone marrow transplantation and transplacental transmission from mother to fetus or via breastfeeding have been reported [6,7]. Laboratory-acquired infection is a well-documented infection route in the United States and Asia [8,9]. Clinical signs of brucellosis in humans are highly variable, non-specific, and the disease has similar or very close symptoms to some diseases that cause fever resulting in misdiagnosis and insufficient treatment. Dissemination of *Brucella* to the genitourinary system, joints, parenchymatous organs and bone may develop chronic brucellosis [10]. Numerous relapses following therapy were reported [11–15]. Still, it is unclear whether this is due to sequestration within infected sites, e.g. in reticuloendothelial cells and bone or the development of acquired or intrinsic resistance against antimicrobial compounds. Thus, prompt diagnosis and appropriate antimicrobial therapy are required to prevent the development of chronic debilitating illness.

In Egypt, brucellosis is an endemic disease in animals and humans. *B. melitensis* remains the primary causative agent, with much higher infection rates in humans and animals compared to *B. abortus* [16,17]. In the livestock, treatment of brucellosis is not attempted because all positive cases are slaughtered, and carcasses are used for human consumption after the disposal of the genital system and associated lymph nodes. Also, occupational brucellosis is on the rise among veterinarians, abattoir workers, and animal breeders [18]. Sporadic cases of AMR and disease relapse have also been reported from Egypt [19], and a rise in the number of isolates with an AMR phenotype became obvious. However, routine susceptibility testing of *Brucella* spp. is not practiced due to treatment ban in veterinary medicine, strict therapy regimes in human medicine and the risk of infection to laboratory personnel. Few studies have been carried out to investigate the antimicrobial resistance in brucellae worldwide. Only two studies were done in Egypt on strains collected from humans [16] and animals [20].

The One-Health approach is playing a significant role in understanding, preventing, control and tackling zoonoses. Therefore, the current study aimed to evaluate and characterize, at the molecular level, *B. melitensis* and *B. abortus* strains isolated from different reservoirs, e.g. humans, milk, and animals in Egypt, and antimicrobial susceptibility of strains against most of the antibiotics used to treat brucellosis in humans.

2. Materials and method

2.1. *Brucella* strains

Thirty-five *Brucella* strains were isolated from humans ($n = 12$), milk samples ($n = 19$) and lymph nodes of seropositive animals ($n = 4$). Strains of human origin were obtained by lysis-concentration technique from blood samples of 12 persons suffering from fever of unknown origin (FUO) and non-specific clinical signs. Among them, eleven persons had direct contact with animals, either at work (farmers, veterinarians and butchers) or those who kept farm animals inside their houses. One male patient had no history of contact with animals, and the infection source stays unknown. All cases were suspected cases from private clinics sent to the public fever hospital in Giza city to confirm diagnosis and treatment. Nineteen milk samples were collected from animals (18 from cattle and one from a goat) with a history of abortion. However, the milk of these animals was still used for human consumption. Four lymph node samples were collected from seropositive animals during slaughter at the abattoir, two from sheep and two from cattle. All samples were collected between December 2018 and January 2020.

The institutional review boards and the Animal Health Research Institute (AHRI) ethical committee in Giza approved the study and the ethical code [Ref. No. 165870] was obtained. The consent of the patients was sought. Written informed consent was obtained from all

participants.

2.2. Identification and biotyping of *Brucella* strains

Genus identification was initially carried out using matrix-assisted laser desorption/ionization (MALDI-TOF-MS) as previously described [21]. Briefly, a single colony from a pure culture of each sample was suspended in 300 μ L of HPLC grade water and was inactivated by 900 μ L of absolute ethanol. Protein extraction was done as described before [22]. The inactivated bacterial pellet was centrifuged and air-dried and then reconstituted in 50 μ L of 70% formic acid and 50 μ L of acetonitrile. The sample was sonicated for 1 min on ice, centrifuged at 11290g for 5 min at room temperature, and one μ L of each supernatant was spotted onto the MALDI target plate. After air-dried, the plate overlaid with 1.0 μ L of saturated α -cyano-4-hydroxycinnamic acid matrix solution and the MALDI measurements were carried out (Bruker Daltonics, Bremen, Germany). MALDI log score values between 2.000 and 2.290 were considered a 'secure genus identification and probable species identification,' and log score values equal to or greater than 2.300 were considered highly probable for species identification.

Biotyping of *Brucella* isolates was carried out based on colony morphology, biochemical reactions (catalase, oxidase and urease), the requirement of CO₂, H₂S production, growth in the presence of thionin and fuchsine dyes, reaction with mono-specific anti-sera (A, M, R), agglutination with acriflavine and crystal-violet as described by Alton [23]. According to the manufacturer's instructions, genetic DNA was extracted from heat-inactivated biomasses using the High-Pure template preparation kit (Roche Applied Sciences, Mannheim, Germany). DNA content of samples was measured, and species diagnosis was molecularly confirmed by Bruce-Ladder PCR [24].

2.3. Phenotyping characterization of *Brucella* strains

Antimicrobial Susceptibility Testing (AST) was carried out to determine the susceptibility against 11 antimicrobial agents regularly used in treating brucellosis in humans. The minimum inhibitory concentration (MIC) was determined via the broth microdilution method (Micronaut, MERLIN Diagnostics GmbH, Bornheim-Hersel Germany) in case of chloramphenicol (CMP), ciprofloxacin (CIP), doxycycline (DOX), gentamicin (GEN), levofloxacin (LEV), rifampicin (RIF), streptomycin (STR), tetracycline (TET) and trimethoprim/sulfamethoxazole (T/S) according to the manufacturer's instructions. The interpretation of MIC values (μ g/mL) for GEN, STR, DOX, TET and T/S was carried out according to CLSI guidelines for potential bacterial agents of bioterrorism (CLSI M100-S20 table 21) [25]. As MIC breakpoints of CMP, CIP, LEV and RIF are not established yet, the MIC values were interpreted according to the CLSI guidelines for the fastidious bacterium *Haemophilus influenzae*. Tigecycline (TGC) and azithromycin (AZM) were tested by the disc diffusion method with 15 μ g compound content (Oxoid Deutschland GmbH, Wesel, Germany) according to the manufacturer's instructions. Strains with an inhibition zone ≤ 15 mm or a single colony's growth within the inhibition zone were considered resistant.

2.4. Whole genomic sequencing of *Brucella* isolates

WGS was carried out for the 35 *Brucella* strains in the current study. The genomic DNA was extracted from heat-inactivated biomasses using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Default genomic library preparation and total genomic DNA sequencing were performed by Eurofins Genomics. The libraries were sequenced using Illumina Novaseq, producing at least 5 million 150 bp paired-end reads. Coverage was at least 100, with an average of 450 for each read. *In-silico* species identification based on contigs produced by shovill (<https://github.com/tseemann/shovill>) was done and by performing a virtual Bruce-ladder PCR by Geneious version 11.1.5 using WGS data. *In-*

in-silico detection of AMR genes and virulence-associated determinants was performed by using different databases, i.e. the Resistance Gene Identifier (RGI) based on the Comprehensive Antibiotic Resistance Database (CARD) [26], the ResFinder database [27], and the NCBI AMR Finder Plus (<https://github.com/ncbi/amr/wiki/Running-AMRFinderPlus>) [28] for the identification of resistance genes and chromosomal mutations mediating antimicrobial resistance. Identifying the potential virulence-associated determinants was retrieved from the virulence factor database (VFDB, <http://www.mgc.ac.cn/VFs/>) using the core dataset [29].

3. Results

3.1. Molecular characterization of *Brucella* strains

A total of 35 *Brucella* strains (27 *B. melitensis* and 8 *B. abortus*) were molecularly characterized by MALDI-TOF, PCR and WGS data in the current study. Identification of *B. abortus* and *B. melitensis* at the species level by MALDI-TOF was inconsistent with PCR and WGS based identification results. For example, by MALDI-TOF, four isolates were identified as *B. canis* ($n = 2$), *B. ovis* ($n = 1$) and *B. abortus* ($n = 1$), with score values of 2.100, were finally diagnosed as *B. melitensis* using PCR and WGS data. Only one *B. melitensis* and six *B. abortus* strains were diagnosed correctly. MALDI-TOF identified the rest twenty-four isolates as *Brucella* spp., while PCR and WGS data confirmed 22 as *B. melitensis* and two as *B. abortus*.

The 27 phenotypic *B. melitensis* strains were isolated from human blood samples ($n = 10$), bovine milk samples ($n = 13$) and lymph nodes ($n = 2$) of cows with a history of abortion, and one each from a milk

sample of a seropositive goat and a lymph node of a seropositive sheep. These strains showed identical Bruce-Ladder PCR results and were also diagnosed as *B. melitensis* using the WGS data. Eight *B. abortus* strains were phenotypically characterized in the current study. Two isolates from human patients, one isolate from a lymph node of a seropositive sheep and five isolates from cow milk showed identical Bruce-Ladder PCR results and were also diagnosed as *B. abortus* using the WGS pipeline. The two strains of human origin were obtained from a farmer who kept small and large ruminants inside his house and from a man who reported no contact with animals at all. These isolates were the first *B. abortus* strains isolated from humans and sheep in Egypt (Table 1). The *in-silico* detection of AMR genes in the 35 genomes of Egyptian *Brucella* strains based on WGS data using the ResFinder, CARD, NCBI, plasmid Finder and AMR Finder Plus databases succeeded in identifying only the *Brucella suis* *mprF* and *bepC*, D, E, F, G genes in the genomes of all strains. Moreover, forty-five genes corresponding to pathogenicity and virulence factors were identified in all isolates (data not shown).

3.2. Phenotyping characterization of *Brucella* spp. to antibiotics

Antibiotic susceptibility testing of *Brucella* strains showed that all strains were susceptible to chloramphenicol, ciprofloxacin, doxycycline, gentamicin, levofloxacin, streptomycin, tetracycline, trimethoprim/sulfamethoxazole and tigecycline. A non-susceptible pattern was seen only for rifampicin and azithromycin. All *B. abortus* strains ($n = 8$) showed MIC (=1 µg/ml) for rifampicin and were considered susceptible, while all *B. melitensis* strains ($n = 27$) showed MIC (=2 µg/ml) for rifampicin and were considered intermediate. Eleven *B. melitensis* strains showed an inhibition zone between 35 and 40 mm (≥ 15 mm) for discs

Table 1

Molecular characterization and susceptibility to RIF and AZM of *B. abortus* and *B. melitensis* strains isolated from human blood samples and milk samples and lymph nodes of animals in Egypt.

ID	Host	Source	Year	Location	Description	MALDI-TOF	Ladder PCR wet/in-silico	WGS	RIF	AZM
20RB21875	Human	Blood	2018	Beni-suef	Male, veterinarian	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21876	Human	Blood	2018	Fayoum	Male, farmer	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21877	Human	Blood	2019	Giza	Male, butcher	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21878	Human	Blood	2019	Giza	Male, farmer	<i>Brucella</i> spp.	<i>B. abortus</i>	<i>B. abortus</i>	S	R
20RB21879	Human	Blood	2019	Giza	Male, sheep breeder	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21880	Human	Blood	2019	Giza	Male, butcher	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21881	Human	Blood	2019	Giza	Male, no contact with animals at all	<i>Brucella</i> spp.	<i>B. abortus</i>	<i>B. abortus</i>	S	R
20RB21882	Human	Blood	2019	Giza	Animal producer	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21883	Human	Blood	2019	Fayoum	Male, farmer	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21884	Human	Blood	2019	Fayoum	Male, farmer	<i>B. ovis</i>	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21885	Human	Blood	2019	Fayoum	Male, farmer	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21886	Human	Blood	2019	Fayoum	Male, farmer	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21887	Cattle	Milk	2019	Fayoum	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21888	Goat	Milk	2020	Fayoum	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21889	Cattle	Milk	2019	Sharkia	History of abortion	<i>B. canis</i>	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21890	Cattle	Milk	2019	Sharkia	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21891	Cattle	Milk	2019	Sharkia	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21893	Cattle	Milk	2019	Sharkia	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21894	Cattle	Milk	2019	Sharkia	History of abortion	<i>B. canis</i>	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21895	Cattle	Milk	2019	Damietta	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21897	Cattle	Milk	2019	Damietta	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21898	Cattle	Milk	2019	Damietta	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	R
20RB21899	Cattle	Milk	2019	Damietta	History of abortion	<i>B. abortus</i>	<i>B. abortus</i>	<i>B. abortus</i>	S	R
20RB21900	Cattle	Milk	2019	Sharkia	History of abortion	<i>B. abortus</i>	<i>B. abortus</i>	<i>B. abortus</i>	S	R
20RB21901	Cattle	Milk	2019	Sharkia	History of abortion	<i>B. abortus</i>	<i>B. abortus</i>	<i>B. abortus</i>	S	R
20RB21906	Sheep	L. N.	2019	Aswan	Seropositive case	<i>B. abortus</i>	<i>B. abortus</i>	<i>B. abortus</i>	S	R
20RB21907	Sheep	L. N.	2019	Aswan	Seropositive case	<i>B. abortus</i>	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21908	Cattle	Milk	2019	Beni-suef	History of abortion	<i>B. melitensis</i>	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21910	Cattle	Milk	2019	Beni-suef	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21912	Cattle	L. N.	2019	Ismailia	Seropositive case	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21913	Cattle	L. N.	2020	Ismailia	Seropositive case	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21914	Cattle	Milk	2020	Behira	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21915	Cattle	Milk	2020	Behira	History of abortion	<i>Brucella</i> spp.	<i>B. melitensis</i>	<i>B. melitensis</i>	I	S
20RB21916	Cattle	Milk	2020	Behira	History of abortion	<i>B. abortus</i>	<i>B. abortus</i>	<i>B. abortus</i>	S	R
20RB21917	Cattle	Milk	2020	Behira	History of abortion	<i>B. abortus</i>	<i>B. abortus</i>	<i>B. abortus</i>	S	R

(MALDI) matrix-assisted laser desorption/ionization; (WGS) Whole-genome sequencing; (*B. abortus*) *Brucella abortus*; (*B. melitensis*) *Brucella melitensis*; (*B. ovis*) *Brucella ovis*; (*B. canis*) *Brucella canis*; (RIF) Rifampicin; (AZM) Azithromycin; (I) Intermediate resistant; (R) Resistant; (S) Susceptible; (L.N) Lymph node.

containing 15 µg azithromycin and were considered susceptible. In contrast, all *B. abortus* strains (n = 8) and 16 *B. melitensis* showed an inhibition zone between 0 and 12 mm (≤ 15 mm) and were considered resistant to azithromycin (Table 2). Breakpoints of ≤ 16 µg/ml for incubation conditions with 5% CO₂ and ≤ 8 µg/ml for incubation conditions without CO₂ are considered the breakpoints representing susceptibility to streptomycin.

4. Discussion

Brucellosis is a worldwide zoonosis that affects a wide range of mammals, including livestock and humans [30]. The disease is prevalent in Middle Eastern countries and the Mediterranean region for thousands of years and still causing significant public health and veterinary public health concerns [4]. The most important aspect of its One-Health is the strong connection of humans, foods and livestock. Accurate diagnosis and species identification of brucellae isolated from human and non-human sources are highly required for prompt treatment. In the current study, MALDI-TOF as a proteomics-based tool combined with PCR and WGS data as DNA-based tools to identify *Brucella* strains recovered from different reservoirs. The results were the same at the genus level while were inconsistent at the species level. MALDI-TOF mass spectrometry appeared to be a rapid and reliable method for the routine identification of brucellae [31]. However, using DNA-based tools is required for species identification. In the past two decades, the identification of brucellae by MALDI-TOF has emerged as a rapid method in routine diagnostic laboratories. However, the construction of sensitive reference libraries needs a significant number of strains from different species and biovars to improve the accurate identification of *Brucella* species [32]. All new isolates and a decent number of historic isolates from the National Reference Laboratories' collections in endemic countries should be sequenced and phenotypically typed to amend the

current database. A combination of DNA-based assays such as PCR or WGS is indispensable in diagnosing *Brucella* at the species level to avoid phenotypic handling and reduce the risk of laboratory-acquired infection.

Brucellosis is endemic in Egypt in humans and animals. However, the research on human brucellosis is neglected and not carried out in a significant range at all [33]. *Brucella melitensis*, particularly biovar 3, is the dominant *Brucella* species in Egypt and its neighboring countries [4]. However, the present study confirmed two cases of human brucellosis and one infection in sheep due to *B. abortus* by isolation. Cross-species infection of *B. abortus* from cattle to non-preferred hosts, i.e. sheep and goats, is evident in the mixed rearing breeding system [34]. All but one human case in the current study had close occupational contact with animals. Low biosafety measures in animal husbandry increase occupational infection risk [35]. One *B. abortus* case had no contact with animals at all. This infection could be caused by the consumption of unpasteurized milk or dairy products, which is still the leading risk factor for human infection in many developing countries, including Egypt [35]. Cattle and buffalo milk is the primary source of human infection in Egypt, and the shedding of *Brucella* spp. in milk poses an increasing threat to consumers [35]. In Egypt, *Brucella* DNA has been detected in non-pasteurized dairy products sold for human consumption produced by individual farmers operating under poor hygienic conditions [36]. All milk samples in the current study were collected from herds producing milk for human consumption despite the fact that those herds have a history of abortion.

Treatment of brucellosis in livestock is not regularly practiced because of its high cost, but the meat of slaughtered animals is used for human consumption in developing countries. In humans, doxycycline with rifampicin or fluoroquinolones with rifampicin are the most common combinations of antibiotics recommended by the World Health Organization to treat brucellosis [10]. Previous studies dealing with antimicrobial susceptibility testing for the genus *Brucella* are scarce, and disc diffusion and E-test are usually applied [20,37]. However, MIC testing using broth microdilution is neglected. Lack of standardization, absence of quality standards for the media used and variety in breakpoints due to cultivation with and without CO₂ make detection of AMR in *Brucella* a considerable challenge [38]. According to our results, *Brucella* has maintained its susceptibility to doxycycline, new glycylicline tigecycline, aminoglycosides (gentamicin), fluoroquinolones (ciprofloxacin and levofloxacin), and rifampicin to some extent. Rifampicin stays in combination with others, the main common treatment option of brucellosis in humans. Susceptibility of 100% of *Brucella* strains to doxycycline, tetracycline, ciprofloxacin, streptomycin, gentamicin, trimethoprim-sulfamethoxazole and levofloxacin was also reported in isolates from Saudi Arabia [39], Turkey [40], China [41], and Norway [42]. These findings are also in complete agreement with a previous study on Egyptian *B. melitensis* of human origin [16]. However, the examination of *B. melitensis* strains of animal origin revealed resistance to ciprofloxacin, rifampicin and streptomycin in 75.2%, 66.7% and 4.8% of strains, respectively [20]. All *B. melitensis* strains in the current study showed intermediate resistance to rifampicin, and 16 of 27 were resistant to azithromycin. A previous study on 355 *B. melitensis* strains of human origin in Egypt showed resistance to rifampicin in 277 (64%) strains [16]. All *B. abortus* strains in the current study were susceptible to rifampicin and resistant to azithromycin. The previous examination of *B. abortus* isolates of animal origin revealed the presence of resistance to rifampicin and ciprofloxacin in 25% and 37.5% of strains, respectively [20]. Rifampicin resistance was seen in 1 out of 85 *B. melitensis* isolates in China using E-Test [41] and 55.3% of thirty *B. melitensis* strains from Iran using disc diffusion test [43]. Examination of 23 *B. melitensis* isolates obtained from patients registered in Norway in the period 1999 to 2016 using broth microdilution revealed that 17 out of 23 isolates (74%) were intermediate resistant for rifampicin (MIC = 2 µg/mL) and 6 (26%) were resistant (MIC = 4 µg/mL) [42]. By E-test, an examination of 73 *B. melitensis* of human origin in Turkey revealed resistance and

Table 2

Interpretation of MIC values (µg/mL), and inhibition zone (mm) of *Brucella* spp., and resistance pattern.

Antibiotic	Concentration µg/ml	MIC interpretive criteria (µg/ml)			Resistance pattern		
		S	I	R ≥	S	I	R
Gentamicin	8-0,004	4	-	-	8/27	0/0	0/0
Streptomycin *	16-0,008	8:16	-	-	8/27	0/0	0/0
Doxycycline	8-0,004	1	-	-	8/27	0/0	0/0
Tetracycline	8-0,004	1	-	-	8/27	0/0	0/0
Chloramphenicol	8-0,5	1	-	-	8/27	0/0	0/0
Rifampicin **	8-0,125	1	2	4	8/0	0/27	0/0
Trimethoprim/Sulfamethoxazole	4/76-0,00195/0,037	2/38	-	-	8/27	0/0	0/0
Ciprofloxacin **	4-0,002	1	-	-	8/27	0/0	0/0
Levofloxacin **	4-0,004	1	-	-	8/27	0/0	0/0
Tigecycline	15 mg	-	-	≤ 15 mm	8/27	0/0	0/0
Azithromycin	15 mg	-	-	≤ 15 mm	0/11	0/0	8/16

S: susceptible; I: intermediate; R: resistant; A: *B. abortus*; M: *B. melitensis*; MIC: minimum inhibitory concentration. *the streptomycin-susceptible breakpoint is ≤ 16 µg/ml for incubation conditions with 5% CO₂ and ≤ 8 µg/ml without CO₂. **CLSI breakpoints interpretation for fastidious bacteria, i.e. *Haemophilus influenzae*.

intermediate resistance to rifampicin in 40 and 33 strains, respectively, and resistance to azithromycin was seen in 34 out of 73 *B. melitensis* and 2 out of 2 *B. abortus* strains in the same study [44].

Antimicrobial resistance is on the rise in *Brucella*. However, the absence of classical AMR genes in the *Brucella* genome raises the interesting question of these genes' existence in brucellae. The absence of resistance genes may be biased because current *Brucella*-specific AMR genes are still unknown or not yet in the available public AMR databases. Additionally, the intracellular lifestyle of brucellae that hinders the penetration of various antimicrobials may impair fast resistance development. The high rates of relapse of brucellosis and the rise in AMR in the phenotype of *Brucella* have become evident in the last years [11–14]. Relapse history was investigated in 127 Saudi patients. The recurrence of clinical symptoms was reported in 21 out of 127 (16.5%) patients [45]. Some studies have shown that relapses due to inadequate therapy may occur in 2% to 40% of patients [46–48]. In Egypt, the relapse rate was 59.3% in patients with osteoarticular brucellosis after treatment with rifampicin + doxycycline for five months [19]. Very few molecular-based studies have been carried out to investigate resistance in brucellae worldwide [41,42,49], and only two studies were identified in Egypt on samples of human [16] and animal origin [20]. Several studies showed that *rpoB* gene mutations contribute to rifampin resistance in many bacterial species, including *Brucella* [50]. However, screening the *rpoB* gene in *B. melitensis* shows complete resistance to rifampicin, despite the absence of any mutation [41]. Examining 85 *B. melitensis* isolates from human patients in China showed no *rpoB* gene mutations present in isolate resistant to rifampin [49].

Brucellae do not bear “classic” virulence factors, e.g. capsules, exotoxins, cytolytins, exoenzymes, proteases, pili or fimbriae and virulence plasmids [51]. Detection of virulence-associated genes in *B. melitensis* bv3 has been attempted by PCR on 52 isolates from Egypt before [52]. This study targeted only three genes (*byfA*, *virB*, *ure*) and revealed the presence of the three genes in almost all isolates. No difference in distribution was seen in strains obtained from different hosts. In the same context, the implementation of WGS for the 35 genomes of *Brucella* identified 45 virulence-associated genes. It is important to note that there is no difference found in the distribution of virulence genes among *B. abortus* and *B. melitensis* strains, even for those isolated from different hosts. The AMR-associated genes and genes encoding virulence factors found are always present in all brucellae. Therefore, assessment mutations that lead to resistance and mutational impact on virulence and pathogenesis are urgent to be explored in brucellae.

5. Conclusion

Brucellosis is still a significant public health problem, and the most important aspect of the One-Health approach is the strong connection with humans, foods and livestock. Brucellosis is a common zoonosis in Egypt, and *B. melitensis* is the dominant *Brucella* species. To the best of our knowledge, *B. abortus* was isolated for the first time from human patients and sheep in Egypt. MALDI-TOF is suggested to be the potential rapid first-line screening tool for *Brucella* identification in routine diagnostic laboratories with minimal time, effort and cost. However, a combination of DNA-based assays such as PCR or WGS is indispensable in diagnosing *Brucella* at the species level. Egyptian *Brucella* strains tested still susceptible to the most important antibiotics used for treatment in humans. Implementation of high-throughput WGS to identify the AMR and virulence-associated genes in *Brucella* isolates revealed no apparent difference in their distribution between *B. abortus* and *B. melitensis* strains or isolates from different hosts. Ongoing investigation on antibiotic susceptibility and updating of breakpoints is required. Investigations of resistance and virulence mechanisms at the proteomic and transcriptomic levels have to be considered in future research.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication. G.W., M.W.P. and H.N. conceived and designed the experiments. M.E., N.A., M.E.R.H., S.M. and M.S. performed the sampling and preliminary identification. G. W. and F.M. performed antibiotic susceptibility testing. K.H. provided *in-silico* analysis. K.H. and W.B. provided funding for WGS. G.W., M.W.P. and H.N. analyzed the data and wrote the manuscript's draft. All authors read and approved the final version.

Funding

Internal funding of the Friedrich-Loeffler-Institut (FLI) and LEAP-AGRI project MUSCBCEA. Number 2817LEAP01 supported this work.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

We want to thank Dr. Jörg Linde, Katja Fischer and Peggy Methner for their excellent technical assistance.

References

- [1] M. Dadar, Y. Shahali, Y. Fakhri, J. Godfroid, The global epidemiology of *Brucella* infections in terrestrial wildlife: a meta-analysis, *Transbound. Emerg. Dis.* (2020), <https://doi.org/10.1111/tbed.13735>.
- [2] N. Zhang, D. Huang, W. Wu, J. Liu, F. Liang, B. Zhou, P. Guan, Animal brucellosis control or eradication programs worldwide: a systematic review of experiences and lessons learned, *Prevent. Vet. Med.* 160 (2018) 105–115, <https://doi.org/10.1016/j.prevetmed.2018.10.002>.
- [3] J.T. Mackie, D. Blyde, L. Harris, W.D. Roe, A.L. Keyburn, Brucellosis associated with stillbirth in a bottlenose dolphin in Australia, *Aust. Vet. J.* 98 (2020) 92–95, <https://doi.org/10.1111/avj.12903>.
- [4] R. Bagheri Nejad, R.C. Krecek, O.H. Khalaf, N. Hailat, A.M. Arenas-Gambo, Brucellosis in the Middle East: current situation and a pathway forward, *PLoS Negl. Trop. Dis.* 14 (2020), e0008071, <https://doi.org/10.1371/journal.pntd.0008071>.
- [5] M.A. Hayoun, E. Muco, M. Shorman, *Brucellosis*, in: StatPearls, StatPearls Publishing LLC, Treasure Island (FL), 2020.
- [6] A. Palanduz, S. Palanduz, K. Güler, N. Güler, Brucellosis in a mother and her young infant: probable transmission by breast milk, *Int. J. Infect. Dis.* 4 (2000) 55–56, [https://doi.org/10.1016/s1201-9712\(00\)90068-7](https://doi.org/10.1016/s1201-9712(00)90068-7).
- [7] I. Giannacopoulos, M.I. Eliopoulou, T. Ziambaras, D.A. Papanastasiou, Transplacentally transmitted congenital brucellosis due to *Brucella abortus*, *J. Infect.* 45 (2002) 209–210, [https://doi.org/10.1016/s0163-4453\(02\)91043-1](https://doi.org/10.1016/s0163-4453(02)91043-1).
- [8] J. Ackelsberg, A. Liddicoat, T. Burke, W.A. Szymczak, M.H. Levi, B. Ostrowsky, C. Hamula, G. Patel, V. Kopetz, J. Saverimuttu, et al., *Brucella* exposure risk events in 10 clinical laboratories, New York City, USA, 2015 to 2017, *J. Clin. Microbiol.* (2020) 58, <https://doi.org/10.1128/jcm.01096-19>.
- [9] C. Wong, S.Y. Ng, S.H. Tan, An accidental laboratory exposure to *Brucella melitensis*: the prospective post-exposure management and a detailed investigation into the nature of the exposure, *J. Med. Microbiol.* 67 (2018) 1012–1016, <https://doi.org/10.1099/jmm.0.000772>.
- [10] WHO, WHO/CDS/EPR/2006.7. Brucellosis in Humans and Animals [Internet] [Cited 2019 Feb 3]. Available from: http://www.who.int/csr/resources/publications/deliberate/WHO_CDS_EPR_2006_7/en/, 2006.
- [11] W. Wang, X. Lu, C. Li, M.J. Ri, W. Cui, A man with recurrent fever, arthritis, and rashes-brucellosis? A case report, *BMC Infect. Dis.* 20 (2020) 18, <https://doi.org/10.1186/s12879-019-4746-0>.
- [12] W. Jiang, J. Chen, Q. Li, L. Jiang, Y. Huang, Y. Lan, Y. Li, Epidemiological characteristics, clinical manifestations and laboratory findings in 850 patients with brucellosis in Heilongjiang Province, China, *BMC Infect. Dis.* 19 (2019) 439, <https://doi.org/10.1186/s12879-019-4081-5>.
- [13] J. Solera, Update on brucellosis: therapeutic challenges, *Int. J. Antimicrob. Agents* 36 (Suppl. 1) (2010) S18–S20, <https://doi.org/10.1016/j.ijantimicag.2010.06.015>.
- [14] M.R. Hasanjani Roushan, Z. Moulana, Z. Mohseni Afshar, S. Ebrahimpour, Risk factors for relapse of human brucellosis, *Global J. Health Sci.* 8 (2015) 77–82, <https://doi.org/10.5539/gjhs.v8n7p77>.
- [15] L.F. Nimri, Diagnosis of recent and relapsed cases of human brucellosis by PCR assay, *BMC Infect. Dis.* 3 (2003) 5, <https://doi.org/10.1186/1471-2334-3-5>.
- [16] M. Abdel-Maksoud, B. House, M. Wasfy, B. Abdel-Rahman, G. Pimentel, G. Roushdy, E. Dueger, In vitro antibiotic susceptibility testing of *Brucella* isolates from Egypt between 1999 and 2007 and evidence of probable rifampin resistance, *Ann. Clin. Microbiol. Antimicrob.* 11 (2012) 24, <https://doi.org/10.1186/1476-0711-11-24>.

- [17] M. El-Diasty, R. El-Said, A. Abdelkhalek, Seroprevalence and molecular diagnosis of sheep brucellosis in Dakahlia governorate, Egypt, *Ger. J. Vet. Res* 1 (2021) 34–39, <https://doi.org/10.51585/gjvr.2021.0006>.
- [18] A.M. Zakaria, S.F. Ahmed, M.S. Motawae, 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* 24 (2018) 55–60, <https://doi.org/10.1080/10773525.2018.1516839>.
- [19] Y.M. El Miedany, M. El Gaafary, M. Baddour, I. Ahmed, Human brucellosis: do we need to revise our therapeutic policy? *J. Rheumatol.* 30 (2003) 2666–2672.
- [20] A.U. Khan, W.S. Shell, F. Melzer, A.E. Sayour, E.S. Ramadan, M.C. Elschner, A. A. Moawad, U. Roesler, H. Neubauer, H. El-Adawy, Identification, genotyping and antimicrobial susceptibility testing of *Brucella* spp. isolated from livestock in Egypt, *Microorganisms* (2019) 7, <https://doi.org/10.3390/microorganisms7120603>.
- [21] A. Karger, F. Melzer, M. Timke, B. Bettin, M. Kostrzewa, K. Nöckler, A. Hohmann, H. Tomaso, H. Neubauer, S. Al Dahouk, 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.* 51 (2013) 3123–3126, <https://doi.org/10.1128/jcm.01720-13>.
- [22] J. Murugaiyan, B. Walther, I. Stamm, Y. Abou-Elnaga, S. Brueggemann-Schwarze, S. Vincze, L.H. Wieler, A. Lubke-Becker, T. Semmler, U. Roesler, Species differentiation within the *Staphylococcus intermedius* group using a refined MALDI-TOF MS database, *Clin. Microb. Infect.* 20 (2014) 1007–1015, <https://doi.org/10.1111/1469-0691.12662>.
- [23] G.G. Alton, L.M. Jones, R.D. Angus, J.M. Verger, *Techniques for the Brucellosis Laboratory*, 17–62, *Institutional de la Recherche Agronomique, Paris*, 1988.
- [24] I. López-Goñi, D. García-Yoldi, C.M. Marín, M.J. de Miguel, P.M. Muñoz, J. M. Blasco, I. Jacques, M. Grayon, A. Cloeckaert, A.C. Ferreira, et al., Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains, *J. Clin. Microbiol.* 46 (2008) 3484–3487, <https://doi.org/10.1128/jcm.00837-08>.
- [25] CLSI. *Clinical and Laboratory Standards Institute (CLSI), Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria*, 3rd edition, 2016 (M45Ed3E, table 21).
- [26] B. Jia, A.R. Raphenya, B. Alcock, N. Wagelchner, P. Guo, K.K. Tsang, B.A. Lago, B. M. Dave, S. Pereira, A.N. Sharma, et al., CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database, *Nucleic Acids Res.* 45 (2017) D566–d573, <https://doi.org/10.1093/nar/gkw1004>.
- [27] E. Zankari, H. Hasman, S. Cosentino, M. Vestergaard, S. Rasmussen, O. Lund, F. M. Aarestrup, M.V. Larsen, Identification of acquired antimicrobial resistance genes, *J. Antimicrob. Chemother.* 67 (2012) 2640–2644, <https://doi.org/10.1093/jac/dks261>.
- [28] M. Feldgarden, V. Brover, D.H. Haft, A.B. Prasad, D.J. Slotta, I. Tolstoy, G. H. Tyson, S. Zhao, C.H. Hsu, P.F. McDermott, et al., Validating the AMRFinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates, *Antimicrob. Agents Chemother.* 63 (2019), <https://doi.org/10.1128/aac.00483-19>.
- [29] B. Liu, D. Zheng, Q. Jin, L. Chen, J. Yang, A comparative pathogenomic platform with an interactive web interface, *Nucleic Acids Res.* 2019 (47) (2019) D687–d692, <https://doi.org/10.1093/nar/gky1080>.
- [30] M. Suárez-Esquivel, E. Chaves-Olarte, E. Moreno, C. Guzmán-Verri, *Brucella* genomics: macro and micro evolution, *Int. J. Mol. Sci.* 21 (2020), <https://doi.org/10.3390/ijms21207749>.
- [31] S. Christoforidou, M. Kyritsi, E. Boukouvala, L. Ekateriniadou, A. Zdragas, G. Samouris, C. Hadjichristodoulou, Identification of *Brucella* spp. isolates and discrimination from the vaccine strain Rev.1 by MALDI-TOF mass spectrometry, *Mol. Cell. Probes* 51 (2020) 101533, <https://doi.org/10.1016/j.mcp.2020.101533>.
- [32] G. Wareth, M.W. Pletz, H. Neubauer, J. Murugaiyan, *Proteomics of Brucella: technologies and their applications for basic research and medical microbiology*, *Microorganisms* 8 (2020), <https://doi.org/10.3390/microorganisms8050766>.
- [33] G. Wareth, A. Hikal, M. Refai, F. Melzer, U. Roesler, H. Neubauer, Animal brucellosis in Egypt, *J. Infect. Dev. Ctries* 8 (2014) 1365–1373, <https://doi.org/10.3855/jidc.4872>.
- [34] G. Wareth, F. Melzer, H. Tomaso, U. Roesler, H. Neubauer, Detection of *Brucella abortus* DNA in aborted goats and sheep in Egypt by real-time PCR, *BMC Res. Notes* 8 (2015) 212, <https://doi.org/10.1186/s13104-015-1173-1>.
- [35] E.W. Abd El-Wahab, Y.M. Hegazy, W.F. El-Tras, A. Mikheal, A.F. Kabapy, M. Abdelfatah, M. Bruce, M.M. Eltholth, A multifaceted risk model of brucellosis at the human-animal interface in Egypt, *Transbound. Emerg. Dis.* 66 (2019) 2383–2401, <https://doi.org/10.1111/tbed.13295>.
- [36] G. Wareth, F. Melzer, M.C. Elschner, H. Neubauer, U. Roesler, Detection of *Brucella melitensis* in bovine milk and milk products from apparently healthy animals in Egypt by real-time PCR, *J. Infect. Dev. Ctries* 8 (2014) 1339–1343, <https://doi.org/10.3855/jidc.4847>.
- [37] F. Torkaman Asadi, S.H. Hashemi, M.Y. Alikhani, A. Moghimbeigi, Z. Naseri, Clinical and diagnostic aspects of brucellosis and antimicrobial susceptibility of *Brucella* isolates in Hamedan, Iran, *Jpn. J. Infect. Dis.* 70 (2017) 235–238, <https://doi.org/10.7883/yoken.JJID.2016.133>.
- [38] D.J. Trott, S. Abraham, B. Adler, Antimicrobial resistance in *Leptospira*, *Brucella*, and other rarely investigated veterinary and zoonotic pathogens, *Microbiol. Spectrum* 6 (2018), <https://doi.org/10.1128/microbiolspec.ARBA-0029-2017>.
- [39] S.M. Qadri, Y. Ueno, Susceptibility of *Brucella melitensis* to the new fluoroquinolone PD 131628: comparison with other drugs, *Chemotherapy* 39 (1993) 128–131, <https://doi.org/10.1159/000239114>.
- [40] T. Dal, R. Durmaz, A. Ceylan, F. Bacalan, A. Karagoz, B. Celebi, E. Yasar, S. Kilic, C. Acikgoz, Molecular investigation of the transmission dynamics of brucellosis observed among children in the province of South - East Anatolia, Turkey, *Jundishapur J. Microbiol.* 11 (2018), e58857, <https://doi.org/10.55812/jjm.58857>.
- [41] Z.G. Liu, X.A. Cao, M. Wang, D.R. Piao, H.Y. Zhao, B.Y. Cui, H. Jiang, Z.J. Li, Whole-genome sequencing of a *Brucella melitensis* strain (BMWS93) isolated from a bank clerk and exhibiting complete resistance to rifampin, *Microbiol. Resour. Announc.* 8 (2019), e01645-01618, <https://doi.org/10.1128/MRA.01645-01618>.
- [42] T.B. Johansen, L. Scheffer, V.K. Jensen, J. Bohlin, S.L. Feruglio, Whole-genome sequencing and antimicrobial resistance in *Brucella melitensis* from a Norwegian perspective, *Sci. Rep.* 8 (2018) 8538, <https://doi.org/10.1038/s41598-018-26906-3>.
- [43] A. Farazi, S. Hoseini, E. Ghaznavirad, S. Sadekhoob, Antibiotic susceptibility of *Brucella melitensis* in Markazi province of Iran, *Int. J. Infect. Dis.* 73 (2018), <https://doi.org/10.1016/j.ijid.2018.1004.3697>.
- [44] M. Parlak, H. Gündüçüoğlu, Y. Bayram, A. Çikman, C. Aypak, S. Kılıç, M. Berktaş, Identification and determination of antibiotic susceptibilities of *Brucella* strains isolated from patients in van, Turkey by conventional and molecular methods, *Int. J. Med. Sci.* 10 (2013) 1406–1411, <https://doi.org/10.7150/ijms.6565>.
- [45] E.S. Abdelbaser, H.A. Mohammed, Brucellosis relapse: a retrospective study of risk factors among saudi patients, *J. Afr. Egypt. Infect. Endemic Dis.* 8 (2018) 149–154, <https://doi.org/10.21608/aeji.2018.13526>.
- [46] W.H. Hall, Modern chemotherapy for brucellosis in humans, *Rev. Infect. Dis.* 12 (1990) 1060–1099, <https://doi.org/10.1093/clinids/12.6.1060>.
- [47] J. Ariza, M. Bosilkovski, A. Cascio, J.D. Colmenero, M.J. Corbel, M.E. Falagas, Z. A. Memish, M.R. Roushan, E. Rubinstein, N.V. Sipsas, et al., Perspectives for the treatment of brucellosis in the 21st century: the Ioannina recommendations, *PLoS Med.* 4 (2007), e317, <https://doi.org/10.1371/journal.pmed.0040317>.
- [48] J. Ariza, F. Gudiol, R. Pallarés, G. Ruff, F. Fernández-Viladrich, Comparative trial of co-trimoxazole versus tetracycline-streptomycin in treating human brucellosis, *J. Infect. Dis.* 152 (1985) 1358–1359, <https://doi.org/10.1093/infdis/152.6.1358>.
- [49] Z.G. Liu, D.D. Di, M. Wang, R.H. Liu, H.Y. Zhao, D.R. Piao, Z.Z. Zhao, Y.Q. Hao, Y. N. Du, H. Jiang, et al., In vitro antimicrobial susceptibility testing of human *Brucella melitensis* isolates from Ulanqab of Inner Mongolia, China, *BMC Infect. Dis.* 18 (2018) 43, <https://doi.org/10.1186/s12879-018-2947-6>.
- [50] V. Sandalakis, A. Psaroulaki, P.J. De Bock, A. Christidou, K. Gevaert, G. Tsiotis, Y. Tselentis, Investigation of rifampicin resistance mechanisms in *Brucella abortus* using MS-driven comparative proteomics, *J. Proteome Res.* 11 (2012) 2374–2385, <https://doi.org/10.1021/pr201122w>.
- [51] P. Glowacka, D. Żakowska, K. Naylor, M. Niemcewicz, A. Bielawska-Drózd, *Brucella* - virulence factors, pathogenesis and treatment, *Polish J. Microbiol.* 67 (2018) 151–161, <https://doi.org/10.21307/pjm-2018-029>.
- [52] M.E.R. Hamdy, H.M. Zaki, Detection of virulence-associated genes in *Brucella melitensis* biovar 3, the prevalent field strain in different animal species in Egypt, *Open Vet. J.* 8 (2018) 112–117, <https://doi.org/10.4314/ovj.v8i1.17>.