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List of Abbreviation

AFI	Acute febrile illness
Spp.	Species
B. melitensis	*Brucella melitensis*
B. ovis	*Brucella ovis*
B. abortus	*Brucella abortus*
B. canis	*Brucella canis*
B. ceti	*Brucella ceti*
B. inopinata	*Brucella inopinata*
B. microti	*Brucella microti*
B. neotoma	*Brucella neotoma*
B. pinnipedialis	*Brucella pinnipedialis*
B. suis	*Brucella suis*
Y. enterocolitica	*Yersinia enterocolitica*
BAPAT	Buffered acidified plate agglutination test
2MET	2Mercapteoethanol test
CFT	Complement fixation test
DBH	Dot blot hybridization assay
LAT	Latex agglutination test
ELISA	Enzyme linked immunosorbant assay
iELISA	Indirect enzyme linked immunosorbant assay
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphisms
MRT	Milk ring test
RBT	Rose bengal test
Riv. T	Rivanol test
S.19	Strain 19
SAT	Serum agglutination test
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
MALDI-TOF/MS	Matrix assisted laser desorption ionization -time of flight mass spectrometry
TSB	Tryptic soy broth
SOD	Superoxide dismutase
LPS	Lipopolysaccharide
IEF	Isoelectric focusing
2DE	Two-dimensional electrophoresis
TBS	Tris buffered saline
TSBT	Tris buffered saline with tween
CBB	Coomassie brilliant blue
IHC	Immunohistochemistry
MLVA	Multiple locus of variable number tandem repeats analysis
CFU	Colony forming unit
HE	Hemalum and Eosin

Dedication

I dedicate this work to the spirit of my father who was always the source of encouragement for me.

****

I dedicate this work to my mother.

****

I dedicate this work to my wife Marwa and my sons Mohamed and Mazen.

****

I dedicate this work to my supervisors.

Gamal wareth

Introduction

Brucellosis is a highly contagious bacterial zoonosis spread worldwide and has different names: Infectious or enzootic abortion and Bang's disease in animals; and Mediterranean or Malta fever, Crimean fever, Undulant fever and Rock fever in humans (Xavier and Paixão, 2010). Sir David Bruce (1855-1931) provided the first description of brucellosis and succeeded to isolate *Micrococcus melitensis*, the causative agent of a disease among British army soldiers in the Mediterranean area. The organism was later renamed *Brucella melitensis* (Nielsen and Yu, 2010). Brucellosis is classified among the top seven world neglected zoonotic diseases (Gorvel, 2014). The bacterium is affecting a wide range of mammals including bovines, small ruminants, pigs, equines, rodents, marine mammals as well as human (Cutler et al., 2005), resulting in tremendous economic losses and sequelae in humans. The genus *Brucella* contains Gram negative, aerobic, non-spore forming, facultative intracellular coccobacilli or short rods (0.6 to 1.5 μm) in length and (0.5 to 0.7 μm) in width. Pleomorphic forms are evident in old culture. Bacteria are usually arranged singly and less frequently in small groups. Taxonomically, brucellae are placed in the α-2 subdivision class of the *Proteobacteria* (Alton et al., 1988). Because brucellae are members of the α-*Proteobacteria* group, they can scuffle in highly diversified ecological niches and are often to a host (Batut et al., 2004). Hence, *Brucella* spp. are non-motile, *B. melitensis* expresses genes corresponding to the distal and basal parts of the flagellum (Fretin et al., 2005). The genus encompasses 11 accepted nomo-species. Each species was named based on antigenic and biochemical characteristics and primary its host species specificity. The 'classical' six species are *B. melitensis, B. abortus, B. suis, B. canis, B. ovis,* and *B. neotomae* which are primarily isolated from small ruminants, bovines, pigs, dogs, sheep and desert wood rats, respectively (Corbel and Brinley, 1984). Two species of marine origin were described (*B. ceti* isolated from dolphins and whales and *B. pinnipedialis* isolated from seals). In middle Europe, *B. microti* was isolated from the common vole *Microtus arvalis* (Foster et al., 2007; Scholz et al., 2008). *B. inopinata* was isolated from a breast implant wound of a North American female patient (Scholz et al., 2010). Recently, *B. papionis* was described from an isolate from baboons (*Papio* spp.) (Whatmore et al., 2014). *B. melitensis, B. abortus, B. suis*, and *B. canis* are pathogenic to humans. Brucellosis can be transmitted either by direct contact with infected animals and animal excreta or indirect contact through ingestion of contaminated food and water containing large quantities of bacteria (Zhang et al., 2014). Contact with soil contaminated with abortion secrets is also source for infection. Brucellae can survive up to 15-25 days on pastures (Richomme et al., 2006), and can survive in soil (20-120 days), in water (70-150 days), and in milk and meat (60 days). However, it is being inactivated within few hours by high temperature and direct sunlight (Zhang et al., 2014).

In Egypt brucellosis may be endemic since thousands of years. Common bone affections of brucellosis such as sacroiliitis, spondylitis and osteoarticular lesions were found in bone remnants of ancient Egyptians (750 BC) (Pappas et al., 2006; Pappas and Papadimitriou, 2007). Nevertheless, the disease 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, buffalo, sheep and goat and is still representing a public health hazard. A comprehensive, evidence-based assessment of literature and officially available data on animal brucellosis for Egypt are missing. Moreover, the epidemiological situation of brucellosis awaits clarification and diagnosis and surveillance of the disease still pose for public health a great challenge (Wareth et al., 2014a).

Therefore, the study aimed to investigate the epidemiological situation of brucellosis in Egypt, study the pathogenesis of the disease and use modern technology to improve the diagnostic procedures for better control and surveillance procedures.

Aim of the study

Based on the previously mentioned information, the aim of present study was to:

1. Provide deeper insight in brucellosis in animal populations of Egypt.
2. Provide facts about seroprevalence, isolation and biotyping of *Brucella* isolated from Egypt to understand the situation of the last decades.
3. Assess the role of milk in transmission of brucellosis and its public health significance.
4. Asses cross species transmission of *Brucella* spp. to non-preferred hosts.
5. Identification of immunodominant proteins from *Brucella abortus* and *Brucella melitensis* that play a role in pathogenesis.
6. Identification of immunodominant proteins from *Brucella abortus* and *Brucella melitensis* that might be used as antigen in serodiagnosis of brucellosis.
7. Study the pathogenesis of newly described *B. microti* in the chicken embryo as a non-mammalian host.
8. Study the possibility of chicken embryo as a model of infection in brucellosis.

CHAPTER 1

Review of Literature

Animal brucellosis in Egypt.

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Review

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Animal brucellosis in Egypt

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Abstract

Brucellosis is a highly contagious zoonosis that affects the public health and economic performance of endemic as well as non-endemic countries. In developing nations, brucellosis is often a very common but neglected disease. The purpose of this review is to provide insight about brucellosis in animal populations in Egypt and help to understand the situation from 1986 to 2013. A total of 67 national and international scientific publications on serological investigations, isolation, and biotyping studies from 1986 to 2013 were reviewed to verify the current status of brucellosis in animal populations in Egypt. Serological investigations within the national surveillance program give indirect proof for the presence of brucellosis in cattle, buffaloes, sheep, goats, and camels in Egypt. Serologic testing for brucellosis is a well-established procedure in Egypt, but most of the corresponding studies do not follow the scientific standards. *B. melitensis* biovar (bv) 3, *B. abortus* bv 1, and *B. suis* bv 1 have been isolated from farm animals and Nile catfish. Brucellosis is prevalent nationwide in many farm animal species. There is an obvious discrepancy between official seroprevalence data and data from scientific publications. The need for a nationwide survey to genotype circulating Brucellae is obvious. The epidemiologic situation of brucellosis in Egypt is unresolved and needs clarification.

Key words: brucellosis; biotyping; Egypt; isolation; seroprevalence.

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1. Introduction

Brucellosis is caused by bacteria of the genus *Brucella*. Brucellae are small Gram-negative, non-motile, non-spore forming, aerobic, facultative intracellular coccobacilli capable of invading epithelial cells, placental trophoblasts, dendritic cells, and macrophages [1]. The genus includes 10 nomo-species based on their different host specificity [2]. The six classical species are *B. melitensis* biovar (bv) 1–3, mainly isolated from sheep and goats; *B. abortus* bv 1–6 and 9, primarily isolated from cattle and buffaloes; *B. suis* bv 1–3, mainly isolated from pigs, bv 4 from reindeer and bv 5 isolated from small ruminants; *B. canis* isolated from dogs; *B. ovis* isolated from sheep; and *B. neotomae* isolated from desert wood rats [3]. Recently, four new species have been described. Two are of marine origin (*B.*

pinnipedialis from seals, and *B. ceti* from dolphins and whales). *B. microti* was isolated from the common vole *Microtus arvalis* [4]. Finally, *B. inopinata* was isolated from a breast implant wound of a female patient [5].

Brucellosis caused by *B. melitensis, B. abortus, B. suis* (except bv 2) and in rare cases *B. canis*, is a highly contagious and zoonotic disease affecting livestock and humans worldwide. In animals, brucellosis causes tremendous economic losses [6]. The disease provokes abortion, stillbirth, mastitis, metritis, and placental retention in females and orchitis and arthritis in males. Infertility may be seen in both sexes. The true incidence of human brucellosis is not easy to estimate globally, but an estimated 500,000 persons are newly infected every year [7]. The World Health Organization considers brucellosis a neglected zoonosis and classifies Brucellae as risk group III agents because they can be easily transmitted via aerosols [8]. Airborne transmission of *B. melitensis* infection has been previously described [9], and Brucellae have previously been used as biological agents in weapons of mass destruction [7].

2. *Brucella* in Egypt

It is likely that brucellosis has been an endemic disease in Egypt for thousands of years. For example, there is evidence in 5.2% of bone remnants from ancient Egyptians (750 BCE) of sacroiliitis in pelvic bones, and evidence of spondylitis and osteoarticular lesions have also been found, both common complications of brucellosis [10]. In 1939, brucellosis was reported in a scientific report from Egypt for the first time [11]. Since then, the disease has been detected at high levels among ruminants, particularly in large intensive breeding farms (Refai, personal communication, 20.07.2013). Consequently, a control program including serological surveys and voluntary vaccination of ruminants was established in the early 1980s [12]. Indirect techniques regularly used in diagnosis of *Brucella* are field tests such as the milk ring test (MRT), serological tests such as the standard agglutination test (SAT) and buffered agglutination test, which are confirmed by the complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA) [13]. Serological diagnosis of Brucellae currently relies mainly on the detection of anti-*Brucella* lipopolysaccharide (LPS) antibodies. In *B. melitensis*, *B. abortus*, and *B. suis*, the LPS is smooth (containing an O-polysaccharide); *B. canis* isolates lack the O-polysaccharide and are considered rough. However, these tests cannot differentiate antibodies originating from vaccine or wild-type strains. The tests are also prone to false-negative and false-positive reactions, the latter caused by cross-reactions with LPS of other Gram-negative bacteria [14].

Isolation of Brucellae is still the gold standard for diagnosis; however, this method often fails due to the delays in symptoms, resulting in incorrect sample types and low bacterial loads in specimens such as blood, milk, or tissue. Biotyping of isolates involves evaluation of a combination of growth characteristics (colonial morphology, oxidase, urease, CO_2 requirement, H_2S production, growth in presence of the dyes Fuchsin and Thionin), lysis by bacteriophage (Tiblisi and R/C), and agglutination with monospecific A, M, and R anti-sera [2, 15]. Although various polymerase chain reaction (PCR) assays have been created to

diagnose Brucellae at the species level (*e.g.*, the Abortus, Melitensis, Ovis, Suis AMOS PCR), these assays are most useful when applied to DNA extracted from a positive culture.

A comprehensive, evidence-based assessment of current literature and of officially available data on animal brucellosis is missing for Egypt. The aim of this review is to provide insight regarding brucellosis in Egypt over the last 27 years and to assist observers interested in Brucellosis to more fully understand the situation in Egypt.

3. Literature search and data collection

National and international publications on serological investigations and on typing studies of brucellosis from 1986 to 2013 were obtained through PubMed, Science Direct, Google, and from Egyptian university libraries such as The Egyptian National Agricultural Library (ENAL) and the Federation of Egyptian University Libraries. The following search terms were used: Brucellosis in Egypt, *Brucella* infection in Egypt, *Brucella* in animals in Egypt, and animal brucellosis in Egypt. Theses dealing with brucellosis available from Egyptian universities were included in this study (1986–2013). The libraries were personally visited or contacted via e-mail. Reports on brucellosis from the General Organization of Veterinary Services in Egypt (GOVS) from January 2006 through December 2011 were investigated. Studies dealing with human infection were excluded.

A full text analysis of each publication was done by at least two reviewers. Publications describing serological investigations were included even if statistical analyses were not sound to avoid loss of data. Publications on cultivation, bio- and genotyping or PCR analyses were included only if state-of-the-art techniques could be verified by the respective material, and if the methods sections and results were clear. To clarify ambiguities, the authors were first contacted by e-mail or phone. If the authors could resolve those ambiguities, the publications were accepted for further assessment. The following data were extracted from the manuscripts, reports, or theses: seroprevalence for brucellosis in host species populations and regional distribution, prevalence of Brucellae in animals or food proofed, and identification of isolates.

4. Data acquisition

A total of 25 scientific papers on seroprevalence [6,12,16-38] and 18 on isolation of Brucellae [11,16,17,20,22,25,26,29,31,33-35,38-43] were identified by online search. Local scientific papers and 10 theses were obtained from Egyptian universities; 28 of them dealt with seroprevalence [44-71] and 16 dealt with isolation of Brucellae [44, 45, 48-51, 53-55, 58, 68, 72-77]. The official data collection of the General Organization of Veterinary Services (GVOS) was evaluated for the years 1999 to 2011. Two publications on serology [31,38] and nine on isolation of Brucellae [17,20,35,38,39,41,48,55,58] were finally excluded from evaluation because ambiguities were identified within the materials and methods sections and the authors could not be contacted to resolve these ambiguities.

5. Serological investigations

Information on serological investigations was provided by the General Organization of Veterinary Service (GOVS), Cairo, Egypt, as official reports from 1999 to 2011. Screening with the Rose Bengal plate agglutination test (RBPT) and Rivanol test followed by confirmatory CFT in screening test-positive animals is the approved technical procedure of the official control program. This procedure is in accordance with the procedures proposed in the World Organization for Animal Health (OIE) manual of standard diagnostic tests and vaccines. Serological investigations within the national surveillance program give indirect proof for the presence of brucellosis in cattle, buffaloes, sheep, and goats in 22 of 27 governorates. Ismailia, Red Sea, North Sinai, South Sinai, and Matroh did not report seropositive animals. The total number of animals steadily increased during the reporting time (Figure 1). Sheep and goats had a higher seroprevalence than did cattle and buffaloes (Table 1). Peaks were seen in 2002/2003 and 2008/2009/2010 (Figure 2). The number of animals tested was always very low when compared to the total number of animal stocks in Egypt according to the Food and Agriculture Organization (FAO) registers (Table 1). Sampling plans were not made available. It cannot be excluded that sampling is biased; therefore, only tendencies should be read. Based on this data, it can be concluded that brucellosis is present in all governorates in cattle, buffaloes, goats, and sheep. The lowest total percentage of seropositive animals was recorded in 2011 with 0.33%. In 2011, the riots and civil commotions of the Arab Spring lead to a depletion of state resources, resulting in low numbers of animals tested, a decrease of the reimbursement funds for owners, and increased animal movement within villages and governorates.

A total of 53 scientific publications and theses on serological investigations were selected for review. Serological studies were made in Qalyobia, Menufiya, Gharbia, Behira, Alexandria, Kafrelsheikh, Dakahlia, Sharkia, Giza, Fayoum, Beni-Suef, El-Minia, Assuit, New Valley, Sohag, Qina, Luxor, and Aswan in bovines, small ruminants, camels, and Nile catfish, rendering positive results. Assuit, Menufiya, Kafrelsheikh, Giza, and Behira have been studied very well; they have been included in more than five investigations (Supplementary Table 1). Most studies were made in response to clinical events such as notice of late abortion, elevated levels of insemination, and mastitis. As such, these studies do not comply with the standards for epidemiological investigations concerning study design or biostatistics. However, they show that in infected animal herds, the prevalence rate may be high independent of the animal species (1%–100%). In cross-sectional studies, approximately 15% of households in a study area kept animals and within a herd, up to 15% (cattle and buffaloes) or even more (sheep and goats) animals could be expected to be seropositive [6,19,32].

Data obtained by sampling animals in slaughterhouses have to be considered biased, as brucellosis-seropositive animals ought to be slaughtered by law. Studies on camels (n=12) demonstrated a high seroprevalence in these animals. It should be noted that camels are imported from Sudan, where brucellosis is endemic. The prevalence of brucellosis in cattle,

buffaloes, sheep, and goats was generally higher in Beni-Suef governorate than in other governorates in Upper Egypt [11, 22]. In the Delta region, the highest prevalence was reported in Behira governorate. Inadequate preventive measures and uncontrolled transport between Egyptian governorates to and from animal markets may play an important role in the incidence of brucellosis.

Figure 1. Total number of animals in Egypt, 1999–2011 (FAO, 2013).

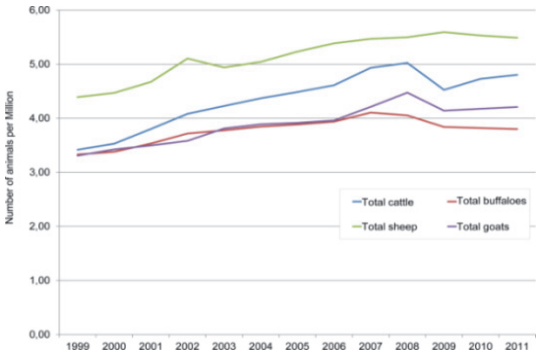
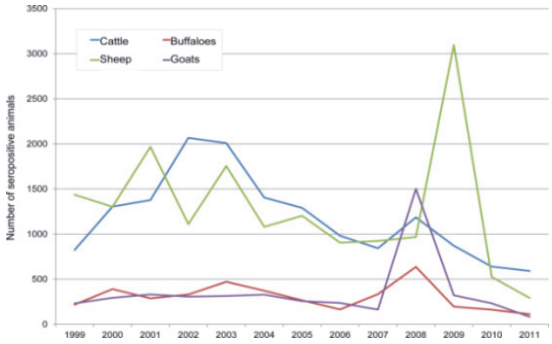


Figure 2. Number of seropositive animals according to the General Organization of Veterinary Service



6. Culture and biotyping

Isolation of *Brucella* is still the gold standard for brucellosis diagnostics, but it has several drawbacks such as hands-on time and low sensitivity, especially in chronic cases. Handling of culture material poses a high risk of infection to the operator. Our analysis shows that this technique is restricted to a few laboratories in Egypt. A total of 35 publications on isolation or biotyping of Brucellae were selected for review. In general, these studies were done within outbreak investigations. Most authors of theses described the techniques used very clearly and comprehensively so that results could easily be checked for plausibility. Strains isolated were regularly determined by investigating CO_2 requirement, H_2S production, growth in the presence of thionin and basic fuchsin dyes, agglutination test with monospecific A and M antisera, and phage lysis test. In contrast, only 15 articles published between 1986 and 2012 followed the complete method of biotyping. *Brucella* strains were isolated from milk, blood, vaginal discharge, and aborted fetuses of infected cattle, buffaloes, sheep, goats, and camels [22,25,72,73], and also from organs including liver, spleen, lung, kidneys, heart, and lymph nodes [22,40,55]. The rationales for sampling, sampling strategy, or statistics of sampling were missing. Hence, the presence of *B. melitensis* bv 1, 2, 3 and *B. abortus* bv 1, 3, and 7 was unambiguously demonstrated. *B. melitensis* bv 3 is the predominant pathovar isolated independent from the host species and bv 1 and 2 were described in a single study in 2004 only. Isolates of *B. melitensis* originated from all farm animal species and also from rats. Vaccine strain Rev. 1 was isolated from ewes in Minufya in 2007. Only 12 publications describe the presence of *B. abortus* in Egypt; bv 3 was found by four author groups in 1986, 1987, and 1990. Five publications also mentioned bv 7, which was later on removed from the nomenclature list as being erroneous.

The presence of *B. abortus* bv 3 has yet to be confirmed. Isolates were obtained from cattle and buffaloes and the erroneous *B. abortus* bv 7 was obtained from a camel one instance. Human pathogenic *B. suis* bv 1 was isolated from pigs in 1996. No Brucellae isolates exist from Red Sea, New Valley, Luxor, North Sinai, or South Sinai. All data are shown in Table 2.

Isolation of *B. melitensis* from cattle and buffaloes was attributed to mixed rearing of sheep and goats with cattle or buffaloes on holdings or in one flock, contamination of pastures by infected sheep and goats, and spreading of disease by these animals to new areas [22]. However, no proof for this assumption was made via genotyping of strains or tracing back investigations. Alarming is the fact that *B. melitensis* bv 3 was also isolated from 4 out of 65 semen samples from bulls (6.2%) and 3 out of 55 (5.5%) samples from rams, respectively, at the Animal Reproduction Research Institute, Giza [43]. Venereal transmission may be responsible for maintaining a bovine brucellosis cycle based on unhygienic serving methods (*i.e.*, that one bull serves cows of various holdings in different neighboring villages). As a consequence, artificial insemination and semen collection have to be done under strict precautions.

7. Molecular diagnostics

Because of the shortcomings of culture, the use of new diagnostic techniques for the direct detection of Brucellae was attempted, although no biovar-specific PCR assays exist. Authors of only 15 publications from 1986 to 2012 used PCR. The sensitivity of PCR proved to be higher than cultivation [78], and even small numbers of Brucellae were detected in samples [25]. *B. melitensis* DNA was found in the semen of bulls and rams [43] and in the milk of cattle, buffaloes, sheep, and goats in Menufiya, Gharbia, Behira, Fayoum, Aswan, Beni-Suef, and Sohag governorates [16,26]. Montasser *et al*. and Zahran found DNA of *B. melitensis* in tissue samples of cattle, sheep, and goats in Assiut and El-Minia governorates, respectively [35,55]. *B. abortus* DNA was detected and identified in Fayoum governorate from seropositive cattle [54]. In Menufiya governorate, the use of PCR restriction fragment length polymorphism (PCR-RFLP) identified four strains of *B. melitensis* bv 3 and two strains of *B. melitensis* Rev. 1 vaccine in tissue samples collected from six seropositive ewes [33]. The first comprehensive report describing the presence of *B. melitensis* DNA in camel milk dates back to 2002 when it was amplified from a milk sample from Giza governorate [25]. *B. melitensis* DNA was found again in Aswan and Sohag governorates in both milk and serum of camels [26]. PCR is a sensitive tool for the diagnosis of brucellosis. Recently, Wareth *et al* identified *B. abortus* and *B. melitensis* DNA in bovine milk collected from apparently healthy animals by species-specific IS711 RT-PCR [79]. These results highlight a special public health hazard for farmers and nomadic peoples who encourage the drinking of raw milk from camels as they believe that it has a soothing and therapeutic effect against digestive tract diseases and liver infections [78].

Table 1. Prevalence of brucellosis in Egypt from January 1999 through December 2011 based on reports from the General Organization of Veterinary Services

	Cattle				Buffalo				Sheep				Goat				Total	
Year	Total no. in Egypt	No. tested	No. +ve	% +ve from tested	Total no. in Egypt	No. tested	No. +ve	% +ve from tested	Total no. in Egypt	No. tested	No. +ve	% +ve from tested	Total no. in Egypt	No. tested	No. +ve	% +ve from tested	Total tested	% +ve from tested
1999	3,417,580	108,622	824	0.76	3,329,700	62,900	218	0.35	4,390,730	62,151	1,437	2.31	3,308,150	17,875	232	1.30	251,548	1.08
2000	3,529,720	145,750	1,305	0.90	3,379,410	66,109	391	0.59	4,469,130	68,342	1,303	1.91	3,424,760	16,685	294	1.76	296,886	1.11
2001	3,801,070	152,436	1,378	0.90	3,532,240	81,302	288	0.35	4,671,240	78,310	1,967	2.51	3,497,000	21,912	331	1.51	333,960	1.19
2002	4,081,000	162,309	2,067	1.27	3,717,000	67,802	331	0.49	5,105,000	99,466	1,111	1.12	3,582,000	23,560	307	1.30	353,137	1.08
2003	4,227,000	168,281	2,009	1.19	3,777,000	67,588	471	0.70	4,939,000	79,565	1,755	2.21	3,811,000	29,576	314	1.06	345,010	1.32
2004	4,369,000	154,984	1,406	0.91	3,845,000	56,041	373	0.67	5,043,000	68,122	1,081	1.59	3,889,000	25,719	329	1.28	304,866	1.05
2005	4,485,000	174,673	1,291	0.70	3,885,000	69,931	266	0.38	5,232,000	69,571	1,203	1.73	3,915,000	25,325	257	1.01	339,500	0.87
2006	4,610,000	199,954	982	0.49	3,937,000	61,595	165	0.27	5,385,000	71,929	905	1.26	3,960,000	26,689	237	0.89	360,167	0.64
2007	4,932,660	161,206	843	0.52	4,104,810	68,548	334	0.49	5,467,470	68,171	924	1.36	4,210,710	33,791	163	0.48	331,716	0.68
2008	5,023,160	182,248	1,186	0.65	4,052,650	59,080	637	0.40	5,498,030	106,215	968	0.91	4,473,490	46,703	1502	3.22	294,246	0.99
2009	4,524,950	175,750	871	0.50	3,838,720	51,924	196	0.38	5,591,850	84,798	3,095	3.65	4,139,260	44,023	322	0.73	356,495	1.25
2010	4,728,720	183,490	640	0.30	3,818,240	53,783	162	0.30	5,529,530	66,412	525	0.79	4,174,990	39,143	233	0.60	342,828	0.5
2011	4,803,000	167,188	592	0.35	3,800,000	55,986	112	0.20	5,488,000	65,849	292	0.44	4,207,400	31,772	83	0.26	320,795	0.33

Table 2. Origin of *Brucella* isolates in Egypt

		B. melitensis					*B. abortus*				*B.suis*
Location	*B. melitensis*	bv3	bv2	bv1	rev.1	*B. abortus*	bv1	bv3	bv7	*B. suis* bv1
Cairo		[49,50,73]					[49]			
Qalyobia		[22,49,50,73]					[49]			
Menufiya	[76]	[22,26,33,34,44,49, 73]	[73]		[33]		[49]	[44]	[44]	
Gharbia		[26,34,49,73]	[73]				[49]			
Behira		[20,22,26,34,49,73]					[49]			
Alexandria		[22,49,73,74]					[49]			
Kafrelsheikh		[17,34,44, ,48,50,49,73,74]					[49]	[44]	[44]	
Demiatta		[49,73]					[49]			
Dakahlia		[34,50]					[74]			
Sharkia		[29,41, 49,73]					[49,77]	[77]		
Suez		[49,73]					[49]			
Ismalia	[42]									
Port-Said		[49,73]					[49]			
Matroh		[73]								
Giza	[16,42]	[22,25,49,50,73]		[73]			[25,49]			
Fayoum		[26,44,49]				[54]	[49]	[44]	[44]	
Beni-Suef	[16,40]	[22,44,73]				[40]		[44]	[44]	
El-Minia		[55,73,74]								
Assiut		[22,31,35,49,72,73]					[49]			
Sohag	[16]	[26,73]								
Qina		[73]								
Aswan		[26]								
Different locations in Egypt		[39,43,44,51,53,75]					[53]	[51, 75]	[44,51,53,58,75]	[68]

8. Environmental contamination with Brucellae

Significant environmental contamination has to be assumed due to local husbandry methods and the lack of effective carcass disposal. Nile catfish have been found to be infected with *B. melitensis*, especially in small tributaries of Nile canals in the governorates of Kafrelsheikh, Menufiya, Gharbiya, and Dakahlia in the Nile Delta region. It was isolated from 5.8%, 4.2%, 5.8%, and 13.3% of liver, kidney, spleen samples and skin swabs, respectively; it was not isolated from samples of farmed fish [34]. It is speculated that disposal of animal waste (carcasses, milk, aborted and parturition materials) into the Nile or its canals plays an important role in the transmission of *Brucella* and is also the reason for the high incidence in these regions. Farmers also wash their animals in these canals or try to reduce the body temperature of diseased animals in the Nile, which may contribute to spreading of Brucellae. Moreover, *B. melitensis* bv 3 was also isolated from rats [44]. Only one study reported Brucellae in fish. This fact is interesting and should be investigated further in the future. The presence of Brucellae in rat and fish indicates high environmental contamination, which is alarming.

9. Surveillance program

Despite 30 years of work and efforts of the General Organization of Veterinary Services to overcome brucellosis in Egypt by testing female cattle and buffaloes older than six months of age and slaughtering serologically positive animals, the vaccination of calves with *B. abortus* S19 and adults with BR51 vaccines and small ruminants with *B. melitensis* Rev 1 vaccine [11], the results are disappointing and brucellosis is still endemic among humans and ruminants in Egypt. Modeling of the currently applied measures suggests that, at best, 4% of the animal stocks (but not more than 5%) are included in the control program [80]. Our data implies that even this number is overestimated. Several authors proposed that, hotspots are located in the Delta region and in Upper Egypt, along the River Nile and south of the Delta containing 32% of the Egyptian large ruminant and 39% of the small ruminant stocks which are often kept in small mixed herds owned by single households [81]. The assumption of hotspots needs further confirmation. A simple sampling bias might be seen. Various authors linked the limited success of the control program to improper diagnosis and spreading of the disease at large animals markets where different animal species of unknown health status from different towns and governorates intermix. Additionally, small ruminant flocks present in high numbers in Egypt are highly migratory [22]. Low compensation for owners results in slaughtering of only 0.2% of seropositive animals [18]. Emotional attachment of owners to animals that they had kept for long time may also be a reason for their unwillingness to slaughter seropositive animals [82].

10. Summary

In summary, it can only be assumed that brucellosis is prevalent nationwide in all farm animal species, in the environment, and in carrier hosts such as rats. The predominant occurrence of *B. melitensis* bv 3 in bovines is in contrast to Egyptian reports published before 1980 which had described the classic epidemiology of brucellosis with *B. abortus* in cattle and buffaloes and *B. melitensis* in small ruminants, respectively. The question must be raised whether a *B. melitensis* clone was able to cross species barriers and was able to establish a permanent reservoir in cattle and buffaloes. A husbandry system favoring mixed populations of cattle, buffaloes, sheep and goats, limited success of the official control program due to unrealistic high sampling numbers, and poor compliance of livestock farmers has contributed to the emergence of brucellosis in Egypt [18]. The need for a nationwide survey to genotype circulating Brucellae is obvious. Thus, the epidemiologic situation of brucellosis in Egypt is cryptic and needs clarification. Consequently, cultivation and biotyping of *Brucella* isolates has to be made available for all governorates to monitor the effect of control programs and to trace back outbreaks. Future seroprevalence studies must meet scientific standards. The current control program is ineffective and a new strategy to combat brucellosis has to be developed, tailored for the parlous situation of Egypt farmers.

The need for an efficient animal registration and marking system is obvious. The sale of *Brucella*-infected animals in the open market is increasing in Egypt. The introduction of a *Brucella*-infected animal into a herd can lead to spread of the infection to the whole herd, causing economic losses. Markets should be controlled by veterinarians and compensation for those selling animals should be satisfied to prevent infected animals from being sold [83]. Slaughter has to be replaced by culling and safe disposal of carcasses to avoid human infection or pollution of the environment. The measures of the control program have to be made mandatory, and a reasonable system of compensation has to be implemented to enhance acceptance. The basic tools for a program such as an adequate number of public veterinarians for field work and state laboratories capable of serological techniques are already available. Information technology solutions and further logistic means such as animal identification techniques are in place in many countries and may be adapted to the special needs of a country like Egypt.

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13. Supplementary Items

Supplementary Table 1. Serology data arranged in tables according to time of publication.

Reference	*Serology Tests*	*Animals Tested*	*Sample No.*	*Sample type*	*Prevalence*	*Location*	*Isolates*	*Inclusion criteria*
[16]	BAPAT* RBT**, TAT*** Riv.T**** MRT***** PCR******	Cow Buffalo Cow Buffalo	32 18 96 54	Serum Serum Milk Milk	100 % 100 % 87.5 % 83.3 %	Sohag, Beni-Suef, Giza	*B.melitensis*	Outbreak investigation
[26]	RBT MRT ELISA# PCR DBH##	Cows Buffalo Sheep Goats Camel Cows Buffalo Sheep Goats She-camel	660 482 194 198 151 302 321 73 121 64	Serum Serum Serum Serum Serum Milk Milk Milk Milk Milk	45.8% 66.6% 37.6% 61.1% 42.1% 51 % 49.8% 56.2% 36.4 % 34.4%,	Menufiya Gharbia, Behira, Fayoum, Aswan, Sohag	*B.melitensis bv3*	Outbreak investigation and trade (camel)
[12]	STAT RBT	Cattle Buffalo Camel Mares Ewes Does	305 1103 381 36 70 40	Serum Serum Serum Serum Serum Serum	7.86 % 4.35 % 7.61 % 2.77 % 5.71 % 10 %	Different localities in lower Egypt		Outbreak investigation
[36]	BAPAT RBT,TAT ELISA LAT§, ICA§§	Cattle Sheep Goats	376 106 158	Serum Serum Serum	5.32% 9.43% 8.86%	Menufiya		Outbreak investigation
[24]	RBT BAPAT Riv.T TAT CFT###	group1 cow suspected	180	Serum	77.2% 79.4% 72.2% 81.1% 72.8%		B.melitensis bv3	Outbreak investigation
	RBT BAPAT Riv.T TAT CFT	group2 free cow group3 cow vaccinated	125 530	Serum Serum	1.6% 3.2% 0.8% 4% 0.8%			
[45]	BAPAT Brucella card CFT	Cattle Buffalo-cow Sheep Goats Cattle bull Buffalo bull	549 338 404 336 217 152	Serum Serum Serum Serum Serum Serum	14.57% 10% 25.4% 30.9% 6.9% 3.9%	Menufiya, Beni-Suef Assuit, Giza, Gharbia, Sharkia, Behira	B.melitensis bv3	Outbreak investigation
[30]	RBT SAT ELISA PCR	Sheep	300	serum	29.3% 27% 28.3% 39%	Kafrelsheik, Gharbiya		Outbreak investigation
[31]	Positive serum samples	Cattle Sheep Goats	32 69 5	L.N Spleen	28.13% 36.23% 100%	Assuit	B.melitensis bv3	No outbreak investigation
[38]	RBT	Swine	230	Serum	12.61%	Cairo	B. suis	No outbreak investigation
[29]	BAPAT RBPT M.P.A.T Riv.T, 2MT ELISA	Cattle Buffalo Sheep Goats	967 462 591 539	Serum Serum Serum Serum	6.72%, 5.62%, 7.61% 10.95%	Sharkia	B.melitensis bv3	Outbreak Investigation
[35]	BAPAT RBT,SAT Riv.T	Cattle Sheep Goats	715 1323 100	Serum serum serum	4.5% 5.2% 5%	Assiut	B.melitensis bv3	Outbreak Investigation

Reference	Serology Tests	Animals Tested	Sample No.	Sample type	Prevalence	Location	Isolates	Inclusion criteria
[18]	RBT CFT	Cattle Buffalo Sheep Goats household	Total 120,077	Serum data from GOVS	0.79% 0.13% 1.16% 0.44% 1.2%	Beni-Suef, El-Minia, Assiut, Sohag, Qina, Luxor, Aswan		Official data
[19]	RBT CFT iELISA	Cattle Buffalo Sheep Goats	188 173 791 383	Milk tank Milk tank Serum Serum	15.1% 15.1% 41.3% 32.2%	Kafrelsheikh		A cross-sectional study was carried out among dairy cattle, buffalos, sheep and goats and a multistage random sampling strategy was used to select cattle milk tanks and individual sheep and goats within the governorate. The first level sampling unit in this study was the village, the second level sampling units were the cattle milk tanks and the individual sheep/goat.
[6]	iELISA	Cattle Buffalo Household	109 46 104	Milk Milk	Total n = 22 (14.6%) 15.5%	Menufiya		A cross-sectional study was carried out in a village. The village was selected due to convenience. The study population comprised all households with lactating cattle and buffalo in the village. There was no sampling frame in the village and all lactating cattle and buffalos were sampled.
[34]	RBT Riv T PCR	Nile catfish	120 from Nile 120 from Farm	Serum Skin liver kidney spleen	8.3% Only from Nile	Kafr-elsheikh, Menufiya, Gharbiya, Dakahlia, Behira	B.melitensis bv3	Samples collected from 17 sites in small tributaries of Nile canals.120 catfish were collected from 7 fish farms from Kafrelsheikh, Behira and Dakahlia governorates and unlikely to be exposed to water contaminated by carcasses and other contaminated animal materials.
[64]	RBT SAT iELISA	Buffalo	452	Serum	12.83% 11.28% 19.25%		B.melitensis bv3	Outbreak investigation
[27]	RBT iELISA	Sheep Goats Cattle Sheep Goats Cattle	Total 1670 45 55 26	Serum Serum Serum Herds Herds Herds	21.20% 14.2% 2.16% 26.66% 18.88% 21.6%			Across sectional study was carried out on different governorates. In each region, blood samples were taken from herds / flocks with no previous history of vaccination against Brucella. The number of samples was collected in simple and\or systemic random sampling as follows: Animals from each herd were randomly selected using a table of random digits. Only female cows older than 6 months of age were sampled. The herd were stratified into three herd sizes: small herds (≤ 50), medium herds (50-150) and large herds (>150).

Reference	*Serology Tests*	*Animals Tested*	*Sample No.*	*Sample type*	*Prevalence*	*Location*	*Isolates*	*Inclusion criteria*
[28]	CFT	Camel	340	Serum	7.35%	Behira	B.melitensis B.abortus	No outbreak investigation
[48]	BAPAT RBT, Riv.T	Cattle Buffalo	7102 2895	Serum	0.20- 0.37% 0.11- 0.38%	Kafrelsheikh	B.melitensis bv3	Outbreak investigation
[23]	SAT BAPAT RBT Riv.T SAT BAPAT RBT Riv.T	Cattle friesian breed Cattle charolaise breed	57 43	Serum Serum	8.77% 10.53% 10.53% 8.77% 6.68% 9.30%, 11.63% 4.65%	Egypt		Breed
[22]	BAPAT RBT SAT Riv.T	Cattle Buffalo Sheep Goats	1966 1237 813 366	Milk Tissue	5.44 % 4.11 % 5.41 % 3.55%	Beni-Suef, Assiut, Alexandria, Giza, Behira Qaliobia, Menufiya.	B.melitensis bv3	No brucellosis history
[17]	BAPAT RBPT, TAT Riv.T, CFT PCR	Baladi does	577	Serum	3.11% To 5.71%	Kafrelsheikh	B.melitensis bv3	Outbreak investigation
[32]	BAPAT RBT TAT Riv.T	Livestock Cattle Buffalo Sheep Goats	350 77 35 29 18	Serum	0-16% 15.6% 14.3% 20.7% 11.1%	Gharbiya 95% CI 1-28 6-30 0-1 3-35		A cross-sectional survey was conducted in two villages. Criteria for inclusions of the villages were easy accessibility for the study team and a population size of approximately 5000 in each village. Each village was divided into small clusters from which one house was randomly selected. Members (aged ≥3 years) and their livestock were enrolled until the sample size was achieved.
[63]	MRT, wTAT wRBPT wBAPAT wRiv.T	Cattle Buffalo	210 50	Raw milk Raw milk	12.38% 0.00%	Assiut		No outbreak investigation
[33]	SAT, RBT Riv.T, CFT PCR	Ewes native breed	32	serum	31.25% 25.00% 21.88% 21.88%	Menufiya	B.melitensis bv3 B.melitensis Rev.1	No outbreak investigation
[61]	RBPT BAPAT TAT, Riv.T ELISA	Cattle Sheep Buffalo Dairy cow	197 129 32 41	Serum Serum Serum Milk	3. 6% 11.6% 0.00% 7.3%	Assiut		No outbreak investigation
[71]	BAPAT RBT SAT Riv.T ELISA	Cattle Sheep Goats Camel Cattle Sheep Goats Camel	180 180 100 100 15 16 36 10	Serum Serum Serum Serum Milk Milk Milk Milk	7.22- 10.56% 2.22-3.89% 6-7 % 0.00% 6.67% 6.25% 2.78% 0.00%	New-Valley		Outbreak investigation

Reference	Serology Tests	Animals Tested	Sample No.	Sample type	Prevalence	Location	Isolates	Inclusion criteria
[57]	RBPT BAPT TAT Riv.T Ewe Rams Does Bucks	Ewe Rams Does Bucks	450 300 220 180 426 210 105 70	Serum Serum Serum Serum Serum Serum Serum Serum	Total 1.26% Total 9.30%	Assiut Sohag		No outbreak investigation
[65]	RBPT STAT ELISA RBPT STAT ELISA	Local camel Imported camel	95 31	Serum Serum	9,47% 5.26% 9.47% 6.67% 9.67% 25.80%	Halaieb, Shalateen, Abo-Ramad triangle		No outbreak investigation
[46]	RBPT, TAT BAPT, Riv.T	Camel	300	Serum	3.04% 0.00%	Assuit New-Valley		No outbreak investigation
[60]	RBPT, SAT, MET§§§, Riv.T DIA	Camel in closed farm Imported camel Camel kept with animal	80 94 72	Serum Serum Serum	0.0-2.5% 8.5-11.70 % 6.94-11.1 %	Giza		No outbreak investigation
[54]	TAT PCR	Friesian cattle	124	Serum	29.8%	Fayoum	B.abortus	Animals were not subjected to any vaccination.
[37]	RBT BAPT TAT MET Riv.T ELISA	Camel	766	Serum	8.74% 9.53% 9.92% 8.09% 8.87% 9.26%	Behira		No outbreak investigation
[34]	RBPT TAT MET Riv.T	Camel	430	Serum	7.67% 8.84% 6.97% 6.75%	Assiut		No outbreak investigation
[55]	RBT SAT Riv.T PCR	Cattle Buffalo Sheep Goats	1783 942 1455 624	Serum Serum Serum Serum	8.5% 7.0% 7.8% 7.0%	El-Minia	B.melitensis bv3	Outbreak investigation
[25]	SAT RBPT MRT#### PCR	Cattle Sheep Goats Camels	52 21 18 12	Milk	n= 29 n= 10 n=13 n=1	Giza	B.abortus bv 1 B.melitensis bv3	Outbreak investigation
[20]	SAT MRT WRBPT WRiv.T	Cattle	150	Serum Milk Milk Milk	10% 8% 4.7% 4%	Behira	B.melitensis bv3	No outbreak investigation
[53]	BAPT RBPT CFT SAT	Camel	750	Serum	3.9% 4.9%	Egypt	B.melitensis bv3 B.abortus bv 1,7	No outbreak investigation
[56]	RBT BAPT TAT, Riv.1	Cattle Sheep Goats	6495 8457 3872	Serum Serum Serum	0.46-0.61 0.85-1.15 0.74-1.1	Assiut		No outbreak investigation
[52]	BAPT RBPT ELISA CFT TAT MRT	Milky Cattle Dry cow Aborted cow Calves Bulls Milky cattle	238 176 9 6 13 238	Serum Serum Serum Serum Serum Milk	28.51%, 28.05%, 24.89%, 22.85% 21.72% 16.39%	Sharkia	isolation from milk was negative	Outbreak investigation
[69]	BAPT RBPT TAT Riv.T	Sheep	21776	Serum	1.6% 1.6% 1.33% 1.4%	Assiut		Samples collected officially
[66]	BAPT RBPT TAT Riv.T	Goats	16285	Serum	0.33% 0.33% 0.15% 0.3%	Assiut		Samples collected officially

Reference	*Serology Tests*	*Animals Tested*	*Sample No.*	*Sample type*	*Prevalence*	*Location*	*Isolates*	*Inclusion criteria*
[67]	BAPT RBPT TAT Riv.T	Cattle	8774	Serum	0.89% 0.87% 0.6% 0.57%	Assiut		Samples collected officially
[70]	BAPAT SAT MRT	Lactating buffalo Lactating buffalo Dry buffalo Bull	295 282 44 18	Serum Milk Serum Serum	19.9% 12.3% 19.9% 25%	Giza	B.abortus	Outbreak investigation
[68]	SAT MET BAPAT RBT Riv.T	Swine	288	Serum	29.2% 24.6% 35.7% 29% 27.4%		B. suis bv 1	No outbreak investigation
[49]	SAT, MET BAPAT RBPT Riv.T	Cattle Buffalo Sheep Goats	1683 1286 2257 532	Serum Serum Serum Serum	8.2% 11.4% 5.1% 11.1%	Alexandria, Assiut, Cairo, Giza, Behira, Demiatta, Fayoum, Gharbiya, Kafrelsheik, Qaliobia, Menufiya, Suez, Port-Said, Sharkia	B. melitensis bv3 B.abortus bv 1	Outbreak investigation
[59]	RBPT	Cattle Buffalo Sheep Goats	176 97 169 20	Serum Serum Serum Serum	2.27% 3.09% 4.73% 0.00%	Kafrelsheikh		No outbreak investigation
[58]	TAT MT TAT CFT indirect haemolysis	Camel	1500	Serum	5.3% 6.33% 6.4% 7.93% 7.27%	Egypt	B.abortus bv7	No outbreak investigation
[47]	STA, RBPT 2ME, MRT CFT, Riv.T semen agglutination	Friesian cattle Native cattle Buffalo	533 302 547	Serum Serum Serum	4.48% 6.43% 2.89%	Menufiya	No isolation	Outbreak investigation
[50]	TAT, Riv.T BAPAT RBPT, MET, MRT	Sheep Goats Sheep Goats	925 560 25 21	Serum Serum Milk Milk	13.3% 7.14% 40% 23.8%	Cairo, Giza, Qaliobia, Kafrelsheik, Dakahlia.	B.melitensis bv 3	Outbreak investigation
[21]	TAT Riv.T RBT TAT Riv.T RBT	Cattle Buffalo	1832 118	Serum Serum	37.9% 32.8% 61.8% 10.2% 7.8% 22.2%		B.melitensis bv3 B.abortus bv 3,7	No outbreak investigation
[44]	CFT TAT BAPAT RBPT Riv.T MRT	Cattle Buffalo Cattle Buffalo Dog Wild Rat	800 300 800 300 108 130	Serum Serum Milk Milk	3% 4% 2.63% 3.67%	Menufiya, Beni-Suef Kafrelsheikh Fayoum	B.melitensis bv3 B.abortus bv 3,7	Outbreak investigation
[51]	TAT Riv.T RBPT MRT	Cattle Buffalo Sheep Goats	1832 118 648 131	Serum Serum Serum Serum	37.99% 10.17% 23.92% 00.00	Alexandria, Assiut, Cairo, Giza, Demiatta, Kafrelsheik, Qaliobia, Menufiya, Port-Said, El-Menia, Beni-suef, Dakahlia.	B.melitensis bv3 B.abortus bv 3,7	Outbreak investigation

Supplementary table abbreviations

*Buffer Acidified Plate Antigen Test (BAPAT)

**Rose Bengal Test (RBT)

***Tube Agglutination Test (SAT)

****Rivanol Test (Riv. T)

*****Milk Ring Test (MRT)

******Polymerase Chain Reaction (PCR)

#Enzyme Linked Immunosorbant Assay (ELISA)

##Dot Blot Hybridization Assay (DBH)

###Complement Fixation Test

####Milk Ring Test

§Latex agglutination test (LAT)

§§Immunochromatographic Assay (ICA)

§§§ Mercapteoethanol test (MET)

CHAPTER 2

Risk of unpasteurized milk in transmission of infection

Detection of *Brucella melitensis* in bovine milk and milk products from apparently healthy animals in Egypt by real time PCR.

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Brief Original Article

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Detection of *Brucella melitensis* in bovine milk and milk products from apparently healthy animals in Egypt by real-time PCR.

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Abstract

Introduction: Brucellosis in Egypt is an endemic disease among animals and humans. In endemic developing countries, dairy products produced from untreated milk are a potential threat to public health. The aim of this study was to detect Brucellae in milk and milk products produced from apparently healthy animals to estimate the prevalence of contamination.

Methodology: Two hundred and fifteen unpasteurized milk samples were collected from apparently healthy cattle (n = 72) and buffaloes (n = 128) reared on small farms, and from milk shops (n = 15) producing dairy products for human consumption. All milk samples were examined by indirect enzyme-linked immunosorbent assay (iELISA) and real-time PCR (RT-PCR) to detect *Brucella* antibodies and *Brucella*-specific DNA, respectively.

Results: Using iELISA, anti-*Brucella* antibodies were detected in 34 samples (16%), while RT-PCR amplified *Brucella*-specific DNA from 17 milk samples (7.9%). Species-specific IS711 RT-PCR identified 16 of the RT-PCR-positive samples as containing *B. melitensis* DNA; 1 RT-PCR-positive sample was identified as containing *B. abortus* DNA.

Conclusions: The detection of *Brucella* DNA in milk or milk products sold for human consumption, especially the highly pathogenic species *B. melitensis*, is of obvious concern. The shedding of *Brucella* spp. in milk poses an increasing threat to consumers in Egypt. Consumption of dairy products produced from non-pasteurized milk by individual farmers operating under poor hygienic conditions represents an unacceptable risk to public health.

Key words: *Brucella melitensis*; bovine; unpasteurized milk and milk products; iELISA; RT-PCR.
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1. Introduction

Brucellosis is a highly contagious bacterial disease of zoonotic importance, causing significant reproductive losses in animals. Members of the genus *Brucella* are Gram-negative, facultative intracellular pathogens that may affect a wide range of mammals including humans, cattle, sheep, goats, pigs, rodents, and marine mammals [1]. Despite the implementation of the National Brucellosis Control Program in Egypt 32 years ago [2], the disease is still endemic among ruminants and humans [3]. Recently, concurrent infections

with acute febrile illness (AFI) of unknown cause have been reported as a common clinical syndrome among patients seeking hospital care in Egypt [4]. Of these patients, 5% are culture-positive for Brucellae and 11% show positive results by serological testing [5]. The total seroprevalence of human brucellosis ranges between 5% and 8%, with no significant effect of seasonal variation [6]. Furthermore, there are reports suggesting that the incidence of human infection may be increasing in these and other populations in Egypt [4, 7, 8].

Brucellosis is an occupational disease that affects individuals who have close contact with infected animals, such as veterinarians, abattoir workers, farmers, and laboratory personnel. Ingestion of unpasteurized milk and dairy products made from this source may expose humans to pathogenic *Brucella* species, and is a common route of infection in humans [9,10]. In particular, immunocompromised persons, including the elderly, pregnant women, infants and young children, are at the highest risk of contracting brucellosis [11]. In dairy animals, *Brucella* spp. replicate in the mammary gland and supra-mammary lymph nodes, and these animals continually excrete the pathogen into milk throughout their lives [12]. Since cow and buffalo milk and milk products are more commonly consumed than the milk of sheep, goats and camels in Egypt, the risk for human infection is mainly confined to cattle and buffaloes

In Egypt and other developing countries, dairy products such as butter, fermented milk, Kareish cheese, and yogurt may be produced from unpasteurized milk collected by individual farmers operating small farms in substandard sanitary conditions. It has also been shown that *B. melitensis* can survive in naturally contaminated unpasteurized milk for up to five days when kept at 4°C and up to nine days at -20°C [14]. In yogurt stored at ambient temperature and at 4°C, *Brucella* organisms can survive four and eight days, respectively. In Kareish cheese manufactured from naturally contaminated unpasteurized milk, the *Brucella* survival rate increased until the eighth day at ambient temperature [14]. Therefore, the occurrence of *Brucella* spp. in these products is to be expected. This preliminary study was performed to assess the presence of Brucellae in fresh milk samples and untreated dairy products (*e.g.*, yogurt), using iELISA and RT-PCR

2. Methodology

A total of 215 raw or unpasteurized milk samples were collected from apparently healthy cows (n = 72) and buffaloes (n = 128) at small farms, and from milk shops (n = 15) that produce dairy products for human consumption. From milk shops, 5 samples were collected from milk tanks, 6 from yogurt, and 4 from cream. All samples were collected from neighboring localities in Menufiya, Qalyobia, and Sharkia governorates of the Delta region, Egypt. These areas are known to be endemic for brucellosis. Cattle and buffaloes are reared there to produce milk for consumption in large cities such as Cairo. Indirect enzyme-linked immunosorbent assay (iELISA) was performed on all milk samples using *Brucella* smooth lipopolysaccharide (S-LPS) as the antigen (IDEXX, Montpellier SAS, France). The iELISA

results were classified as positive or negative using the cutoff values recommended by the manufacturer. DNA was extracted from milk, cream, and yogurt samples using the High Pure PCR Template Preparation Kit (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions. RT-PCR assays were used to confirm the presence of the genus *Brucella* and to identify *B. abortus* and *B. melitensis* in the extracted DNA samples. Assays were performed in single runs for genus and species identification as described previously by Probert *et al*. [15]. All samples were tested in duplicate; cycle threshold (ct) values below 40 cycles were interpreted as positive.

3. Results

As shown in Table 1, 38 milk samples were positive in at least one test and 177 samples were negative either with iELISA or PCR assay for *Brucella*. The iELISA detected *Brucella* antibodies in 18, 13 and 3 milk samples from cows, buffaloes and milk tanks, respectively. Genus-specific *bcsp*31 PCR amplified *Brucella*-specific DNA from 9, 7 and 1 milk samples obtained from cows, buffaloes and a milk tank, respectively. Species-specific IS711 RT-PCR confirmed the presence of *B. abortus*-specific DNA in 1 cow milk sample, while in 16 samples, *B. melitensis*-specific DNA was detected. In 18, 17 and 3 milk samples from cows, buffaloes and milk tanks in dairy shops, respectively, *Brucella* antibodies and/or *Brucella*-specific DNA were detected. All cream and yogurt samples were negative.

4. Discussion

Brucellosis remains an endemic disease of ruminants and humans in most Middle Eastern countries and in various countries of the Mediterranean basin [2]. Recently, brucellosis cases have increased sharply in persons living in areas located far away from *Brucella*-endemic areas. Brucellosis can also be easily transmitted from endemic rural pockets to non-endemic urban areas [16]. The explanation for this is in part may be that raw milk and dairy products of animals infected with *Brucella* are now being transported over very long distances and consumed by an at-risk population. In Egypt, huge investments in surveillance and eradication of brucellosis were made in the last 25 years with only limited success. Endemic countries suffer from loss of productivity and an adverse impact on human health [1].

Isolation and phenotyping of *Brucella* is still the gold standard for diagnosis, but it is time consuming, potentially hazardous, and requires well-trained personnel [17]. Molecular diagnosis of brucellosis by PCR techniques has increasingly been used as a supplementary method [18, 19]. Genus-specific PCR assays are inexpensive tests for screening and have the capability to detect low concentrations of DNA. Our findings are completely in agreement with previous reports that *B. melitensis* DNA can be amplified from bovine milk samples [20].

Table 1. iELISA and PCR results of milk samples showing a positive result in at least one test.

No.	Type of sample	Source of sample	iELISA	BCSP 31 PCR	IS711 *B. abortus* PCR	IS711 *B. melitensis* PCR
			O.D	ct value		
1	Milk	Cow	+/2.93	+/36.3	+/37.45	-/No Ct
2	Milk	Buffalo	+/3.11	+/35.55	-/No Ct	+/36.8
3	Milk	Cow	+/2.99	+/36.78	-/No Ct	+/37.9
4	Milk	Cow	+/2.95	+/35.57	-/No Ct	+/36.42
5	Milk	Cow	+/2.65	+/35.44	-/No Ct	+/36.1
6	Milk	Cow	+/3.02	+/34.78	-/No Ct	+/35.9
7	Milk	Cow	+/3.01	+/36.57	-/No Ct	+/38.36
8	Milk	Cow	+/2.66	-/44.4	-/No Ct	-/No Ct
9	Milk	Cow	+/2.24	-/45.3	-/No Ct	-/No Ct
10	Milk	Cow	+/2.23	+/35.57	-/No Ct	+/36.44
11	Milk	Cow	+/2.01	+/36.55	-/No Ct	+/37.25
12	Milk	Cow	+/3.02	-/No Ct	-/No Ct	-/45.55
13	Milk	Cow	+/3.14	+/36.4	-/No Ct	+/37.55
14	Milk	Buffalo	+/2.65	-/No Ct	-/No Ct	-/48.88
15	Milk	Buffalo	+/2.58	-/No Ct	-/No Ct	-/47.95
16	Milk	Buffalo	+/2.88	+/35.33	-/No Ct	+/34.44
17	Milk	Buffalo	+/3.07	+/34.45	-/No Ct	+/33.2
18	Milk	Milk Tank	+/3.15	-/No Ct	-/No Ct	-/46.35
19	Milk	Milk Tank	+2.24	+/36.55	-/No Ct	+/35.54
20	Milk	Milk Tank	+/2.45	-/No Ct	-/No Ct	-/No Ct
21	Milk	Cow	+/3.10	-/No Ct	-/No Ct	-/No Ct
22	Milk	Buffalo	+/2.56	-/No Ct	-/No Ct	-/No Ct
23	Milk	Buffalo	+/3.07	-/No Ct	-/No Ct	-/No Ct
24	Milk	Buffalo	+/3.19	-/No Ct	-/No Ct	-/No Ct
25	Milk	Buffalo	+/2.18	-/No Ct	-/No Ct	-/No Ct
26	Milk	Cow	+/2.14	-/No Ct	-/No Ct	-/No Ct
27	Milk	Cow	+/2.25	-/No Ct	-/No Ct	-/No Ct
28	Milk	Buffalo	+/2.13	-/No Ct	-/No Ct	-/No Ct
29	Milk	Buffalo	+/3.10	-/No Ct	-/No Ct	-/No Ct
30	Milk	Buffalo	+/3.00	-/No Ct	-/No Ct	-/No Ct
31	Milk	Buffalo	+/2.24	-/No Ct	-/No Ct	-/No Ct
32	Milk	Cow	+/2.65	-/No Ct	-/No Ct	-/No Ct
33	Milk	Cow	+/2.58	-/No Ct	-/No Ct	-/No Ct
34	Milk	Cow	+2.97	-/No Ct	-/No Ct	-/No Ct
35	Milk	Buffalo	-/0.024	+/36.29	-/No Ct	+/35.69
36	Milk	Buffalo	-/0.011	+/33.44	-/No Ct	+/32.84
37	Milk	Buffalo	-/0.95	+/36.49	-/No Ct	+/35.19
38	Milk	Buffalo	-/0.051	+/36.30	-/No Ct	+/34.35
Total No.	38		34	17	1	16

ELISA-positive samples showing cutoff values (≥ 2);
PCR-positive samples showing ct value ($ct \leq 40$).

Our data show that these assays can be used for risk analysis investigation during routine control of milk, especially as they were able to detect *Brucella* DNA in ELISA-negative samples. Failure of PCR in ELISA positive milk samples can be explained by the fact that antibody titers remain elevated for a long time after infection, independent of circulating bacteria or DNA. However, false positive ELISA results due to cross-reactions with the LPS of other bacteria (*e.g.*, *Yersinia enterocolitica* O:9) would coincide with true negative PCR results. *Yersinia enterocolitica* is known to be widespread in dairy herds worldwide, but its prevalence in Egyptian cattle herds is unknown. Further investigations are needed to illuminate the true cause of these findings. Failure of PCR to detect *Brucella* DNA in cheese or yogurt might be explained by the fact that these products were indeed not contaminated or simply by the fact that the purification method used by us was inadequate for these matrices. A more dedicated study is needed to determine the risk for the consumer posed by these foods.

Mastitis in animal brucellosis is uncommon, but persistent infection of the udder accompanied by intermittent shedding of the organism in milk has been reported [21]. Cows infected with *B. abortus* usually abort only once, and following that give birth to healthy or weak calves. Some cows may not exhibit any clinical signs of the disease and give birth to healthy calves [22]. Those animals can be the source of continual infection [23]. In infected herds, RT-PCR may be a very valuable tool in reducing the time to eradicate the disease by identifying anergic shedders or newly infected animals that should be removed from the herds immediately. *B melitensis* is one of the major causes of abortion in small ruminants; other ruminants may be infected occasionally [24]. It is also the main agent responsible for brucellosis in humans, as it is highly virulent for humans. Circulation of this species in untypical hosts like cattle or buffaloes is of special concern to public health; control or eradication programs have to be adapted to this special situation accordingly. As such, species-specific PCRs are valuable tools in screening programs to identify the prevalent *Brucella* species.

Transmission of *Brucella* through contaminated milk and milk products is an increasing threat not only for individuals, but also for whole families in urban and rural settings of endemic countries [25]. In these areas, trade of non-pasteurized fresh milk and raw dairy products should be strictly controlled and limited to certified *Brucella*-free farms. Our data show that PCR is a sensitive tool for the control of brucellosis in raw milk. Basic health education with respect to the nature of the disease and the modes of transmission through milk products is required for local farmers and consumers. Additionally, a traditional belief that raw milk is better than pasteurized milk must be addressed in light of the current scientific information.

5. Conclusions

Consumption of potentially contaminated raw milk and unpasteurized dairy products is a serious risk with great public health significance. General health education on the nature of the disease and the modes of transmission through milk products is generally required to avoid infection or spread of the pathogens.

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CHAPTER 3

Interspecies transmission of *Brucella* in Egypt

Detection of *Brucella abortus* DNA in aborted goats and sheep in Egypt by real-time PCR.

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SHORT REPORT Open Access

Detection of *Brucella abortus* DNA in aborted goats and sheep in Egypt by real-time PCR.

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Abstract
Background: Brucellosis is a major zoonoses affects wide range of domesticated as well as wild animals. Despite the eradication program of brucellosis in Egypt, the disease is still endemic among cattle, buffaloes, sheep, goats, and camels.
Results: In the present study, abortion occurred naturally among 25 animals (10 cows, 5 buffaloes, 9 Egyptian Baladi goats and one ewe) shared the same pasture were investigated by real-time polymerase chain reaction (RT-PCR). DNA of *Brucella (B.) abortus* was detected in serum of goats and sheep which has aborted recently by species-specific real time-PCR. The results suggest cross-species infection of *B. abortus* from cattle to non-preferred hosts raised in close contact.
Conclusion: This article will renew our knowledge about the *Brucella* agent causing abortion in small ruminants in Egypt. Information provided in this study is important for surveillance program, because eradication programs and vaccination strategies may have to be adapted accordingly.
Key wards: *Brucella abortus*; Cross-species transmission; Real-time PCR; Small ruminants.

1. Background

Brucellosis is a serious zoonosis transmitted by direct contact to secretions of animals which have aborted or contaminated dairy products [1]. The genus *Brucella* (B.) is a facultative intracellular pathogen that currently includes 11 accepted nomo-species. Based on the primary host species specificity. The 'classical' six species are *B. melitensis, B. abortus, B. suis, B. canis, B. ovis,* and *B. neotomae* which are primarily isolated from small ruminants, bovines, pigs, dogs, sheep and desert wood rats, respectively [2]. Two species of marine origin (*B. pinnipedialis* from seals, and *B. ceti* from dolphins and whales). *B. microti* was isolated from the common vole *Microtus arvalis* in middle Europe [3, 4]. *B. inopinata* was isolated from a breast implant wound of a North American female patient [5]. Recently, *B. papionis* was isolated from baboons (Papio spp.) [6].

In Egypt, brucellosis is still endemic and infects a wide range of animal species causing tremendous economic losses [7]. *B. melitensis* was isolated from cattle, buffalo, sheep, goat and Nile catfish in the past [8, 9]. In contrast, *B. abortus* was isolated from cattle, buffalo and camel [10-12], but was not recorded in small ruminant [13]. Host specificity of *Brucella* pathovars has been recognized for a long time and was used to phenotype isolates in the past. Goats and sheep are considered the classical and preferred hosts for *B. melitensis*. The clinical, pathological and epidemiological picture of caprine brucellosis due to *B. melitensis* is similar to *B. abortus* infection in cattle [1]. Due to existence of mixed livestock shelters and uncontrolled animal flock movements in Egypt [8], it was considered necessary to investigate the ability of *Brucella* isolates to be transmitted to and replicate outside its preferred host species in field conditions. Therefore, the present study was performed to investigate whether interspecies transmission of *B. abortus* may occur naturally and may cause clinical disease in small ruminants. This is of important once, because current eradication programs and vaccination strategies may have to be adapted if trans-species infections play a relevant role.

2. Results

A storm of abortion occurred naturally among 10 cows (*Bos taurus*), 5 buffaloes (*Bupalus bubalis*), 9 Egyptian Baladi goats (*Capra hircus*) and one ewe (*Ovis orientalis aries*). Aborted animals submitted to veterinary clinic after abortion for diagnosis and treatment in a small village at Minufya governorate, Delta region, Egypt. All aborted animals shared the same pasture, but were owned by different peasants from neighboring localities. Serum samples were collected from animals after receiving permission from the owners. Samples from aborted fetus were not available. Sera were analyzed using the rose bengal test (RBT), the complement fixation test (CFT) and enzyme linked immunosorbent assay (ELISA) (IDEXX Brucellosis serum X2 AB test, Montpellier SAS, France).

Genomic DNA was extracted with the High Pure template preparation kit (DNA HP kit, Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions. Specific real-time PCR assays for genus and species described by Probert et al. were performed in single runs [14]. The primers and probes were obtained from TIB MOLBIOL (Berlin, Germany) (Table 1). Each amplification reaction mixture was contained 0.75 µl of each primer (0.3 µM), 12.5 µl TaqMan™ Universal Master Mix (Applied Biosystems, USA), 0.25 µl probe (0.1µM), 2 µl of DNA template and was filled up to a total volumes of 25 µl with HPLC grade water. Positive controls that contained *Brucella* DNA and No Template Controls (NTC) that contained PCR-grade water instead of DNA were used in all assays. Real-time-PCR assays were performed with the following cycling conditions, decontamination at 50^{o} C for 2 min, one cycle with initial denaturation at 95^{o} C for 10 min, and 50 cycles with 95^{o} C for 25 sec and 57°C for 1 min. All samples were tested in duplicates; cycle threshold (ct) values below 40 cycles were interpreted as positive.

Serum samples collected very recently after abortion from four buffaloes and six goats gave negative results in serology. Contrastingly, samples collected three weeks after abortion produced strong positive reactions in RBT, CFT and ELISA. Real time-PCR assays resulted in a higher numbers of positive cases than serology. All examined serum samples (n=25) revealed positive results in PCR, while only ten samples were positive in serology (Figure 1). All serum samples collected from aborted cows (n=10), buffaloes (n=5), ewe (n=1) and goats (n=9) were positive with the genus specific bcsp31 real-time PCR assays. Interestingly, *B. abortus* DNA was identified in all serum samples collected from cows, buffaloes, ewe and goats. It is worth mentioning that one ovine serum contained both, *B. abortus* and *B. melitensis* DNA (Table 2). Bacterial isolation failed to isolate *Brucella.*

Table 1. Primers and specific probes used in the real-time multiplex PCR assay for the detection of *Brucella* spp., *B. abortus*, and *B. melitensis.*

PCR Identification	Primer and probe	Sequence (5' to 3')
Brucella spp	Forward primer 5′-3′	GCT-CGG-TTG-CCA-ATA-TCA-ATG-C
	Reverse primer 5′-3′	GGG-TAA-AGC-GTC-GCC-AGA-AG
	Probe 5′-3′	6FAM-AAA-TCT-TCC-ACC-TTG-CCC-TTG-CCA-TCA-BHQ1
B.abortus	Forward primer 5′-3′	GCG-GCT-TTT-CTA-TCA-CGG-TAT-TC
	Reverse primer 5′-3′	CAT-GCG-CTA-TGA-TCT-GGT-TAC-G
	Probe 5′-3′	HEX-CGC-TCA-TGC-TCG-CCA-GAC-TTC-AAT-G-BHQ1
B.melitensis	Forward primer 5′-3′	AAC-AAG-CGG-CAC-CCC-TAA-AA
	Reverse primer 5′-3′	CAT-GCG-CTA-TGA-TCT-GGT-TAC-G
	Probe 5′-3′	Cy5-CAG-GAG-TGT-TTC-GGC-TCA-GAA-TAA-TCC-ACA-HQ2

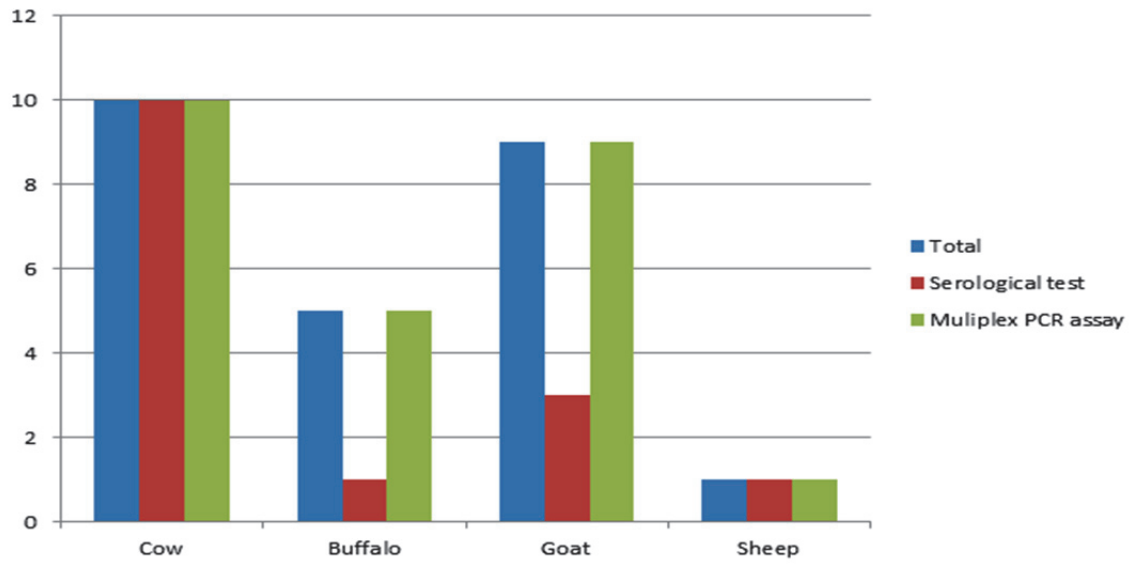

Figure 1. Serological and Multiplex PCR assay result in cow, buffalo, goat and sheep.

Table 2: Serology and real-time PCR results of serum samples collected from animals, which had aborted recently and positive in at least one test.

Case no.	Host	History of samples collection after abortion	Serological assay			PCR[d]		
			RBT[a]	CFT[b]	ELISA[c]	bcsp 31	IS711 *B.abortus*	IS711 *B.melitensis*
1	Cow	4 weeks	+	+	+	+	+	-
2	Cow	3weeks	+	+	+	+	+	-
3	Cow	6weeks	+	+	+	+	+	-
4	Cow	4weeks	+	+	+	+	+	-
5	Cow	3weeks	+	+	+	+	+	-
6	Cow	3weeks	+	+	+	+	+	-
7	Cow	6weeks	+	+	+	+	+	-
8	Cow	4weeks	+	+	+	+	+	-
9	Cow	4weeks	+	+	+	+	+	-
10	Cow	3weeks	+	+	+	+	+	-
11	Buffalo	4weeks	+	+	+	+	+	-
12	Buffalo	1week	-	-	-	+	+	-
13	Buffalo	1week	-	-	-	+	+	-
14	Buffalo	1week	-	-	-	+	+	-
15	Buffalo	1week	-	-	-	+	+	-
16	Goat	4weeks	+	+	+	+	+	-
17	Goat	3weeks	+	+	+	+	+	-
18	Goat	4weeks	+	+	+	+	+	-
19	Goat	1week	-	-	-	+	+	-
20	Goat	1week	-	-	-	+	+	-
21	Goat	1week	-	-	-	+	+	-
22	Goat	1week	-	-	-	+	+	-
23	Goat	1week	-	-	-	+	+	-
24	Goat	1week	-	-	-	+	+	-
25	Sheep	4weeks	+	+	+	+	+	+
Total positive			**15**	**15**	**15**	**25**	**25**	**1**

[a]Considered positive when showing any degree of agglutination.

[b]Positive samples (≥20 IU/mL).

[c]positive samples showing cut off values (≥ 2)

[d]Positive samples showing ct value (ct ≤ 40)

3. Discussion

In developing countries such as Egypt, conventional tests done on serum are used for screening of brucellosis and play an important role in surveillance programs of the disease [13]. Based on previous publication about brucellosis in Egypt, this study is the first to record *B.abortus* DNA in sera samples of sheep and goat. *Brucella* organisms were not isolated in this study. *Brucella* culturing is hazardous, and the technique is restricted to few laboratories in Egypt. Isolation rate is very low even in experienced laboratories [13]. The probability of successful isolation of *B. abortus* is markedly reduced when a few organisms are present in the samples or the material is heavily contaminated. Negative culture results cannot exclude infection with *Brucella* [15]. Nevertheless, clinical presentation i.e. abortion and strong seropositive results finally led to the diagnosis of brucellosis. Serological diagnosis from freshly aborted animals may fail because antibody titers against *B. abortus* rise only 1-2 weeks after infection [16], however circulating *Brucella* DNA may be detected with molecular techniques. These facts can explain the absences of antibody titres in some animals.

Serological diagnosis of brucellosis is presumptive evidence of infection and laboratory confirmation of brucellosis requires isolation of bacteria or detection of *Brucella* DNA by PCR. Thus, the diagnostic window of *Brucella* serology should be complemented by bacteriological or molecular diagnosis [17]. PCR assay able to detect *Brucella* DNA in seronegative animals and it was proposed to use PCR even as a tool for routine diagnosis [18]. Our results corroborate this proposal.

All *Brucella* species are closely related and can be considered as pathovars of a single species [19]. Thus, it is not unexpected that host specificity of *Brucella* spp. is not 'absolute' but 'relative' [1]. Although ruminants in general are susceptible to *B. abortus*, the infection in small ruminants is rare [1]. Experimental infection of pregnant ewes with *B. abortus* produced late term abortions. The aborted ovine fetuses developed lesions due to systemic infections similar to those reported in bovine fetuses after natural and experimental infections [20]. *B. abortus* infections have been reported in sheep in the USA [21], in Nigeria [22, 23] and in Iran [24]. The protective efficacy of vaccines against *B. abortus* infections has not been studied in small ruminants and may play a role for the persistence of brucellosis in cattle [1, 25, 26]. In Egypt, *B. abortus* bv 1 and 3 have been reported in cattle and buffaloes [12, 27]. Cross species transmission of *B. melitensis* to cattle and buffalo from small ruminants that shared the same stables and farmyards was recognized in Egypt [10, 28, 29]. Recently, *B. melitensis* DNA was also detected in milk samples collected from apparently healthy cattle and buffaloes by real-time PCR [30]. However, no reports could be found that *B. abortus* or its DNA was ever found in small ruminants in Egypt. To the best of our knowledge; this is the first report of sheep and goat brucellosis caused by *B. abortus* in Egypt. Accidental *B. abortus* infections in small ruminants may even play an understanding role for the persistence of brucellosis in cattle [1].

Detection of both, *B.abortus* and *B.melitensis* DNA, in one animal observed in this study demonstrated that one host can be infected with two different species of *Brucella* at the same time. The potential host range of Brucellae may also depend on breeding conditions [19]. Co-habitation and close contact of different animal species increase the risk of a pathogen to cross the species barrier [31]. Infection of small ruminants with *B. abortus* can occur as result of natural exposure to infected materials from another species or indirectly through contact with soil contaminated with abortion secrets. Brucellae can survive up to 15-25 days on a pasture depending on environmental conditions e.g. intensity of UV-light [31]. It is likely that the Egyptian Baladi goats and sheep which had aborted had contact with either the fetus or infective fluids from cattle abortion. Isolation of *B. abortus* DNA from a doe that aborted corroborates a cross-species transmission of the *Brucella* spp.

4. Conclusion

In summary, clinical presentation i.e. abortion and presence of *Brucella* DNA finally led to the diagnosis of brucellosis caused by *B. abortus* in Egyptian Baladi does *(Capra hircus)* and sheep *(Ovis orientalis aries)*. To the best of our knowledge, our study is the first record on brucellosis caused by *B. abortus* in small ruminants in Egypt. Our findings indicate also that, in endemic areas like Egypt, where both *Brucella spp*. are present and small ruminants are raised with cattle in close contact in the same pasture, transmission of host specific *Brucella species* to non-preferred hosts may occur. These results should be taken in account while assessing the epidemiological situation in an area and during implementation of control measures. Trials to isolate the bacteria and molecular typing such as multi-locus variable number of tandem repeats (MLVA) to obtain an epidemiological evidence of transmission between animals is required.

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CHAPTER 4

Role of proteomics in pathogenesis of *Brucella*

Proteomics based identification of immunodominant proteins of Brucellae using sera from infected hosts points towards enhanced pathogen survival during the infection

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CHAPTER 5

Immunodominant proteins for the serodiagnosis of *Brucella*

Identification of immunodominant proteins using fully virulent *Brucella abortus* and *Brucella melitensis* field strains and circulating antibodies in the naturally infected host.

Under review

Identification of immunodominant proteins using fully virulent *Brucella abortus* and *Brucella melitensis* field strains and circulating antibodies in the naturally infected host

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Abstract

Background: Brucellosis is a debilitating zoonotic disease affecting humans and animals. The diagnosis of brucellosis is challenging as rapid and accurate identification of the causative *Brucella* (*B.*) species is not possible with any of the diagnostic methods based on serology currently available. The present study aimed at identifying proteins, which might induce *Brucella* species-specific antibodies in different host species.
Methods: Whole cell protein of a *B. abortus* and a *B. melitensis* field strain were extracted and separated using two-dimensional gel electrophoresis. Subsequent Western blotting was done using sera from naturally infected host species, i.e. cattle, buffalo, sheep and goat. Proteins matching western blot signals were subjected to MALDI TOF MS analysis.
Results: Twenty five and 20 specific proteins were identified for *B. abortus* and *B. melitensis*, respectively. Dihydrodipicolinate synthase, glyceraldehyde-3-phosphate dehydrogenase and lactate/malate dehydrogenase assigned to *B. abortus,* amino acid ABC transporter substrate-binding protein assigned to *B. melitensis*, and fumarylacetoacetate hydrolase domain-containing protein 2 found in both species, were reactive with the sera of all *Brucella*-infected host species.
Significance: The identified proteins appear to be useful candidates for a future serological assay capable of detecting pan-*Brucella*, *B. abortus* and *B. melitensis* specific antibodies.

Author Summary

Brucellosis is a severely debilitating zoonotic disease affecting animal and man. The diagnosis is tedious as cross-reactivity with other Gram-negative bacteria and within the species of the genus hamper serological diagnosis. The results presented here open up new possibilities for the serodiagnosis of brucellosis by providing *Brucella* species-specific immunodominant protein candidates reacting only with sera collected from naturally infected cattle, buffalo, sheep and goat. The study provides information on new protein candidates and could help to improve the serological diagnosis of brucellosis.

1. Introduction

Brucellosis is a zoonosis affecting a wide range of mammals including humans [1]. The genus *Brucella* currently includes 11 species, with *Brucella abortus* and *Brucella melitensis* representing the species in the majority of notified human cases. These two species possess strikingly similar genomes [2] but display differences in host specificity, their proteomes [3] and immunodominant proteins [4]. *B. melitensis* is the most virulent species of all Brucellae, one of the major causes of abortions in small ruminants and the causative agent of severe infections in humans [5]. *B. abortus* infections occur in cattle while infections in small ruminants and camels are not common [6]. In humans the course of *B. abortus* infections is milder [5].

The conventional methods for species identification include cultivation, as well as genome-based and serological methods [7]. All these methods are hazardous, time-consuming and not suitable for 'high-throughput analysis'; moreover, the routinely utilized bacterial lipopolysaccharide (LPS) - based serological method suffers from reduced sensitivity due to cross reactivity with the LPS of other Gram-negative bacteria such as *Yersinia enterocolitica*, *Salmonella* spp, and *Escherichia coli* O:157 [8]. Furthermore, serological tests cannot distinguish between *B. abortus* and *B. melitensis* infection or between naturally infected and vaccinated animals [9, 10].

The aim of this study was to identify bacterial species-specific proteins by immunoblotting using the circulating antibodies in the naturally infected animal host species. This approach identified several immunodominant proteins from *B. abortus* and *B. melitensis* that can be used to design a new tool for brucellosis diagnostics.

2. Materials and Methods

Bacterial strains, antisera selection and protein extraction

The *B. abortus* and *B. melitensis* field strains used in the present study were obtained from the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Jena, Germany. Identification, biotyping of *Brucella* isolates, and antisera selection was done as previously described [4]. The whole cell protein of *B. abortus* and *B. melitensis* was extracted in HEPES lysis buffer as described by Wareth *et al* [4].

Two-dimensional electrophoresis

2-D electrophoresis (DE) was performed as described [11]. Briefly, the first dimension of 2-DE was performed by applying 100 μg of acetone-precipitated protein per sample to 4-7.7 cm immobilized pH gradient (IPG) strips (Immobiline^{TM} Dry Strip, GE Healthcare Bio-sciences AB; Uppsala, Sweden). The strips were rehydrated overnight at room temperature with 135

µL DeStreak Rehydration Solution. Isoelectric focusing (IEF) was performed by using the Ettan™ IPGphor3™ Unit (GE Healthcare Europe; Freiburg, Germany) and carried out at 20°C for 6.5 h at 5000 V and 50 µA/strip.

Then the strips were sequentially equilibrated for 20 min in 2 ml equilibration buffer 1 (0.05 M trichloroethylene HCl (pH 8.8), 6 M Urea, 30% Glycerol, 4% SDS, 2% DTE, 0.002% bromophenol blue) and equilibration buffer 2 (0.05 M trichloroethylene HCl (pH 8.8), 6 M urea, 30% Glycerol, 4% SDS, 2.5% Iodoacetamid, 0.002% bromophenol blue). Standard molecular weight prestained protein ladder marker (10-250 kDa; Page Ruler™ Plus, ThermoScientific; Germany) and IPG strips were loaded onto homogeneous 12% polyacrylamide gels and sealed with 1% agarose solution. Electrophoresis was carried out at room temperature and 10 mA/gel until the tracking dye reached the bottom of the gels (1.5h). 2-D protein profiles were visualized using Coomassie blue stain as previously described [12].

2-D Western blotting

2-D Immunoblotting was carried out as previously described [13] with minor modifications. Briefly, proteins were separated on 2-DE gels and transferred at 80 mA/gel for 90 min to nitrocellulose membranes (0.2 µM Bio-Rad laboratories; München; Germany) using Towbin transfer buffer (0.025 M Tris, 0.192 M glycine, 2.33% SDS, 20% (v/v) methanol, pH 8.3). The nitrocellulose membrane was blocked overnight at room temperature and gentle shaking in 1% skimmed milk in Tris buffered saline (TBS). The membrane was washed twice using TBS with Tween (TBST; 20 mM Tris, pH 7.5; 500 mM NaCl; 0.05% Tween-20; 10 min). Next, the nitrocellulose membrane was placed for 90 minutes at room temperature in a diluted solution of the respective antisera in TBST. Bovine sera (1:200 dilution) and small ruminants sera (1:5000 dilution) were used as primary antibody source while 1:1000 diluted anti-bovine IgG (H&L) (Chicken) peroxidase-conjugated, anti-sheep IgG (H&L) (Donkey) peroxidase-conjugated and anti-goat IgG (H&L) (Chicken) peroxidase-conjugated antibody served as secondary antibody source. All the secondary antibodies were obtained from Biomol-Rockland, Hamburg, Germany. After washing the nitrocellulose membrane twice with TBST for 10 min, the detection of signals was carried out using the TMB kit™ (3,3′,5,5′-tetramethylbenzidine liquid substrate; Sigma-Aldrich; Steinheim, Germany) according to the manufacturer's description.

In-gel trypsin digestion and MALDI-TOF MS/MS

Following the selection of the spots of interest, the protein spots corresponding to the western blots were excised from the gel, destained and subjected to overnight trypsin digestion (0.01 µg/µL) (Promega; Mannheim, Germany) as previously described [4]. The digested precipitates were reconstituted in 3.5 µL 5% acetonitrile in 0.1% TFA (trifluoroacetic acid; Merck; Darmstadt, Germany). The reconstituted precipitates were then

spotted on to target plates for matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) on a Bruker Ultraflex II instrument (Bruker Daltonik; Bremen, Germany) using HCCA (α-Cyano-4-hydroxycinnamic acid; Sigma-Aldrich; Steinheim, Germany) as matrix. A database search was conducted against all entries using the MS/MS ion search mode (MASCOT, http://www.matrixscience.com) as previously described [14]. Protein identification was considered valid if more than two peptides matched and the MOWSE score was significant ($p < 0.05$).

Comparison of the identified proteins and other cross reactive bacteria

BLAST search was done as previously described [3] to compare the identified proteins against *Brucella* spp*., B. suis, B. ovis, Ochrobactrum* spp*., Yersinia enterocolitica, Yersinia pseudotuberculosis, Salmonella enterica,* and *Escherichia coli* O:157, the latter five species being the most cross-reactive bacteria with *Brucella*. Query cover and identity values were evaluated and cut-off values set between 31-54%.

3. Results

Detection of immunoreactive proteins of *B. abortus*

A total of 50 immunoreactive protein spots, corresponding to 25 proteins, were detected by 2D-immunoblotting with a cell lysate of a *B. abortus* field strain and sera from naturally infected cows, buffaloes, sheep and goats (Fig. 1). Total numbers of proteins identified were 24, 19, 29 and 15 for cow, buffalo, sheep and goat, respectively. Subsequent Western Blot matching revealed 10 spots (A01-05, A15, A26, A47, A49, A50), corresponding to 5 proteins, which were detected in all four tested animal species. There was no unique host-specific immunodominant protein for buffalo and goat, whereas two (A43; A21) and four proteins (A08; A10-A12) were specific for cow and sheep, respectively (Table 1).

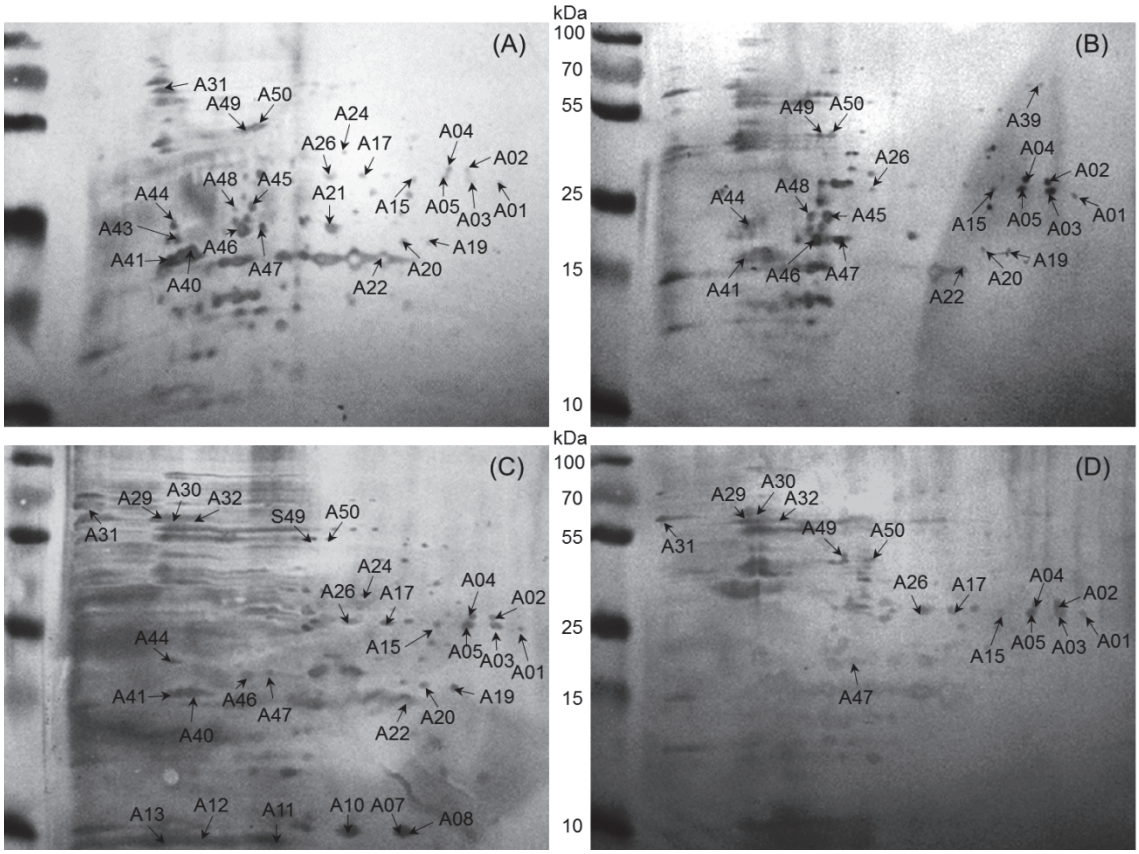

Figure 1: Representative 2D immunoblotting images of whole cell proteins from *B. abortus* extracts separated on a 12% polyacrylamide gel. The blot was developed using the TMB kit after immuno-blotting with serum from A) cattle, B) buffalo, C) sheep, and D) goat and the respective peroxidase-conjugated secondary antibodies.

Table 1: Immunoreactive proteins from *B. abortus* using 2D western blot and MALDI-TOF-MS.

No	spot ID	Acc.ID	Protein	MW	MOWSE score[a]	*PI*	Sequence coverage (%)	No of peptides matching	Host	Reference
1	A01	gi|256369084	dihydrodipicolinate synthase	31892	244	6,26	67	13	Cow, Buffalo, Sheep, Goat	
	A03	gi|256369084	dihydrodipicolinate synthase	31892	426	6,26	67	13	Cow, Buffalo, Sheep, Goat	
	A05	gi|493692811	dihydrodipicolinate synthase	33539	132	7,08	68	14	Cow, Buffalo, Sheep, Goat	
	A15	gi|495149454	dihydrodipicolinate synthase	31753	75	5,94	28	5	Cow, Buffalo, Sheep, Goat	
2	A02	gi|496823699	glyceraldehyde-3-phosphate dehydrogenase	36385	356	6,26	48	13	Cow, Buffalo, Sheep, Goat	[15]
	A04	gi|4165122	glyceraldehyde-3-phosphate dehydrogenase	36344	107	5,89	38	8	Cow, Buffalo, Sheep, Goat	[15]
3	A26	gi|226887955	chain A, Crystal structure of lactate malate dehydrogenase	34152	243	5,24	31	5	Cow, Buffalo, Sheep, Goat	
4	A47	gi|493015116	hypothetical protein (fumarylacetoacetate hydrolase family protein)	29383	343	5,09	36	7	Cow, Buffalo, Sheep, Goat	M25
5	A49	gi|17987134	phosphopyruvate hydratase	45462	421	4,99	53	18	Cow, Buffalo, Sheep, Goat	
	A50	gi|148560469	phosphopyruvate hydratase	45431	494	5,03	47	16	Cow, Buffalo, Sheep, Goat	
6	A20	gi|148558534	metal-dependent hydrolase	25257	103	5,58	44	7	Cow, Buffalo, Sheep	
7	A17	gi|82700282	choloylglycine hydrolase	36868	108	5,62	29	9	Cow, Sheep, Goat	
8	A22	gi|490830157	hydrolase	27731	134	6,07	50	8	Cow, Buffalo, Sheep	[16] M43
	A19	gi|490830157	hydrolase	27731	383	6,07	48	8	Cow, Buffalo, Sheep	[16]
9	A44	gi|320161003	putative DNA processing protein	40919	56	5,85	42	7	Cow, Buffalo, Sheep	
10	A46	gi|489055332	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase	30092	380	5,08	48	8	Cow, Buffalo, Sheep	
11	A41	gi|493691811	sugar ABC transporter substrate-binding protein	33258	440	5,11	52	11	Cow, Buffalo, Sheep	M36
12	A45	gi|384211119	lysine-arginine-ornithine-binding periplasmic protein	36684	440	5,09	57	15	Cow, Buffalo	M27
13	A48	gi|152013695	ADP/ATP translocase	20876	63	9,63	31	4	Cow, Buffalo	
14	A39	gi|62317242	urocanate hydratase	61589	173	6,04	19	11	Buffalo, Sheep	
15	A24	gi|493147262	sulfate transporter subunit	37727	132	5,92	38	11	Cow, Sheep	
16	A31	gi|493053174	catalase	55556	223	6,62	36	18	Cow, Goat	
17	A40	gi|17988780	D-ribose-binding periplasmic protein precursor	31030	193	5,60	57	9	Cow, Sheep	[17, 19] M24
18	A29	gi|148558491	chaperonin GroEL	57505	99	5,08	18	7	Sheep, Goat	[8,20,22]
	A30	gi|14855849	chaperonin GroEL	57505	92	5,08	20	9	Sheep, Goat	[8,20,22]
19	A32	gi|144108	heat shock protein	57534	94	5,33	27	12	Sheep, Goat	
20	A43	gi|492987884	protein grpE	24883	128	4,70	40	12	Cow	
21	A07	gi|384446825	Superoxide dismutase, copper/zinc binding protein	17255	370	6,10	64	7	Sheep	[8,13, 21,22] M01
	A8	gi|489058379	superoxide dismutase copper/zinc binding protein	18205	242	6,24	54	5	Sheep	[8,13, 21,22]
22	A10	gi|17989230	19 kDa periplasmic protein	20238	68	6,06	8	1	Sheep	M05
23	A11	gi|222447132	chain A, crystal structure of ferritin (Bacterioferritin)	20895	68	6,05	33	5	Sheep	
	A13	gi|222447132	chain A, crystal structure of ferritin (Bacterioferritin)	20895	183	5,05	36	4	Sheep	M12
24	A12	gi|493690773	bacterioferritin, partial	16118	220	4,81	33	3	Sheep	[21,22]
25	A21	gi|89258175	31 kDa cell surface protein	31084	293	5,5	38	9	Cow	[21] M21

a) Significant MOWSE score ($P<0.05$)

Detection of immunoreactive proteins of *B. melitensis*

Forty-three immunoreactive protein spots corresponding to 20 proteins were identified for *B. melitensis*. Total numbers of proteins identified were 27, 19, 15 and 12 using sera from sheep, goat, cow, and buffalo, respectively (Fig. 2). Subsequent Western Blot matching revealed 12 spots (M12; M19; M20; M24; M25; M26; M27; M36; M37; M38; M40; M22) common to all four tested animal species, corresponding to 10 proteins. There was no unique host specific immunodominant protein for buffalo and cow, whereas three (M32; M21; M23) and six proteins (M01; M02; M05; M07; M08; M43) were specific for sheep and goat, and sheep only (Table 2).

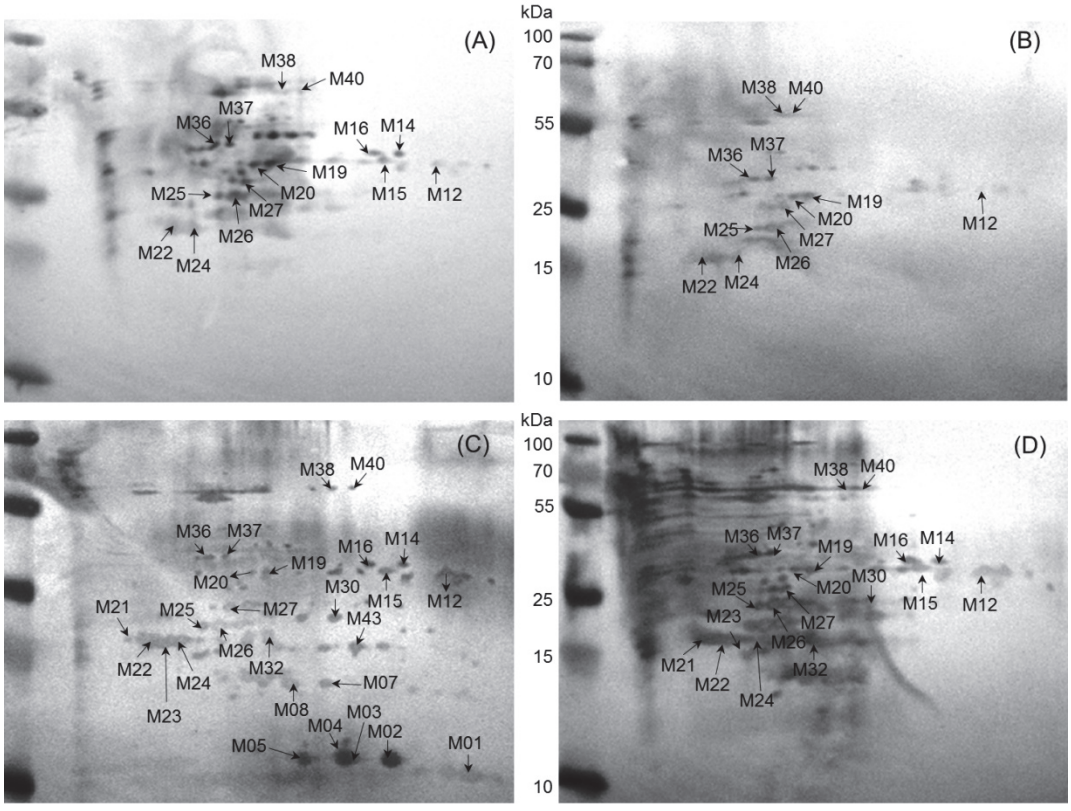

Figure 2: Representative 2D immunoblotting images of whole cell proteins from *B. melitensis* extracts separated on a 12% polyacrylamide gel. The blot was developed using the TMB kit after immuno-blotting with serum from A) cattle, B) buffalo, C) sheep, and D) goat and the respective peroxidase-conjugated secondary antibodies.

Table 2: Immunoreactive proteins from *B. melitensis* using 2D western blot and MALDI-TOF-MS.

No	spot ID	Acc.ID	Protein	MW	MOWSE score [a]	*PI*	Sequence coverage (%)	No of peptides matching	Host	Reference
1	M12	gi|222447132	chain A, crystal structure of ferritin (Bacterioferritin)	20895	183	5,05	36	4	Sheep, Goat, Cow, Buffalo	A13
2	M19	gi|225852817	sulfate ABC transporter substrate-binding protein	37151	324	5,51	44	12	Sheep, Goat, Cow, Buffalo	
3	M20	gi|17986956	thiosulfate-binding protein precursor	37152	34	5,31	5	1	Sheep, Goat, Cow, Buffalo	
4	M24	gi|17988780	D-ribose-binding periplasmic protein precursor	31030	280	5,60	29	5	Sheep, Goat, Cow, Buffalo	[17-19] A40
5	M25	gi|225851771	fumarylacetoacetate hydrolase domain-containing protein 2	30118	471	5,00	61	11	Sheep, Goat, Cow, Buffalo	A47
	M26	gi|225851771	fumarylacetoacetate hydrolase domain-containing protein 2	30118	492	5,00	56	11	Sheep, Goat, Cow, Buffalo	
6	M27	gi|384211119	lysine-arginine-ornithine-binding periplasmic protein	36684	240	5,09	31	10	Sheep, Goat, Cow, Buffalo	A45
7	M36	gi|516360216	sugar ABC transporter substrate-binding protein, partial	44963	121	5,15	50	15	Sheep, Goat, Cow, Buffalo	A41
8	M37	gi|493172683	amino acid ABC transporter substrate-binding protein	31331	178	5,24	48	7	Sheep, Goat, Cow, Buffalo	[21]
9	M40	gi|384410242	amidohydrolase 3	63567	265	5,47	42	20	Sheep, Goat, Cow, Buffalo	
10	M22	gi|493003797	hypothetical protein closed to amino acid ABC transporter substrate-binding protein	21946	90	5,06	33	4	Sheep, Goat, Cow, Buffalo	
	M38	gi|493155701	hypothetical protein closed to ABC transporter substrate-binding protein	58947	437	4,97	43	24	Sheep, Goat, Cow, Buffalo	
	M32	gi|492818336	hypothetical protein closed to ABC transporter substrate-binding protein	31905	113	5,57	33	7	Sheep, Goat	
11	M14	gi|490823297	alcohol dehydrogenase	36537	116	6,07	21	7	Sheep, Goat, Cow	
	M16	gi|489059662	alcohol dehydrogenase	43149	99	7,66	25	9	Sheep, Goat, Cow	
12	M15	gi|493009422	thiamine-binding periplasmic protein	36829	164	5,71	43	9	Sheep, Goat, Cow	
13	M21	gi|89258175	31 kDa cell surface protein	31084	96	5,50	52	10	Sheep, Goat	[21] A21
	M30	gi|89258175	31 kDa cell surface protein	31084	166	5,50	16	5	Sheep, Goat	[21]
14	M23	gi|225686619	rhizopine-binding protein	33294	257	5,11	55	11	Sheep, Goat	
15	M01	gi|384446825	Superoxide dismutase, copper/zinc binding protein	17255	222	6,10	51	6	Sheep	[8,13, 21,22] A07
	M03	gi|384446825	Superoxide dismutase, copper/zinc binding protein	17255	86	6,1	57	6	Sheep	[8,13, 21,22]
16	M02	gi|118137288	chain A, Cu/Zn superoxide dismutase	16176	297	6,11	63	7	Sheep	
	M04	gi|551701922	chain A, Cu/Zn Superoxide Dismutase	16176	83	6,11	61	6	Sheep	
17	M05	gi|384446516	19 kDa periplasmic protein	18735	219	5,65	20	4	Sheep	A10
18	M07	gi|495782928	transaldolase	23554	264	5,47	41	8	Sheep	[13]
19	M08	gi|493009465	fructose-6-phosphate aldolase	23554	244	5,47	22	5	Sheep	
20	M43	gi|490830157	hydrolase	27731	371	6,07	48	8	Sheep	A22

a) Significant MOWSE score ($P<0.05$)

Spot ID: Spot identification; A: B. abortus; M: B. melitensis; NCBI Acc. Nr: accession number at NCBI; sequence in NCBI databank; MOWSE score: -10*Log (P), where P is the probability that the observed match is a random event. This list includes only bands with a MOWSE score greater than (P<0.05); MW: molecular weight; pI: isoelectric point.

Identification of cross-reactive proteins between *B. abortus* and *B. melitensis*

The cell lysates of the *B. abortus* and *B. melitensis* field strains generated a total of 61 immunoreactive spots which could be assigned to 36 proteins. Nine proteins (A47/M25; A22/M43; A41/M36; A45/M27; A40/M24; A07/M01; A10/M05; A13/M12; A21/M21) were detected in cell lysates of *B. abortus* and *B. melitensis* (Table 3), while 16 and 11 proteins were only detected in cell lysates of *B. abortus* or *B. melitensis*, respectively (Tables 1; 2). Spot ID A47/M25 (fumarylacetoacetate hydrolase domain-containing protein 2) was found in cell lysates of *B. abortus* and *B. melitensis* and reacted with the sera of all four tested animal species (Table 3; Table 4). All immunogenic spots reacted only with sera of *Brucella*-positive animals and no reactions were detected with sera from *Brucella*-negative animals.

Table 3: Cross reactive proteins identified in cell lysates of both *B. abortus* and *B. melitensis* (A: *B. abortus*; B: *B. melitensis*)

No	Acc.ID	Protein	*B. abortus*		*B. melitensis*	
			Spot ID	Host	Spot ID	Host
1	gi|493015116	fumarylacetoacetate hydrolase family protein	A47	Cow, Buffalo, Sheep, Goat	M25	Cow, Buffalo, Sheep, Goat
2	gi|490830157	hydrolase	A22	Cow, Buffalo, Sheep	M43	Sheep
3	gi|493691811	sugar ABC transporter substrate-binding protein	A41	Cow, Buffalo, Sheep	M36	Cow, Buffalo, Sheep, Goat
4	gi|384211119	lysine-arginine-ornithine-binding periplasmic protein	A45	Cow, Buffalo	M27	Cow, Buffalo, Sheep, Goat
5	gi|17988780	D-ribose-binding periplasmic protein precursor	A40	Cow, Sheep	M24	Cow, Buffalo, Sheep, Goat
6	gi|384446825	superoxide dismutase, copper/zinc binding protein	A07	Sheep	M01	Sheep
7	gi|384446516	19 kDa periplasmic protein	A10	Sheep	M05	Sheep
8	gi|222447132	chain A, crystal structure of ferritin (Bacterioferritin)	A13	Sheep	M12	Cow, Buffalo, Sheep, Goat
9	gi|89258175	31 kDa cell surface protein	A21	Cow	M21	Sheep, Goat

Table 4: Comparative Blast search between the identified proteins obtained from *B. abortus* and *B. melitensis* and proteins of other possibly cross-reacting bacteria (A: *B. abortus*; B: *B. melitensis*)

No	spot ID	Acc.ID	Protein	Locus, Query cover (QC) and Identity (I)									Host
				100% identity	***Brucella spp.***	***B. suis***	***B. ovis***	***Ochrobactrum spp***	***Yersinia enterocolitica***	***Yersinia pseudotuberculosis***	***Salmonella enterica***	***Escherichia coli* O:157**	
1	A47/ M25	gi|493015116 **MW 29383**	**FAHD2**	WP_006093223 *B. abortus* QC 100% I 100%	WP_006162877 QC 80% **I 96%**	WP_006200925 QC 100% I 96%	YP_001258270 F. hydrolase QC 100% I 96%	WP_006470802 QC 100% I 92%	Not found 16.04.2014	YP_001401380 QC 98% I 62% FAHD	YP_001588666 QC 78% **I 41%**	Not found 16.04.2014	Cow, Buffalo, Sheep, Goat
2	A01	gi|256369084 **MW 31892**	dihydrodipicolinate synthase	YP_003106592 *B. microti* CCM 4915	WP_006165259 **QC 100% I 99%**	NP_697660 QC 100% I 99%	YP_001257393QC 98% I 29%	WP_021587874 QC 100% I 95%	YP_006003506 QC 99% **I 46%**	YP_071290 QC 99% **I 46%**	WP_023259918 QC 99% **I 45%**	NP_311367 QC 99% **I 45%**	Cow, Buffalo, Sheep, Goat
3	A02	gi|496823699 **MW 36385**	glyceraldehyde-3-phosphate dehydrogenase	WP_009374365 *Brucella* spp. QC 100% I 100%	WP_009374365 **QC 100% I 100%**	NP_698712 QC 100% I 99%	NP_698712.1 QC 100% I 99%	WP_021588015 QC 100% I 96%	WP_019083593 Q C 98% **I 54%**	YP_071698 QC 99% **I 46%**	WP_000218344 QC 99% **I 46%**	ELW37260 QC 97% **I 52%**	Cow, Buffalo, Sheep, Goat
4	A26	gi|226887955 **MW 34152**	chain A, of lactate malate dehydrogenase	WP_002970355 *B. abortus* QC 98% I 100%	3GVH_A *B. melitensis* **QC 100% I 100%**	YP_001628354 QC 98% I 99%	YP_001259751 QC 98% I 99%	WP_007872232 L/M dehydrogenase QC 98% I 98%	WP_019080697 QC 67% **I 33%**	YP_069003 QC 67% **I 33%**	YP_218284.1 QC 86% **I 31%**	ELV66131 QC 86% **I 31%**	Cow, Buffalo, Sheep, Goat
5	A49	gi|17987134 **MW 45462**	phosphopyruvate hydratase	NP_539768 *B. melitensis* QC 100% I 100%	YP_008839865 **QC 100% I 99%** enolase	NP_698137 QC 100% I 99%	YP_001259054 QC 100% I 99%	YP_001370601 QC 100% I 97%	YP_001005091. QC 99% **I 60%**	YP_069296.1 QC 99% **I 60%**	WP_016735109 QC 99% **I 61%**	ELV67289 QC 99% **I 61%**	Cow, Buffalo, Sheep, Goat
6	M20	gi|17986956 **MW 37152**	thiosulfate-binding protein precursor	NP_539590.1 *B. melitensis* QC 100% I 100%	WP_008934207 **QC 100% I 99%**	WP_020628554 QC 100% I 100%	YP_001259236 QC 100% I 99%	WP_021586689 QC 100% I 91%	AHM75213.1 QC 98% **I 55%**	YP_071244.1 QC 99% **I 55%**	WP_000290287 QC 93% **I 57%**	NP_288986 QC 92% **I 57%**	Sheep, Goat, Cow, Buffalo
7	M37	gi|493172683 **MW 31331**	amino acid ABC transporter substrate-binding protein	WP_004685846 *B. melitensis* QC 100% I 94%	WP_006161567 *Brucella* spp **QC 100% I 95%**	NP_698767 putative branch QC 100% I 95%	NP_698767 putative branch QC 100% I 95%	WP_006467797 QC 100% I 90%	WP_019080170 QC 95% **I 40%**	Not found 16.04.2014	WP_000822979 leucine branch QC 95% **I 40%**	ELV65532 leucine specific QC 95% **I 42%**	Sheep, Goat, Cow, Buffalo
8	M40	gi|384410242 **MW 63567**	amidohydrolase 3	YP_005602224 *B. melitensis* M5 QC 100% I 100%	YP_005114197 *B. abortus* **QC 100% I 99%**	WP_004689025 QC 100% I 99%	YP_001257534 amidohydrolase QC 88% **I 31%**	YP_001371888 QC 100% **I 53%**	Not found 16.04.2014	Not found 16.04.2014	WP_023220860 amidohydrolase QC 90% **I 26%**	Not found 16.04.2014	Sheep, Goat, Cow, Buffalo
9	M22	gi|493003797 **MW 21946**	hypothetical protein (amino acid ABC transporter substrate-binding protein)	WP_023080384 *B. melitensis* QC 100% I 100%	WP_006085596 *B. abortus* **QC 100% I 100%**	WP_023080435 QC 100% I 84%	YP_001258837 ABC transporter QC 100% I 100%	WP_006466755 ABC transporter QC 99% I 94%	YP_001006291. ABC transporter QC 98% **I 39%**	Not found 16.04.2014	Not found 16.04.2014	Not found 16.04.2014	sheep, goat, cow, buffalo
10	M38	gi|493155701 **MW 58947**	hypothetical protein (ABC transporter substrate-binding protein)	WP_006256535 *B. melitensis* QC 100% I 99%	WP_006164780 **QC 100% I 100%**	WP_006197818 QC 100% I 99%	WP_006157758 QC 99% I 70%	WP_010658797 ABC transporter QC 100% I 89%	WP_019083182 ABC transporter QC 97% **I 40%**	Not found 16.04.2014	WP_023210061 ABC transporter QC 93% **I 40%**	Not found 16.04.2014	sheep, goat, cow, buffalo

Comparative protein BLAST search

In order to identify similar or identical epitope structures between *Brucella* spp., *Ochrobactrum* spp. and putative cross-reacting bacterial species, five proteins (spot ID A47; A01; A02; A26; A49) reacting with *B. abortus* and five proteins (spot ID M20; M37; M40; M22; M38) reacting with *B. melitensis* in the sera of the naturally infected animal host species were submitted to a comparative protein BLAST search (Table 4).

With the exception of the proteins (spot ID) A01, M22, M38 and M40, all proteins displayed identity values ≥ 95% for *Brucella* spp, *B. suis*, *B. ovis* and *Ochrobactrum* spp. Identity values of all ten proteins with the possibly cross-reacting bacterial species *Yersinia enterocolitica*, *Y. pseudotuberculosis*, *Salmonella enterica* and *E. coli* O:157 were between 26 and 62%.

4. Discussion

Diagnosis of brucellosis in veterinary medicine is still a challenging process as it is based on herd serology and the isolation of the agent [7]. The serological assays have their limitations with regard to sensitivity and specificity, as they are neither standardised nor able to distinguish between infected and vaccinated animals [9]. Hence, the aim of this study was to identify immunodominant proteins in both *B. abortus* and *B. melitensis* by immunoproteomic screening to detect specific proteins which can be implemented in a diagnostic assay. Four proteins obtained from *B. abortus* and five obtained from *B. melitensis* cell lysates, and one protein present in both *B. abortus* and *B. melitensis* cell lysates were selected from a total of 61 immunoreactive protein spots identified from the proteome profiles of *B. abortus* and *B. melitensis* using MALDI-TOF MS and the MASCOT data base searching.

In contrast to previous studies on the and *Brucella* proteome which focussed mainly on vaccine or museum strains with altered immunogenic properties, i.e. diminished or loss of virulence [3,8,13,16,18,20,23], the present study used a fully virulent *B. abortus* and a fully virulent *B. melitensis* field strain from a naturally infected cow and sheep, respectively. Sera obtained from naturally infected ruminants which had recently aborted and shown strong positive reactions in the CFT and ELISA, were subsequently tested against both field strains. Since naturally infected hosts generally show a stronger immunoreaction than hosts challenged with inactivated antigen [8], it can be assumed that the sera used in the present study contained antibodies against all immunoreactive proteins involved in infection.

Each *Brucella* species can be associated with a specific host, i.e. *B. abortus* usually infects bovines, whereas *B. melitensis* is the most predominant species in sheep and goat [6]. Despite the close genetic relationship among *Brucella* spp. one could speculate that certain proteins induce a host species-specific immunoreaction. This hypothesis is corroborated by the

findings of Zhao et al., who demonstrated that some proteins are themselves immunogenic and induce high immunogenicity in the host species but not in others [23].

The present study identified a total of 61 immunoreactive protein spots from the proteomic profiles of *B. abortus* and *B. melitensis* using MALDI-TOF MS and the MASCOT data bank corresponding to 36 proteins. By performing the data base search against the sequence information of all entries in MASCOT and the likelihood of identifying suitable proteins was significantly increased by considering MS/MS matched to at least one unique peptide. This approach contrasts the studies of Connolly et al., Yang et al. and Al Dahouk et al., who searched only against data sets of the *Brucella* species used in their experiments [8,16,20] and Zhao et al, who selected proteins containing more than five peptide matches [23]. The sera obtained from sheep were the most reactive, with 56 identified immunogenic protein spots, whereas 39, 31 and 34 spots were found in the sera of cow, buffalo and goat, respectively. Previous studies using the same immunoproteomic techniques as in the present study identified a range of immunoreactive proteins in different *Brucella* spp. and animal species [8, 13, 16, 20, 23]. These observed differences can be attributed to the technical procedures during protein preparation and the source/type of sera samples used, i.e. field or experimental, early or late stage of infection. These findings are in agreement with the idea of a host species specific immunoreaction.

Ten immunogenic proteins specific either from *B. abortus* (n=4), *B. melitensis* (n=5) or both were reactive in all four tested host species i.e. cattle, buffalo, sheep and goat. The mitochondrial catalytic enzyme, Fumarylacetoacetate hydrolase domain-containing protein (FAHD2) was found in both *B. abortus* and *B. melitensis* cell lysates. Four proteins were identified in *B. abortus* only, i.e. dihydrodipicolinate synthase (DHDPS), essential for bacterial growth and involved in the lysine biosynthesis pathway [24]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a protein of the *Brucella*-containing vacuole (BCV) and essential for *B. abortus* virulence [25]. Studies using recombinant GAPDH induced both humoral and cellular immune responses during experimental infection with *B. abortus* in natural hosts (cattle and sheep) and mice [15]. However, when used as DNA vaccine it provided only partial protection against experimental *B. abortus* infection in mice [15]. Chain A of lactate/malate dehydrogenase (MDH) is considered a promising candidate for serodiagnosis and vaccine development [26]. Phosphopyruvate hydratase proteins participate in glycolysis, but their importance as a possible diagnostic candidate is not known [27].

Five proteins found only in *B. melitensis* cell lysates were immunoreactive in all four host species: thiosulfate-binding protein precursor, which specifically binds thiosulfate and is involved in its transmembrane transport; amidohydrolase 3, a member of the amidohydrolase superfamily. These proteins catalyse the hydrolysis of amide or amine bonds in a large number

of different substrates [28]; amino-acid ABC transporter substrate-binding protein, a transmembrane protein previously found via proteome analysis in *B. melitensis* and *B. ovis* [29]; two hypothetical proteins closely related to ABC transporter substrate-binding proteins. The function of these differentially expressed proteins in natural *B. melitensis* infection is not known to date.

BLAST search to assess the similarity of the identified immunoreactive proteins between various *Brucella* species and other bacteria which could show a cross reaction in serological assays revealed that by combining various proteins it is possible to design a pan-*Brucella* test as well as a species differentiating assay. For instance, glyceraldehyde-3-phosphate dehydrogenase, lactate/malate dehydrogenase, thiosulfate-binding protein precursor, the amino acid ABC transporter substrate-binding proteins and FAHD2 are suitable candidates for designing a pan-*Brucella* test. Aminohydrolase 3 on the other hand, might be useful for the differentiation of *B. ovis* and *Ochrobactrum* spp. from *B. abortus*, *B. melitensis* and *B. suis*. The ten proteins identified in the present study are promising candidates for a future serological assay which will be able to detect pan-*Brucella* and *B. abortus* and *B. melitensis* specific antibodies. Moreover, these proteins might also be suitable for vaccine development.

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CHAPTER 6

Chicken embryo as a model of infection in brucellosis

Experimental infection of chicken embryos with recently described *Brucella microti*: Pathogenicity and pathological findings.

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General Discussion

Discussion

Brucellosis is a debilitating and zoonotic disease affecting livestock and man worldwide resulting in huge economic losses (Aparicio, 2013). The disease is caused by Gram-negative bacteria of the genus *Brucella.* In developing countries such as Egypt, the disease is very common but neglected. A retrospective analysis of animal brucellosis research of the last 27 years in Egypt was performed in this thesis. There is an obvious discrepancy of official seroprevalence data obtained from general organization of veterinary service and data published in scientific publications (Wareth et al., 2014a). The disease has been detected nationwide in cattle, buffaloes, sheep, goats, equines, camels and Nile cat fish (El-Tras et al., 2010; Refai, 2002), but no comprehensive reliable data on sero-prevalence were available. Diagnosis of brucellosis is currently based on serological assays which are well-established within the Egyptian national surveillance program, while isolation and identification is available only in few laboratories. All isolation and identification studies were done within the framework of outbreaks investigation. Classical and routine identification of *Brucella* mainly based on phenotyping characteristic i.e. CO2 requirement, H2S production, urea hydrolysis, dye sensitivity, agglutination with monospecific anti-sera and phage lysis (Alton et al., 1988). These techniques are unable to trace back the source of brucellosis in an outbreak effectively, because they only differentiate species and biovars, but cannot be used in areas where specific genotypes are prevalent (Al-Dahouk et al., 2007). Precise strain identification at the subspecies level has become a necessity to design secure control programs (Grissa et al., 2008). Recently, the multiple locus of variable number tandem repeats analysis (MLVA) typing assay has been discussed as a good tool for the *Brucella* species identification combined with a higher discriminatory power between the *Brucella* isolates originating from restricted geographic areas proofing its potential as an epidemiological tool (Le Fleche et al., 2006). Due to insufficient isolation and identification procedures of brucellae, the use of molecular diagnostic techniques was attempted. But only 15 publications used PCR from 1986 to 2012 in Egypt (Wareth et al., 2014a).

The surveillance programs in Egypt are of limited success due to improper diagnosis and prevalence of the disease nationwide (Wareth et al., 2014a). *B. melitensis* bv 3 and *B. abortus* bv 1 are the most commonly isolated agents (Samaha et al., 2009). It may be speculated, that small cattle or buffalo herds sustain a brucellosis cycle specific for Egypt i.e. the circulation of *B. melitensis* bv 3 in bovines. This assumption is corroborated by the fact, that *B. melitensis* bv 3 has been isolated from semen of bulls (and also rams) demostrating venereal transmission (Amin et al., 2001).

Contact of herds of bovines with infected small ruminants may not be necessary anymore to cause disease especially if an infected bull serves herds of various villages. These herds may consequently be a continuing source of human infection and may contribute to disease burden with an unknown impact on public health as *B. melitensis* is regularly isolated from patients (Tiller et al., 2009). In contrast *B. abortus* leads to considerable losses for farmers due to late abortion but is rarely isolated from humans. Hence, the virulence of these 'bovine' *B. melitensis* strains for humans and consequently the risk infected herds pose to public health is unknown for the moment.

Apparently healthy animals living in endemic areas seem to be the source of human infection via intermittent shedding of the brucellae into milk. *B. melitensis* DNA was detected in milk samples from apparently healthy animals which produce milk for human consumption (Wareth et al., 2014b). Consumption of potentially contaminated raw milk and unpasteurized dairy products is a serious risk with great public health significance. Transmission of *Brucella* through contaminated milk and milk products is increasing in urban and rural settings of endemic countries (Chen et al., 2014) as a result of trade of non-pasteurized milk. Thus, raw dairy products and raw milk should be controlled and purchase has to be limited to *Brucella* free farms.

All *Brucella* species are closely related and can be considered as pathovars of a single species (Martirosyan et al., 2011). Thus, it is not astonishing that host specificity of *Brucella* spp. is not 'absolute' but 'relative' (Aparicio, 2013). *B. melitensis* is the most virulent species of all the brucellae and one of the major causes of abortions in small ruminants, and may be infect also other ruminants. Its virulence is partly measured by its capacity to cause brucellosis in cattle and human beings who are not considered as natural or preferred hosts (Blasco and Molina-Flores, 2011). Cross-transmission of *B. melitensis* from small ruminants to cattle has been reported previously (Refai, 2002; Samaha et al., 2008). *B. suis* can infect untypical host as well. Although *B. suis* bv 1–3 are primarily isolated from pigs, bv 3 was isolated from fistulous withers in horses (Cvetnic et al., 2005). Unexpected infection of cows with *B. suis* biovar 2 was reported in Belgium (Fretin et al., 2013) and in Poland (Szulowski et al., 2013), and could play a role in the epidemiology of brucellosis in bovines. Co-habitation, mixed rearing and close contact of different animal species increase the risk of cross species transmission (Richomme et al., 2006). *B. abortus* causes primarily disease in cattle. Cattle are considered the preferential host, but the organism can be transmitted to other mammal as well. Ruminants in general are susceptible to *B. abortus* (Aparicio, 2013). Even though infection with *B. abortus* is rarely reported in small ruminants, it has been reported in

sheep in the USA (Kreeger et al., 2004), in Nigeria (Ocholi et al., 2005; Okoh, 1980) and in Iran (Behroozikhah et al., 2012).

In the present work *B. abortus* DNA was detected in serum samples collected from naturally aborted goats and ewes in an endemic area of Egypt (Wareth et al., 2015b). Accidental *B. abortus* infections in small ruminants may even play an underestimated role for the persistence of brucellosis in cattle (Aparicio, 2013; Fosgate et al., 2011; Gomo et al., 2012). These results highlighted the cross-species infection of *Brucella* to non-preferred hosts raised in close contact and should be taken in consideration during eradication and the vaccination strategies have to be adapted accordingly.

Pathogenesis of *Brucella* in a certain host is depending on the ability of bacteria to invade and replicate within the host cells. *Brucella* is a facultative intracellular bacterium, has marked tropism for the reproductive tract of pregnant hosts. Epithelial cells of the mucosal membrane of digestive, genital and respiratory tracts are the mainly portal of entry for *Brucella* (Poester et al., 2013). The bacteria enter the host cell through interaction with the cell surface lipid rafts, which play significant roles in internalization and intracellular replication of *Brucella* (Watarai et al., 2002). *Brucella* has the ability to survive and replicate within phagocytic and non-phagocytic epithelial cells. The intracellular replication of *Brucella* results in chronic infection and hampers therapy. A comprehensive applications of OMICS (including proteomics, genomics, and transcriptomics) and bioinformatics technologies were used in the past decade to understand the mechanisms of *Brucella* pathogenesis and host immunity (He, 2012). During their intracellular life, brucellae persevere to survive. They appear to express some immunodominant proteins for their survival in the host system during infection (Wareth et al., 2015a). Even though, brucellae display similar genome homogeneity (Wattam et al., 2009), the host specificity and their virulence factors are not clearly described, yet (He, 2012). *B. abortus* and *B. melitensis* appear to express different immunodominant proteins. Some of the identified heat shock proteins, binding proteins and enzymes in this work play a significant role in the rapid turnover of proteins and are associated with cellular metabolism during the infection (Wareth et al., 2015a). However, their contribution to host specificity is not clear.

Diagnosis of brucellosis is based mainly on serology and isolation of brucellae (Alton et al., 1988). Indeed, the current serological assays are based on the detection of anti-*Brucella* lipopolysaccharide (LPS) antibodies. The diagnostic use of LPS antigen from *Brucella* is of low specificity due to cross reactions with other gram negative bacteria e.g. *Yersinia enterocolitica, Salmonella* spp, and *Escherichia coli* O:157 (Al-Dahouk et al., 2006).

Moreover, it does not allow the discrimination of brucellae and is hampering the application of a DIVA approach (Differentiating Infected from Vaccinated Animals). Isolation and identification of the causative agent is still considered to be the gold standard, but has many drawbacks. A perfect antigen having 100% sensitivity and specificity has not been discovered till now and a vaccine which does not interfere with serodiagnosis has not been developed yet (Grillo et al., 2012; Poester et al., 2010). Thus, the identification of immunodominant protein antigens is required for designing serological or diagnostic tools for the accurate diagnosis of brucellosis. A combination of the proteome and immunoproteome using powerful, currently available techniques such as two dimensional electrophoresis (2DE) immunoblotting and mass spectrometric protein identification (MS) would provide a better understanding of the *Brucella* proteome and will speed up the development of better diagnostics tests and promising recombinant vaccines (Zhao et al., 2011). The results presented here open up new possibilities for the serodiagnosis of brucellosis by providing *Brucella* species-specific immunodominant protein candidates reacting only with positive sera collected from naturally infected cattle, buffaloes, sheep and goats. The study provides information on new protein candidates and could help to improve the serological diagnosis of brucellosis.

Brucellae are characterized by great affinity to the pregnant uterus of ruminants. This tropism is enhanced by presence of erythritol in the uterus of pregnant ruminants and its high concentration stimulates bacterial growth (Keppie et al., 1965). Necrotic placentitis with neutrophilic infiltrates is the most microscopic finding that has been seen in brucellosis in addition to the presences of the bacterium inside macrophages and trophoblasts (Xavier et al., 2009). In aborted fetuses, the lesions mainly include fibrinous pleuritis, bronchopneumonia, peritonitis, splenitis and fibrinous pericarditis (Xavier et al., 2009).

The pathogenesis of brucellosis in wildlife and in domestic animals is similar. The similarities encompass both, tropism for reproductive and mammary tissues and histopathological lesions, especially found in the genital tract. However, differences in the disease course are existing due to differences in the immunology and behavior of host species (Rhyan, 2013). *Brucella microti* was originally isolated from a common vole (*Microtus arvalis*) in the Czech Republic in 2000 and had been isolated also from red foxes and soil (Audic et al., 2009; Scholz et al., 2008a, 2008b). Diversity of reservoir species of *B. microti* may also play an important role in the epizootic spread of this bacterium. Virulence of *B. microti* for chicken embryos (CE) was investigated. *B. microti* multiplied rapidly in the chicken embryo and provoked severe gross and histopathological lesions.

The study demonstrated the proliferation in and pathogenicity of *B. microti* for non-mammalian host. CE is a useful diagnostic tool to recover *Brucella* from samples with low numbers of bacteria (Detilleux. et al., 1988; Pulido-Camarillo et al., 2011). Comparatively to other models of infection, CE has several advantages. It provides sterile conditions, is easy to handle and offers different routes of inoculation. Moreover, it is cheap and does not require ethical approval yet. It could be a useful experimental tool to study the pathogenesis, pathogen interaction and immunopathology of brucellae.

Summary of Thesis

Summary

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Molecular Epidemiology of Brucellosis in Egypt, Diagnostic Procedures, Proteomics and Pathogenesis Studies.

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Keywords: Brucellosis, *Brucella,* Molecular Diagnosis, Proteomics, Immunodominant Proteins, MALDI-TOF, Pathogenesis, Model of Infection, Epidemiology, Egypt.

Brucellosis is a zoonotic disease occurring worldwide in animals as well as in humans leading to huge economic losses. The infection is caused by Gram-negative bacteria of the genus *Brucella*. The disease is a very common in developing countries, but is often neglected. In Egypt, brucellosis was reported in a scientific report for the first time in 1939. Since then the disease emerged and remained endemic at high levels among ruminants, particularly in newly established large intensive breeding farms. The disease is prevalence nationwide in all farm animal species, in carrier hosts e.g. rats and in the environment. Serological investigations within the national surveillance program give indirect proof for the presence of brucellosis in cattle, buffaloes, sheep, goats and camels. Even though serologic assays for brucellosis are a well-established procedure but most of the corresponding studies still miss scientific standards. *B. melitensis* bv 3 and *B. abortus* bv 1 are the predominant isolates in Egypt and have been isolated from farm animals and Nile catfish. The epidemiologic situation of brucellosis in Egypt is complicated and needs clarification (Chapter 1).

The disease is characterized by high morbidity but low mortality. However, the disease mainly transmitted via direct contact with infected animals, the most common way of infection is ingestion of contaminated milk or milk products and meat. DNA of *B. melitensis* was detected in milk samples that collected from apparently healthy animals' produces milk for human consumption by molecular assays. The shedding of *Brucella* spp. especially the highly pathogenic species *B. melitensis* in milk poses an increasing threat to consumers and this is of obvious concern (Chapter 2).

In endemic countries like Egypt, transmission of host specific *Brucella* spp. to non-preferred hosts may occur due to the mixed rearing of farm animals. The interspecies transmission of *B. melitensis* from small ruminants to cattle and buffalo was reported. It is worth mentioning that, *B. abortus* DNA was identified in serum samples collected from aborted ewe and goats by real time PCR. This study is the first record on brucellosis caused by *B. abortus* in small ruminants in Egypt. Interestingly that, both *B. abortus* and *B .melitensis*. DNA was detected in one ovine serum. These results should be taken in consideration during implementation of control measures (Chapter 3).

Among the 11 known *Brucella* spp., *B. melitensis* is the most virulent one and is the major causes of abortions in small ruminants. It causes also the severe form of human brucellosis. While, *B. abortus* infectious occurs in cattle preferably among cows. These two species having similar genomes, while are differences in host specificity and display different proteomes. A comprehensive identification of immunodominant proteins of these two species using antibodies present in the serum of naturally infected ruminants provided insight on the mechanism of their infection in different hosts. A number of heat shock proteins, binding proteins, enzymes, and hypothetical proteins were identified using western immunoblotting and MALDI-TOF MS/MS in both *B. abortus* and *B. melitensis*. Brucellae appear to express these proteins mainly for their survival in the host system during infection (Chapter 4).

Diagnosis of brucellosis is still challenging in animals and humans and is based mainly on serology and isolation of *Brucella.* All serological tests have limitations concerning specificity and sensitivity. Cross-reactivity with other Gram-negative bacteria and within the species of the genus is the major hindrance for the specific serological diagnosis of brucellosis. The present study suggest a number of new immunogenic protein candidates of *B. abortus* and *B. melitensis* that had immunoreactivity against only sera collected from cattles, buffaloes, sheep and goats, respectively. Among of them five proteins, (Dihydrodipicolinate synthase, glyceraldehyde-3-phosphate dehydrogenase, lactate malate dehydrogenase, amino acid ABC transporter substrate-binding proteins, and fumarylacetoacetate hydrolase domain-containing protein 2) have prominent immunogenic features. They may be cloned, purified and expressed in recombinant form to be used as specific antigen in serodiagnosis of brucellosis in the future. These proteins can be used to replace the classical LPS antigen preparation in *Brucella* serodiagnosis, will help to specify the causative species and will reduce false positive reactions resulting from cross-reaction with other Gram-negative bacteria (Chapter 5).

Summary of Thesis

Brucellae are intracellular stealthy pathogens causing disease in humans and in a wide range of domestic and wild animals. Rapid multiplication and cytoarchitectural damages induced in liver, kidney, lung, spleen, gastrointestinal tract, spinal meninges, yolk sac and chorioallantoic membrane after egg inoculation of *B. microti* in chicken embryos demonstrated the proliferation and pathogenicity of *B. microti*. This study provides the first results on the multiplication of the mouse pathogenic *B. microti* in chicken embryos and describes gross and histopathology associated with the infection. Our results suggest that, even though chicken are no mammals, they are useful tools to study the pathogenesis, pathogen interactions and immunopathology of brucellae (Chapter 6).

Zusammenfassung

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Molekulare Epidemiologie von Brucellose in Ägypten, Diagnoseverfahren, Proteomics und Untersuchungen zur Pathogenese.

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Stichwort: Brucellose, *Brucella,* Molekulare Diagnostik, Proteomics, Immundominante Proteine, MALDI-TOF, Pathogenese, Infektionsmodell, Epidemiologie, Ägypten.

Brucellose ist eine weltweit vorkommende, zoonotische Erkrankung bei Tieren und Menschen und führt zu großen wirtschaftlichen Verlusten. Die Infektion wird durch Gram-negative Bakterien der Gattung *Brucella* verursacht. In Entwicklungsländern ist die Krankheit sehr häufig, wird aber oft vernachlässigt. In Ägypten wurde Brucellose erstmals 1939 in einem wissenschaftlichen Bericht erwähnt. Seitdem ist die Krankheit als endemisch einzustufen und die Fallzahlen bewegen sich bei Wiederkäuern auf hohem Niveau. Dies ist von besonderer Bedeutung in neu aufgebauten, großen Intensivzuchtbetrieben. Der Erreger kommt landesweit bei den meisten landwirtschaftlichen Nutztieren, in Vektoren wie z.B. Ratten und in der Umwelt vor. Serologische Untersuchungen im Rahmen des nationalen Surveillance-Programms wiesen Brucellose bei Rindern, Büffeln, Schafen, Ziegen und Kamelen nach. Obwohl serologische Assays für Brucellose gut etablierte Verfahren sind, sind die meisten dieser Studien bisher nicht nach den notwendigen wissenschaftlichen Standards durchgeführt worden. *B. melitensis* Biovar (bv) 3 und *B. abortus* bv 1 sind die vorherrschenden Pathovare in Ägypten und wurden aus Nutztieren und Nilwelsen isoliert. Die epidemiologische Situation der Brucellose in Ägypten ist auch auf Grund der vielen Kleinsthaltungen von landwirtschaftlichen Nutztieren schwierig einzuschätzen und bedarf der Klärung (Kapitel 1).

Die Krankheit beim Menschen ist gekennzeichnet durch hohe Morbidität aber geringe Letalität. Die Krankheit wird vor allem über direkten Kontakt mit infizierten Tieren oder den Verzehr von kontaminierter Rohmilch oder Rohmilchprodukten und nicht ausreichend gegartem Fleisch übertragen. DNA von *B. melitensis* wurde in Milchproben, die von

scheinbar gesunden Tieren stammten, mittels molekularbiologischer Testmethoden nachgewiesen. Die Ausscheidung von *Brucella* spp. vor allem der hochpathogenen Art *B. melitensis* in Milch stellt eine wichtige Bedrohung für die Verbraucher dar. (Kapitel 2).

In endemischen Ländern wie Ägypten ist der möglichen Übertragung von wirtsspezifischen *Brucella* spp. auf ansonsten nicht präferierte Wirte durch die häufig gemischte Haltung von Nutztieren besondere Beachtung beizumessen. Beispiele sind die Übertragung von *B. melitensis* von kleinen Wiederkäuern auf Rinder und Büffel. Mittels Real-Time PCR konnte *B. abortus* DNA in Serumproben von Ziegen und Schafen nach Aborten nachgewiesen werden. Damit gelang der erste Nachweis von „Rinderbrucellose" bei kleinen Wiederkäuern in Ägypten. Interessanterweise wurde *B. abortus* und *B. melitensis* DNA in ein und demselben Schafserum nachgewiesen. Die Möglichkeit einer solchen Parallelinfektion sollte bei der Durchführung von Kontrollmaßnahmen berücksichtigt werden (Kapitel 3).

Unter den bisher 11 bekannten *Brucella* spp. ist *B. melitensis* die am höchsten virulente Spezies für den Menschen und gilt als wichtigster Aborterreger bei kleinen Wiederkäuern. Dagegen infiziert *B. abortus* vor allem Rinder und spielt bei Milchkühen eine große Rolle. Diese beiden *Brucella*-Arten haben ähnliche Genome, aber unterschiedliche Proteome und weisen verschiedene Wirtspräferenzen auf. Eine umfassende Identifizierung immundominanter Proteine dieser beiden Bakterienspezies unter Nutzung von Antiseren natürlich infizierter Wiederkäuer gibt einen Einblick in den Infektionsverlauf bei unterschiedlichen Wirten. Eine Reihe von Hitze-Schock-Proteinen, sogenannte binding Proteins, Enzyme und hypothetische Proteine wurden mittels Immunoblotting (Western-Blot) und MALDI-TOF MS/MS bei *B. abortus* und *B. melitensis* identifiziert. Brucellen scheinen diese Proteine während der Infektion für ihr Überleben im Wirtsorganismus zu exprimieren (Kapitel 4).

Die Diagnose der Brucellose bei Tier und Mensch stellt immer noch eine Herausforderung dar und basiert im Wesentlichen auf serologischen Methoden und Erregerisolierung. Alle serologischen Tests haben Einschränkungen hinsichtlich Spezifität und Sensitivität. Die Kreuzreaktivität mit anderen gramnegativen Bakterien und innerhalb der Arten der Gattung *Brucella* stellt ein großes Problem für die serologische Diagnose der Brucellose dar. Die vorliegende Studie beschreibt eine Reihe von immunogen Kandidatenproteinen von *B. abortus* und *B. melitensis*, die eine Immunreaktivität nur gegen seropositiven Proben von Rindern, Büffel, Schafen und Ziegen zeigten. Unter ihnen sind fünf Proteine (Dihydrodipicolinate-Synthase, Glycerinaldehyd-3-phosphat-Dehydrogenase, Laktat-Malat-Dehydrogenase, Aminosäure-ABC-Transporter-Substrat-bindende Proteine und

Fumarylacetoacetate Hydrolase Domäne-haltiges Protein 2), mit wichtigen immunogen Eigenschaften, die kloniert, gereinigt und in rekombinanter Form exprimiert werden könnten, um sie als spezifische Antigene in der serologischen Brucellose-Diagnostik verwenden zu können. Diese Proteine könnten klassische LPS Antigen-Präparationen ersetzen und durch ihre höhere Spezifität falsch positive serologische Reaktionen einschränken (Kapitel 5).

Brucellen sind intrazelluläre Pathogene, die Erkrankungen beim Menschen und bei einer Vielzahl domestizierter und von Wildtieren verursachen. Die experimentelle Inokulation von Hühnerembryonen mit *B. microti* könnte ein *in ovo* Modell sein, um die Interaktion zwischen Erreger und Wirtszellen zu erforschen. Die schnelle Vermehrung des Erregers und pathologische Veränderungen in Leber, Niere, Lunge, Milz, Magen-Darm-Trakt, spinalen Meningen, Dottersack und Chorioallantoismembran nach Inokulation von *B. microti* in embryonierte Hühnereier demonstrierte die Ausbreitung und Pathogenität dieser Spezies in Hühnerembryonen. Die vorliegende Studie beschreibt somit erstmals die Vermehrung von mauspathogenen *B. microti* in Hühnerembryonen und die pathologisch-anatomische und histopathologische Veränderungen, die mit der Infektion assoziiert sind. Unsere Daten legen nahe, dass, obwohl Hühner keine Säugetiere sind und bisher nicht als Wirtstiere für Brucellen galten, das Hühnerembryonenmedell, geeignet ist, Untersuchungen zur Pathogenese, Pathogen-Wirtsinteraktion und Immunpathologie der Brucellen durchzuführen (Kapitel 6).

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List of Publications

A) Publications in peer-reviewed journals:

1. **Wareth, G**., Melzer, F., Weise, C., Neubauer, H., Roesler, U., Murugaiyan, J (2015): Proteomics-based identification of immunodominant proteins of Brucellae using sera from infected hosts points towards enhanced pathogen survival during the infection. Biochem Biophys Res Commun 456 (1): 202 – 206. . http://dx.doi.org/10.1016/j.bbrc.2014.11.059

2. **Wareth, G**., Böttcher, D., Melzer, F., Shehata, A.A., Roesler, U., Neubauer, H., Schoon, H.-A (2015): Experimental infection of chicken embryos with recently described *Brucella microti*: Pathogenicity and pathological findings. Comp Immunol Microbiol Infect Dis. 41. 28–34. http://dx.doi.org/10.1016/j.cimid.2015.06.002

3. **Wareth, G.,** Melzer, F., Tomaso, H., Roesler, U., Neubauer, H (2015): Detection of *Brucella abortus* DNA in aborted goats and sheep in Egypt by real-time PCR. BMC Research notes. 8:212. Doi. 10.1186/s13104-015-1173-1

4. **Wareth, G**., Hikal, A., Refai, M., Melzer, F., Roesler, U., Neubauer, H (2014): Animal brucellosis in Egypt. J Infect Dev Ctries 8(11):1365-73. Doi:10.3855/jidc.4872

5. **Wareth G**, Eravci, M., Melzer F, Weise C, Roesler U, Sprague LD, Neubauer, H., Murugaiyan, J (2015): Identification of immunodominant proteins using fully virulent *Brucella abortus* and *Brucella melitensis* field strains and circulating antibodies in the naturally infected host. PLOS Negl Trop Dis (revised manuscript).

6. **Wareth, G**., Melzer, F., Elschner, M. C., Neubauer, H., Roesler, U (2014): 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(10):1339-43. Doi: 10.3855/jidc.4847.

7. **Wareth, G**., Melzer, F., Weise, C., Neubauer, H., Roesler, U., Murugaiyan, J (2015): Mass spectrometry data from proteomics-based screening of immunoreactive proteins of fully virulent *Brucella* strains using sera from naturally infected animals. Data in Brief 4: 587-590. http://dx.doi.org/10.1016/j.dib.2015.07.029

8. **Wareth G**, Melzer F, Böttcher D, El-Diasty M, El-Beskawy M, et al. (2015): Molecular typing of isolates obtained from aborted foetuses in an Egyptian *Brucella*-free Holstein dairy cattle herd after immunisation with *Brucella abortus* RB51 vaccine. BMC Microbiology (Under review).

9. **Wareth G**, Murugaiyan J, Khater DF, Moustafa SA (2014): Subclinical pulmonary pathogenic infection in camels slaughtered in Cairo, Egypt. J Infect Dev Ctries 8: 909-913. Doi: 10.3855/jidc.4810.

10. **Wareth G**. and Moustafa SA (2013): Pulmonary Leiomyoma in a Dromedary Camel (*Camelus Dromedarius*), International Journal of Veterinary Medicine: Research &Reports, Vol. 2013 (2013). Article ID 773813, DOI: 10.5171/2013.773818.

Publication 1, 2, 3, 4, 5 and 6 have been produced in my doctoral thesis

B) Publications in academic conferences:

1. **Wareth G**., Murugaiyan J., Weise C., Melzer F., Elschner M., Neubauer H and Roesler U. 2014. Identification of novel proteins from *Brucella abortus*. Brucellosis 2014 International Research Conference Including the 67th Annual Brucellosis Research Meeting 9 – 12 September 2014., p46. Berlin, Germany (Poster).

2. **Wareth G.,** Elschner M., Neubauer H., Roesler U and Melzer F. 2014. A retrospective analysis of animal brucellosis research in Egypt in the last 25 years. Brucellosis 2014 International Research Conference Including the 67th Annual Brucellosis Research Meeting 9 – 12 September 2014., p202. Berlin, Germany (Poster).

3. **Wareth G**., Murugaiyan J., Weise C., Melzer F., Elschner M., Neubauer H and Roesler U. 2014. Specific immunogenic proteins of *B. melitensis* for the serodiagnosis of small ruminant brucellosis. Junior Scientist Symposium FLI 2014. 19th – 22nd of August 2014 in Mariensee, Germany (Oral Presentation).

4. **Wareth G.,** Böttcher D., Elschner M., Shehata A., Schoon H., Roesler U., Neubauer H., and Melzer F. 2014. Chicken Embryo as a Model of Infection in Brucellosis. German Symposium on Zoonoses Research 2014 and 7th International Conference on Emerging Zoonoses, 16-17 October, Berlin (Poster).

5. Ali, S., Melzer, F., Khan, I., Ali, Q., Abatih, E.N., Ullah, N., Irfan, M., **Wareth, G.,** Akhter, S., Neubauer, H., 2012. Seroprevalence of brucellosis in high risk professionals in Pakistan. Tagung der DVG-Fachgruppe "Bakteriologie und Mykologie", Leipzig, Germany; 06/2012 (Poster).

6. **Wareth G.,** Melzer F., Roesler U; Sprague LD and Neubauer H., 2015. Investigation and molecular typing of *Brucella* isolated from dairy cattle herd immunized with *Brucella abortus* RB51 vaccine in Egypt. 3rd international congress of bacteriology and infectious disease, August 4-6, Valencia, Spain (Oral Presentation).

C) ProteomeXchange Dataset

1. **Wareth, G.,** Melzer, F., Weise, C., Neubauer, H., Roesler, U., Murugaiyan, J. 2014. Identification of immunodominant antigen among *Brucella* species (*B. abortus* and *B. melitensis*) using hyper immune serum from naturally infected hosts**.** DOI http://dx.doi.org/10.6019/PXD001270.

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Selbständigkeitserklärung:

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

Berlin, den 03.09.2015 Gamal Wareth Abdelaziz Mohamed