

Brucellosis in humans: why is it so elusive?

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Brucella spp. are small, slow-growing, Gram-negative coccobacilli that are responsible for brucellosis, the most common zoonotic disease worldwide. Brucellosis is a notifiable disease in most countries. Brucellosis is also considered as an occupational, laboratory and travel-acquired disease. *Brucella* spp. are transmitted through consumption of raw animal products (food-borne brucellosis) and animal contact. They may be spread through droplets in the air; they are traditionally classified as a class B bioterrorism agent. Brucellosis incidents have been reported in relation to domestic animals. *Brucella* strains have been isolated from terrestrial and marine mammals. *Brucella melitensis*, *B. suis* and *B. abortus* differ in host range, pathogenicity and virulence. *B. canis* infections have rarely been reported. Current global trends on the incidence of brucellosis are reviewed. Brucellosis is often overlooked and can mimic other conditions; it may be acute, subacute or chronic in presentation, and may involve various body sites. Recent studies on endocarditis, osteoarticular, haematological, neurological and other involvements are reviewed. Relapses in brucellosis should also be considered. Collaboration between the microbiologist and the clinician is important for diagnosis, since diagnosis of brucellosis is based on laboratory testing by serology and, ultimately by culture, in the context of clinical presentation and history of recent or past exposure. Advanced PCR-based techniques may also be used to diagnose brucellosis. Today a combination of antibiotics are recommended in treatment, whereas further therapeutic approaches are possible. New challenges posed by international travel, animal trade, animal movement, and occupational migration to/from endemic countries may increase incidence of human cases and the risk of re-emergence of brucellosis in previously brucellosis free regions such as Northern and Central Europe. Animals are considered to be lifelong carriers of *Brucella* spp. providing a large continuous source of human infection. A lower incidence of human brucellosis is likely to result from a decrease in incidence of animal brucellosis. Control and surveillance strategies may depend on the level of healthcare development and the prevalence of reservoir hosts in the affected region.

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Introduction

Human brucellosis is a predominantly zoonotic disease that is notifiable in many countries [1]. One may also consider it to be an occupational, food-borne, travel or

laboratory-acquired illness. It is an infectious disease, (International Classification of Diseases) ICD-9 023 or ICD-10 A23, that carries high morbidity but low mortality, the latter being usually attributed to endocarditis. Undulant fever is the main symptom in the acute

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phase. Chronicity with or without focal complications, that is noncaseating granulomata, somewhat reminiscent of tuberculosis, may also develop. The causative agents of human brucellosis are small, nonmotile, catalase and urease-positive, slow-growing Gram-negative coccobacilli of the genus *Brucella*. Despite its long incubation period, its high virulence and contagiousness recognizes *Brucella* as a bioweapon and class B bioterrorism agent [2].

Evolution and classification of *Brucella*

Brucella belongs to the family Brucellaceae within the class alpha-proteobacteria. *Brucella* 16S rRNA displays a phylogenetic relationship with other members such as *Ochrobactrum*, *Agrobacterium*, *Rhizobium*, as well as *Bartonella*, and *Rickettsia*. More recent studies corroborate a prior hypothesis that the *Brucella* ancestor, a probable soil/plant arthropod-associated alpha-protobacterium, may have had structural features already representing a first step towards innate immune evasion [3]. On the basis of host preference, antigenic and phenotypic characteristics, six classical nomenclatures (with their biovars) were designated and recently reappraised, irrespective of taxonomic considerations [4]. *Brucella melitensis* (biovars 1–3, found in goats, sheep and camels) is the most virulent to humans, followed by *B. abortus* (biovars 1–6 and 9, found in cattle, camels, cervids, yaks and buffalo) and *B. suis* (biovars 1–3, found in swine; biovar 4, found in reindeer and caribou; biovar 5, found in wild rodents) [5,6]. *B. canis* (found in dogs) and *B. suis* biovar 2 (found in brown hares) may cause human infections sporadically, whereas *B. ovis* (found in sheep) and *B. neotomae* (found in desert rats) are nonpathogenic to humans. Only recently, three novel species have been identified: one terrestrial species, *B. microti*, recognized in voles, and two species, *B. ceti* and *B. pinnipedialis*, isolated from marine mammals [7,8]. Additional species or subspecies within marine *Brucella* isolates were proposed more recently [8] and it is tempting to speculate that they might differ in pathogenicity. Also, novel *Brucella* strains have been isolated from human and nonhuman primates [9,10]. Still, the differences in host range, pathogenicity and virulence of brucellae remain unclear. Recent mapping of the genome of zoonotic *B. melitensis*, *B. abortus* and *B. suis*, and nonzoonotic *B. ovis* [11], may provide a better understanding of host–pathogen interactions.

Epidemiology

Reservoirs and transmission

Brucella can survive in groundwater. Further corroboration of *Brucella* survival/persistence in nature is provided by studies showing trans-stadial or stage-to-stage (the passage from larva to nymph to adult) and transovarial (the passage via eggs from parent to offspring) infection of *B.*

melitensis in ticks [12], although the reported role of arthropods in the epidemiology of human brucellosis may be small [13]. Terrestrial domestic animals such as cattle, small ruminants, camels, and so on can serve as *Brucella* reservoirs, whereas terrestrial and marine animals in the wild may also play a role. *Brucella* is transmitted from animals to humans, although other modes such as rare human-to-human transmission or other means cannot be excluded. Workers and professionals, that is farmers, shepherds, slaughterhouse workers, butchers, marine animal workers and veterinarians may be infected through skin cuts and conjunctiva. Animal contact still remains the predominant transmission mode in rural and nomadic populations, practising traditional husbandry [1]. In recent years, however, there has been a shift towards food-borne brucellosis transmitted by raw animal product consumption in urban populations, practising modern animal husbandry in a settled environment [14]. In endemic zones, outbreaks occur when guidelines for processing milk products are not followed, as in a very recent outbreak reported in Mexico from March 2009 onwards due to fresh cheese consumption [15]. In nonendemic areas, however, outbreaks can occur when raw milk products are imported from an endemic area [16], whereas cases of infected returnees from an endemic area are occasionally reported. Apart from travellers, military personnel are at risk of being exposed to *Brucella* when deployed in endemic countries [2]. Even at a low infective dose of 10–100 microorganisms, airborne transmission may support the use of *Brucella* as a biological weapon [2]. Inhalation of particles, accidental inoculation and ingestion or direct contact may be responsible for the potential hazard of *Brucella* to laboratory personnel.

Worldwide incidence and current trends

At least half a million new cases of brucellosis annually are estimated by the World Health Organization (WHO) to occur globally. In developed countries, declared animal brucellosis free, a few human cases may be imported or laboratory acquired. Such countries include fourteen Northern and Western European countries, Great Britain, Australia, New Zealand, Japan, and Canada. Texas was the last state in the United States to be declared free in 2008, whereas Eire is now officially free of bovine brucellosis [15,17]. In comparison to a previous review on the situation in the Mediterranean, once considered to be a region with high incidence rates, a progressive improvement has taken place between 2006 and 2008 [17]. Incidence of brucellosis in Portugal, Spain and Italy has now dropped below 10 per million of population and has, respectively, reached 5.3, 3.0 and less than 2.0 per million of population in 2008 [18]. Although most of the Greek islands can perhaps be considered brucellosis-free ([15], Vassalos, personal communication), mainland Greece has still remained an area with high brucellosis incidence rates. Data from Greece and other countries, which reported high human brucellosis incidence over the period 2006–2008, are presented in Table 1

Table 1. Countries^a that reported high human brucellosis incidence rate per million of population from 2006 to 2008.^b

	2006	2007	2008		2006	2007	2008
Europe				Middle East			
Greece	26.6	14.3	31.8	Afghanistan		100.7	24.9
Western Balkans				Iran [18,20]		308.6	255.8
Albania	246.7	261.6	221.7	Iraq		37.2	55.8
Bosnia and Herzegovina [15,18]	37.8 (R)		300 (E)	Israel	18.6	24.6	19.4
Former Yug. Rep. of Macedonia	151.0	185.8	235.2	Jordan	22.9	37.7	19.3
Caucasus				Kuwait	14.9		
Armenia			95.8	Lebanon			41.0
Azerbaijan	54.9	21.4	18.8	Oman	22.9	29.3	
Georgia	0.6	32.5	34.2	Saudi Arabia [21]	90 (P)		
Central Asia				Syria		2159.0	1372.2
Kazakhstan		150.0	169.7	Turkey	154.9	169.4	140.9
Kyrgyzstan		784.0		North Africa			
Tajikistan	206.0	107.9		Algeria	258.3	233.9	162.6
Turkmenistan	37.6 (E)			Tunisia	45.7	51.0	28.3
Uzbekistan	17.1 (E)	13.8 (E)	15 (E)	Sub-Saharan Africa			
Eastern Asia				Kenya		1.2	135.5
China	14.6	15.1		Americas			
Mongolia [19]	209.7 (R)	150.8 (R)		Mexico	17.3	19.3	20.3
				Peru	14.5		

E, estimated; P, referred to eastern part of Saudi Arabia; R, registered cases.

^aCountry names as currently used by the Office International des Epizooties (OIE) [18].

^bData were provided from the OIE [18]; the other sources, for example, Program for Monitoring Emerging Diseases [15], National Statistic Office of Mongolia [19], MehrNews Agency [20] and medical study in J Clin Infect Public Health [21] were determined.

[15,18–21] and compared to those previously reviewed [22]. The disease has most recently re-emerged in Bulgaria [15]. From our results, Western Balkans, Caucasus, Central Asia, including Southwestern Siberia and hyperendemic Kyrgyzstan, and Mongolia should currently be considered the most important foci of brucellosis. In China brucellosis spread has continued and a small number of deaths have been reported, whereas for Southeast Asia and the Indian subcontinent no recent data are available, despite a resurgence of brucellosis being reported in Thailand and the notion that India should be considered a potential endemic region [15,23]. In the Middle East, whereas high rates have been precisely reported in Afghanistan and Iraq, Syria and Iran should still be considered the major foci of brucellosis in the region. Although the situation is causing alarm in Syria, a 1.5-fold decrease in brucellosis incidence has occurred and things appear to get slightly better in Iran [18,20,22]. The situation seems to be unchanged in the Arabian peninsula. In North Africa, an increase in incidence has occurred, whereas hyperendemic microfoci of brucellosis were first reported in Egypt [24]. Between 2006 and 2008, six sub-Saharan African countries namely Ivory Coast, Mozambique, Rwanda, South Africa and Sudan, have been added to those with a history of brucellosis. In South and Central America the situation has been unchanged compared to the previously reviewed period [22]. However, for the first time Brazil has reported the existence of human brucellosis [18]. In North America, *B. melitensis* infection has been reported to affect the Hispanic population in the areas of the United States neighbouring Mexico [22]. Hence, the slight decrease in the high incidence, noted in Mexico during the period

reviewed here, might be considered a small improvement in this region, despite the aforementioned outbreak [15,22]. Nevertheless, it was suggested that incidence rate may poorly estimate the brucellosis burden in a region, since the disease could affect more persons [25]. Data concerning brucellosis in Greece from 1997 to 2005 [26], which are presented in Fig. 1, demonstrate that the trend of new cases appears to correspond to the trend of cases discharged from hospital, with the latter reflecting perhaps the wide range of manifestations and complications of brucellosis, the chronicity or relapses of this disease. Taken together, trends of the incidence of brucellosis may reflect, if not accurately represent, the

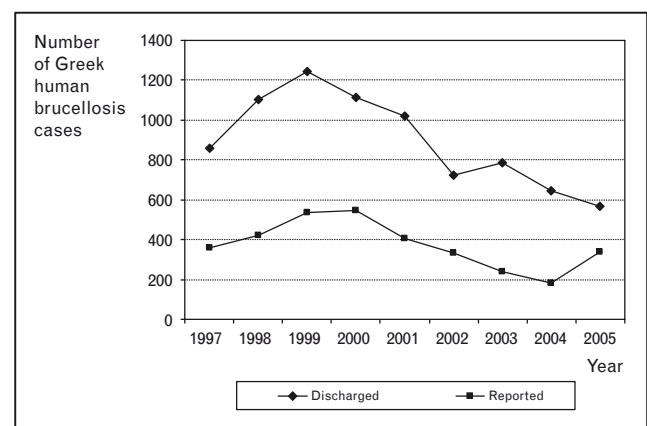


Fig. 1. Trends in human brucellosis cases, reported and discharged from hospital, in Greece from 1997 to 2005. Data were extracted from tables provided from the National Statistical Service of Greece [26].

situation of the disease in a region. In the developing endemic countries, under-reporting affects accurate estimation of the incidence of brucellosis. In the Western Balkans, Caucasus, Central Asia, Middle East and Africa, worsening socioeconomic and administrative status or political turmoil seems to be responsible for high brucellosis rates. Similar to the pattern of other emerging or re-emerging zoonoses [27], transboundary animal trafficking or trade and occupational immigration are more likely to be incriminated in the spread of the disease from neighbouring endemic countries, as in the case of northern [22] and central Greece (Vassalos, personal communication), Bulgaria, Thailand, or Southern United States [15,22].

Pathogenesis and immune response

Intracellular survival of *Brucella*

Brucellae are facultative intracellular bacteria and can become secluded within the endoplasmic reticulum of cells and thereby avoid lysosome fusion. By controlling the maturation of the brucellosome (*Brucella*-containing vacuole) at the onset of infection, unopsonized brucella can enter, survive and replicate in a variety of cells, including dendritic cells and macrophages [5], to evade the host innate immune response before activation of anti-*Brucella* mechanisms by adaptive immunity.

Role of virulence factors

Virulence in *Brucella* should generally be considered as an evolutionary adaptation to an intracellular lifestyle, since classical virulence factors (i.e. endotoxins, fimbriae and flagella, plasmids), apoptosis inducers, pathogenic islands and complete types I, II, and III secretion systems have not been identified [5]. Recent studies using urease-producing strains showed that urease can protect orally acquired brucellae during transit through the stomach [23]. Also, brucella can release adenine and guanine monophosphates that inhibit myeloperoxidase–peroxidase and consequently phagolysosome fusion. Internalization of brucellae into host macrophages may be facilitated by bacterial binding to phagosomal membrane lipid rafts. Cyclic glucan, which is encoded by the cyclic glucan synthetase (*gcs*) gene and able to extract the main constituent of lipid rafts (cholesterol) from cell membrane, may interfere with maturation of the *Brucella* vacuole by disrupting lipid rafts, thus preventing the brucellosome from fusion with lysosomes. This suggestion needs to be further elucidated [28]. Lipopolysaccharide (LPS) is regarded as an essential component of the pathogenic brucellae; it comprises lipid A, a subtly modified core, and an O-polysaccharide chain. Owing to its noncanonical structure, LPS virulence characteristics may contribute to brucella's aforementioned intracellular survival strategy [29]. LPS endotoxicity appears to be less than that of other bacteria; host-recognizable

pathogen-associated LPS molecular patterns are reduced. Interaction with Toll-like receptors (TLRs) has little impact on cytokine production, a native hapten polymer is incorporated, the absence of core uronic acid is responsible for brucella's resistance to cell cationic peptides. Once brucella is internalized LPS alters the infected cell's capacity to present the major histocompatibility complex (MHC) class II Ag (antigen) to specific CD4⁺ (cluster of differentiation 4⁺) cells [30]. Furthermore, LPS is bound to 'smooth' brucellae (versus rough morphology) in culture. The LPS O-chain seems to protect virulent brucellae from oxygen metabolites and complement-mediated lysis and prevent the synthesis of immune mediators. Smooth *Brucella* LPS (S-LPS) has an O-chain, whereas rough-strain LPS (R-LPS) do not contain an O-chain. Perhaps S-LPS may be involved in inhibition of apoptosis, whereas R-LPS strains do not appear to confer host cell resistance to apoptosis [28]. Evasion from host immune responses does not occur in attenuated, rough strains. Smooth *Brucella* strains with mutations in the phosphoglucomutase gene, which is involved in O-chain synthesis, showed attenuation of virulence [28]. More recently, the loss of genomic island-2 was found to alter virulent brucellae with S-LPS, leading to an attenuated, rough phenotype and a potential for altering the TLR signal [31]. As regards differences in virulence of *Brucella* species, cell penetration of smooth *B. suis* was shown to depend on lipid rafts and activate impairment of phagolysosome fusion during the first hours of infection, whereas rough (mutant) *B. abortus* could not enter using lipid rafts [30]. Also, rough *B. abortus* could be killed by macrophages, whereas rough *B. melitensis* were found able to replicate inside the host cells [32]. *B. melitensis* showed more resistance to intracellular killing by human neutrophils than *B. abortus* [32]. The brucella BvrR/BvrS two-component regulatory system seems more likely to be implicated in outer membrane structural homeostasis necessary for brucella's resistance to cidal cationic peptides, binding and cell invasion following escape from the lysosomes [5]. These genes were found to encode outer membrane proteins, Omp25 and Omp22, as well as gene products that modify LPS lipid A composition. The *bvR* and *bvS* mutants were found to be less invasive than the wild-type strain [28]. More recently, a possible role for BvrS/BvrR control over the expression of Omp25, suggested to be a down-regulator of tumour necrosis factor (TNF)-alpha, has been suggested to limit dendritic cell maturation [33]. Although its role in cell adherence and entry remains poorly understood, the brucella VirB type IV secretion system, encoded by the *virB* operon, may have a role in modulation of intracellular trafficking, survival and replication of virulent brucellae. To date, no putative effectors, controlling the fate of the brucellosome, have been identified in brucella-infected cells [33]. It has been hypothesized, however, that brucella VirB apparatus might secrete specific effector molecules into the host cell in an attempt to sustain interactions and fusion with the

endoplasmic reticulum necessary for late brucellosome maturation events, neutralize the pH (acidification is a VirB expression inducer) and allow the virulent strains to undergo cell division within their safe endoplasmic reticulum-derived replication niche [28]. Despite initial spreading inside the host, the *virB* mutants are more likely to be attenuated; however, *virB* mutant replication can possibly be rescued when simultaneous infection with wild-type strains occurs. More recently, quorum-sensing regulators have been considered to modulate brucella's virulence factors, such as the VirB system [34]. Perhaps the type IV secretion system may have some role to play in mounting innate immune responses. Differences in early T-helper (Th) 1 activation were most recently observed among the wild-type and the *virB* mutant strains. Of note, the VirB-dependent stress protein, hsp60, a chaperonin expressed on the surface of virulent brucellae but not on *virB* mutants, seems to play a part in cell adherence by binding to the normal cellular isoform of prion protein, PrP^C, a glycosylphosphatidylinositol-anchored protein attached to cell membranes [35]. PrP^C associates with lipid rafts and appears to serve as a receptor for brucellae promoting their internalization into macrophages [35]. The first step of phosphatidylethanolamine synthesis is controlled by the phosphatidylserine synthase (*ppsA*) gene. In *ppsA* mutant, the loss of phosphatidylethanolamine was shown to result in impaired intracellular survival and brucellosome maturation [36]. Phosphatidylcholine, a typical eukaryotic phospholipid, is regarded as the major phospholipid in the outer membrane of *Brucella* (and other alpha-proteobacteria) so that the host fails to recognize the bacteria in case they interact with eukaryotes [28]. When phosphatidylcholine synthase (Pcs) is mutated, attenuated *pcs* mutants display a reduced ability to avoid fusion with lysosomes. Nevertheless, the role of lipid composition in *Brucella* remains elusive. According to a more recent hypothesis, there may be a link between membrane lipid composition and the type IV secretion system [36].

Host immune response

The host response against *Brucella* involves both innate and adaptive immunity. In the early stages after entry, there is a lack of any proinflammatory response with little activation of complement due to poor binding of S-LPS. A reduced neutrophilicidal activity with inhibition of myeloperoxidase-induced peroxide has been reported hampering neutrophil degranulation and phagolysosome fusion while superoxide dismutase is produced, blocking free radical formation. In the case of acute brucellosis, natural killer cell activity and cytotoxic function and macrophage generation of reactive oxygen metabolites were found to be impaired [5]. Knowledge of the role of TLR in the immune response to *Brucella* infection is far from complete [37]. More recently, down-regulation of maturation of dendritic cells and proinflammatory cytokine secretion was described [33]. In humans, elimination of virulent *Brucella* seems to depend upon activated macrophages, requiring principally the Th1

cytokine, interferon (IFN)-gamma. There does not appear to be a negative regulatory effect on T cell activation through T cell receptor (TCR) signalling in human brucellosis, unlike tuberculosis. CD4⁺ cells may have a limited role, whereas numbers of lymphocytes bearing the gamma delta TCR were increased in brucellosis [5]. Only recently have type 1 natural killer cells with anti-*Brucella* activity through the Fas pathway been described. A recent study indicates that TNF-alpha-308 polymorphism may contribute to susceptibility to *Brucella* infection, but yet the role of TNF-alpha in human brucellosis has not been clearly elucidated [38]. IFN-gamma is considered to play a key role in brucellosis pathogenesis and levels of serum IFN-gamma and interleukin (IL)-12 were increased. However, impairment of IFN regulator was also noted. Persons who are homozygous for the IFN-gamma +874A allele were found to be more prone to brucellosis [5]. Recent results have shown that C alleles controlling Th2 cytokine IL-10 gene polymorphism could possibly affect IL-10 inhibition of macrophage activation in healthy individuals [39]. A more recent study from Spain suggests that transforming growth factor-beta gene polymorphism may be involved in susceptibility to brucellosis and in developing focal disease [40]. The host response to high local brucella concentrations, intact during the early stages of infection but then destroyed by mononuclear cells appears to be formation of a granuloma similar to tuberculosis. However, neutrophil activation was recently shown in localized tissue injury and inflammation, whereas a proinflammatory response, whether direct or indirect, was observed in osteoarticular complication and chronic inflammation [41]. In chronic brucellosis, decreased Th1 cytokine production can contribute to T cell unresponsiveness [42]. Brucellae should be considered persistent bacteria as they can display long-term survival inside host endoplasmic reticulum cells [43]. As there is no lifelong immunity after infection, recurrent or chronic brucellosis may result, particularly if there is dysregulation of the balance between *B. melitensis* pathogenicity and the host immune system [43]. Although humoral antibody responses to *Brucella* antigens have been proven very useful in diagnostic tests, their role in the host response is still being investigated. Results from studies using volunteers who had been vaccinated with the Rev 1 vaccine against *B. melitensis* indicated that immunoglobulins (Ig) M appeared during the first week of infection, followed by IgG as early as the second week; however, IgM and IgG reached peak levels during the fourth week and persisted for nearly a year, whereas IgG and IgA lasted for longer than 6 months in the case of chronic brucellosis [5]. Opsonization with IgG-specific antibodies would be considered another option for virulent brucellae to enter the host cell and survive within phagosomes that remain in the endosomal pathway, whereas survival and replication of virulent strains within these vesicles would most likely play an important role in maintaining chronic infections [29].

Clinical profile

As the clinical presentation of brucellosis may mimic that of many infectious and noninfectious conditions [23], the disease is often misdiagnosed. The most common symptom of brucellosis is fever, whether continuous, intermittent or irregular. The temperature can vary from normal in the morning to 40°C in the afternoon. Symptoms such as chills, night sweating, arthralgia, malaise, weakness, anorexia, weight loss, and so on, and signs such as enlarged lymph nodes, hepato-megaly and spleno-megaly, are also seen [28]. Uncomplicated, acute brucellosis, presenting with fever, chills and profuse sweating, should be differentiated from other febrile illnesses, especially in endemic areas [23]. A relapse, that is reappearance of fever and other symptoms, may occur after completion of treatment. In the case of *B. abortus* infection, symptoms may be subacute. In accidental infection caused by attenuated vaccine strains, symptoms may be benign in contrast to those caused by the wild *Brucella* strains. Brucellosis can last from a few weeks

to several years [44]. A patient with chronic brucellosis may present with mild symptomatology (persisting >1 year)/localized forms, low brucellaemia, and a history of past exposure to *Brucella*. A complicated, focal brucellosis presentation may vary depending on the specific site of infection. Findings from brucellar complication reports published from 2007 onwards are presented in Table 2 and are generally consistent with those in previous reviews [5,23,28] and those of most recent studies [43,45–53]. Musculoskeletal involvement was the most common complication. It is worthwhile noting that life-threatening brucellar endocarditis with valvular vegetations was ranked second, whereas haematological findings were frequently presented. In case of neurobrucellosis, a range of neurological findings were also reported. Complications in other target organs were relatively rare. Only two cases of noncaseating granulomatous infiltrations were reported. Among the cases reviewed, multiple organ or system involvement was a rather common complication of brucellosis.

Table 2. Reported clinical complications (mid-2007–2009).^a

Common brucellar complications	No.	(%)	Rare brucellar complications	No.	(%)
Osteoarticular/soft tissue	19	(30.2)	Respiratory	3	(4.7)
Spondylitis	4		Exudative pharyngitis	2	
Spondylodiscitis	1		Brucellosis of lung	1	
Sacroiliitis	2		Gastrointestinal	3	(4.7)
Chronic osteomyelitis	1		Infective colitis	1	
Sacroiliitis/osteomyelitis	1		Primary peritonitis	1	
Spinal epidural granuloma	1		Secondary peritonitis ^c	1	
Vertebral fracture	1		Renal	3	(4.7)
Arthritis of knee joint	2		Renal failure	1	
<i>Brucella</i> infection in total knee arthroplasty	1		Interstitial nephritis	1	
Iliac abscess/olecranon bursitis/sacroiliitis	1		Glomerulonephritis	1	
Psoas abscess	1		Cutaneous/subcutaneous	3	(4.7)
Gluteal abscess	1		Maculopapular rash	1	
Obturator abscess	1		Cellulitis	1	
Acute myositis	1		Panniculitis	1	
Endocarditis/vascular	10	(15.9)	Ocular	1	(1.6)
Endocarditis	5		Uveitis	1	
Atrial septal defect	1		≥2 organ/system involvement		
Mitral valve endocarditis	1		Reported	6	(9.5)
Prosthetic valve endocarditis	1		Peripheral neuropathy/macular rash	1	
Myocardial infarction	1		Peripheral neuropathy/interstitial nephritis	1	
Infected abdominal aortic aneurysm	1		Endocarditis/splenic abscess	1	
Haematological	6	(9.5)	Endocarditis/sacroiliitis/pyelonephritis/thyroiditis	1	
Pancytopenia/capillary leak syndrome	1		Lung and liver nodules	1	
Thrombocytopenia	1		Pancytopenia/liver abscess	1	
Haemolytic anaemia	2		Total	63	(100.0)
Cryoglobulinaemia	1				
Myelofibrosis	1				
Neurological	5	(7.9)			
Central aneurysms/subarachnoidal haemorrhage	1				
Intracranial hypertension	1				
Thalamic infarction	1				
Meningitis	1				
Paraparesis	1				
Genitourinary	4	(6.6)			
Orchitis	1				
Epididymoorchitis	3				

^aSearch strategy: English literature published from mid-2007 to mid-2009 was searched in MEDLINE; key words such as brucell, brucellosi, clinical, complicat, case were used.

Diagnosis of infection

Several serological tests, conventional and commercial, have been developed for brucellosis screening and serodiagnosis for over a century (Table 3). Cut-off titres differ according to the local epidemiology. In endemic areas, threshold titres are considered to be high ($\geq 1:320$); thus, agglutination tests may lack sensitivity. In non-endemic countries, clinical data and complete history (including recent or past exposures) are indispensable prerequisites for diagnosis, especially in the case of travel-acquired infections or exposed military personnel previously deployed in endemic zones [2]. In the United Kingdom – a nonendemic country – the combination of in-house and commercial immunoassays was recently found to increase the chance of obtaining a positive serodiagnosis [54]. In countries such as Spain where incidence of the disease is progressively decreasing, recent human exposure should be distinguished from past exposure to *Brucella* (Table 3). From our experience in an area with relatively stable incidence of *B. melitensis* infection, serological results should be interpreted in the context of clinical presentation and history. Conventional and commercial tests could be used for screening and

serodiagnosis of active brucellosis, providing positive titres of at least 1:160, whereas enzyme-linked immunosorbent assay (ELISA) could be used among others to detect relapsing disease or diagnose chronic brucellosis ([55–57], Table 3). Despite its low diagnostic yield, culture should also be performed; it is the ultimate diagnosis, particularly if the humoral immune response is poor ([58], Vassalos, unpublished data). *Brucella* can be recovered in culture of blood or nonblood samples in 7–21 days on average, whereas bone marrow culture is considered the gold standard. The biphasic Ruiz-Castañeda system is preferred. To gain a saving in time, however, advanced radiometric and nonradiometric methods or lysis centrifugation technique have replaced traditional culture methods. Using culture-based biotyping, identification of *Brucella* to species level is traditionally carried out on the basis of antigenic, phenotypic and phage susceptibility profiles [5]. However, collaboration between the clinician and laboratory staff should be encouraged so that suspected brucellosis could be reported to laboratories and laboratory tests could be ordered and interpreted appropriately. It remains to be seen to what extent the polymerase chain reaction (PCR) might possibly be used as a routine tool for

Table 3. Laboratory testing in brucellosis diagnosis.

Screening	Test	Interpretation
First screening	RBT Dipstick test LFA ^a	Rapid detection of agglutinating Abs Rapid detection of IgM specific to <i>Brucella</i> spp. Rapid detection of IgM/IgG
Confirmation of first screening	SAT	Agglutinating Ab detection
Presumptive diagnosis	Test	Interpretation
Active infection	RBT/SAT ^b and	Agglutinating Ab detection
Acute phase	Immunocapture agglutination test ^c	Total Ab detection
Recent versus past exposure	Dipstick test	Rapid detection IgM specific to <i>Brucella</i> spp.
Treatment monitor	ELISA IgM	High titres
Chronicity	ELISA: paired samples	Higher titres in secondary sampling
Relapse	ELISA IgG avidity ^d	Low avidity in recent exposure, high avidity in past exposure
Active versus inactive infection	ELISA ELISA IgG, IgA Immunocapture agglutination test ^c ELISA IgG, IgA ELISA against cytosolic proteins	Decrease in Igs titres Elevated IgG and IgA titres ^e Total Ab detection (high titres) IgG and IgA titre resurge High titres in active infection
Definite diagnosis	Method	Interpretation
<i>Brucella</i> infection	Culture	Positive blood culture x2 or Positive bone marrow culture x1
Seronegativity	Culture	Positive blood culture x2 or Positive bone marrow culture x1
Inactive infection	PCR ^f	<i>Brucella</i> DNA load presence
Species identification	PCR ^f Method Culture-based biotyping PCR ^f	<i>Brucella</i> DNA load presence

Ab, antibody; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LFA, immunochromatographic IgM/IgG lateral flow assay; PCR, polymerase chain reaction; RBT, Rose Bengal test; SAT, serum agglutination test.

^aAmong the bedside tests, LFA has only recently been first evaluated for potential use in endemic areas, whereas fluorescent polarization immunoassay is under investigation.

^bSAT titres decrease when turning into chronic phase.

^cAlternative to Coombs' antihuman globulin test.

^dUnder investigation.

^eIgM low.

^fNonvalidated data.

brucellosis diagnosis, especially in endemic regions. For amplification, insertion sequence (IS)711, unique to *Brucella*, 16S–23S rRNA interspace region, *beps31* gene, encoding a 31-kDa immunogenic Omp, and genes of *omp2* locus have been targeted [28]. PCR is considered a fast, convenient method more sensitive than culture or serology [28]. Using serum samples, a guanidine hydrochloride method was most recently shown capable of isolating *Brucella* DNA [59], whereas a probe-based real-time PCR may be used to diagnose brucellosis infection [60]. Although a rapid and simple PCR–enzyme immunoassay was proven to be sensitive and specific for the direct diagnosis of acute human brucellosis [61], quantitative real-time PCR (Q-PCR) assay was found to facilitate initial diagnosis and discrimination between active human brucellosis and past infection [62]. Also, Q-PCR could be used to monitor *Brucella* DNA load throughout treatment and post-treatment follow-up, as in asymptomatic patients with the presence of IgG and IgA and microbiologically undetectable brucellaemia ([43], Table 3). Post-treatment PCR was also found to identify relapses earlier than conventional tests in a recent Mexican study using a cohort of children with brucellosis [63]. A multiplex real-time PCR has been developed to concomitantly detect both *Brucella* and *Mycobacterium tuberculosis* complex DNA and differentiate focal brucellosis from extrapulmonary tuberculosis [64]. To identify *Brucella* species and biovars, IS711 typing or DNA polymorphism at *omp2* locus might be used, but a cross-reaction with *Ochrobactrum* is possible [65]. Instead, *Brucella* typing by amplified fragment length polymorphism or a sensitive *recA* gene-based multiprimer single-target PCR might be used [28]. A hypervariable octameric oligonucleotide fingerprint (HOOF) assay might efficiently discriminate among *Brucella* isolates [5,66]. Most recently, a novel PCR assay, highly specific for rapid *Brucella* detection and species identification, was developed [67]. A recent Lebanese study showed that multilocus variable-number tandem-repeat analysis (MLVA), considered to be a discriminatory PCR-based method, could be used for *Brucella* genotyping and might be useful in larger epidemiological studies in the future [68]. In Turkey, single-nucleotide polymorphism of *rpoB* gene for *Brucella* genotyping has most recently proven efficient for epidemiological studies of *B. melitensis* [69]. It is the first time that loop-mediated isothermal amplification was suggested as a possible *Brucella* screening tool in environmental or food samples in epidemiological settings [70]. A battery of PCR-based tests could detect the *Brucella* DNA signature among other bioterrorism agents [2]. Collectively, in comparison to conventional methods, PCR assays seem more likely to diagnose acute brucellosis faster, whereas post-treatment PCR may possibly be employed to detect low-level brucellaemia, microbiologically undetected in cases of inactive brucellosis ([43], Table 3), or relapses of the disease. PCR testing may be used in the diagnosis of focal brucellosis.

Treatment

Antibiotic susceptibility testing

In-vitro susceptibility testing of brucellae is not routinely required. *Brucella* is susceptible to most of the traditional antibiotics, that is tetracyclines, aminoglycosides, used to treat brucellosis. However, in-vitro susceptibility does not correlate well with in-vivo efficacy. There has been no standardized method for susceptibility testing recommended by the Clinical and Laboratory Standards Institute (CLSI) for *Brucella* spp. The disc diffusion test is considered unsuitable for testing slow-growing bacteria. Using the epsilometric (E)-test, a recent study showed that among 74 *Brucella* strains from Greece, Cyprus and Syria, two isolates were inhibited by 1.5 mg/l rifampicin [71]. But in a recent evaluation of antibiotic efficacy against *B. melitensis*, the minimum inhibitory concentrations (MICs) determined by E-test were lower than the MICs determined with the broth dilution method [72].

Present therapeutic approaches

The WHO-recommended two-antibiotic treatment for acute brucellosis in adults consisting of doxycycline 100 mg twice daily orally for 6 weeks and 1 g streptomycin daily parenterally for 2–3 weeks, or 100 mg doxycycline twice daily and rifampicin 600–900 mg can be given orally as an alternative for at least 6 weeks. A recent meta-analysis suggests that triple rather than dual antibiotic combination may be considered optimal treatment and cost-effective; tetracycline may replace doxycycline [73]. Likewise, the recent Ioannina consensus recommendation is to accept gentamicin (5 mg/kg once daily parenterally for 7 days) as an alternative to streptomycin [74]. The use of trimethoprim-sulphamethoxazole (160 mg TMP–800 mg SMX twice daily for 6 weeks) in treatment needs to be further investigated, due to the development of TMP-SMX resistance. In children less than 8 years of age, 8 mg/kg TMP and 40 mg/kg SMX twice daily should replace 4 mg/kg doxycycline, orally administered in combination with 10 mg/kg rifampicin in children at least 8 years of age. Doxycycline is contraindicated in young children and in pregnant women for possible adverse effects on the foetus. In uncomplicated brucellosis in pregnancy, rifampicin monotherapy for at least 45 days has proven to be well tolerated [74]. Most brucellar complications are adequately treated with standard regimens; however, in some patients, therapy should be individualized. In the cases of spondylitis, quinolones, which penetrate and achieve increased concentrations in bone and soft tissue, are supposed to shorten the length of treatment, even though, generally, they were deemed inferior to other drugs [73,75]. Also, surgical intervention is necessary in patients with cerebral or epidural abscess, endocarditis, splenic abscess, hepatic brucelloma, and so on. Owing to the dearth of therapeutic data on neurobrucellosis, there are currently no recommendations. More data are needed to introduce an effective treatment for endocarditis [74].

Management

Still, the ideal brucellosis treatment remains elusive owing to the spectrum and complications of brucellosis and the inability to eradicate *Brucella* [43]. In an attempt to avoid treatment failure, it is proposed that treatment should start as early as possible and combination therapy [73] rather than monotherapy with the currently accepted antibiotics should be given for an adequate length of time. On the contrary, prolonged duration of treatment and possible side effects may lead to poor compliance, whereas antibiotic resistance is considered only partly responsible for treatment failure [28]. However, with the doxycycline–rifampicin combination, there is a risk of developing resistance to rifampicin in areas where both brucellosis and tuberculosis are endemic, such as Saudi Arabia [76]. In an attempt to avoid possible resistance development, the antibiotic susceptibilities of *Brucella* should periodically be determined.

Prevention

As of today, there is no vaccine, whether live attenuated or nonliving, available for prevention of human brucellosis, whereas immunostimulants such as levamisole, interferon, steroids, and so on do not seem to prevent manifestations in the dysregulated setting of chronic disease [15]. Prevention of brucellosis can be best achieved by minimizing human contact with infected animals or their products. Education on personal and food hygiene, safe working practices, and environmental protection may improve health literacy of populations at risk; however, community social status, culture, beliefs, and so on should be taken into account [15]. Hand washing, eye protection, protective clothing, tool/surface disinfection, infected animal slaughter and destruction are some of the more important measures to be taken in farms and other premises. Raw milk and meat consumption should be avoided to prevent infection of the general population. Pasteurized dairy and cooked meat product consumption is strongly recommended. Biosafety level 3 precautions are required to isolate *Brucella* in a laboratory [2], whereas protective gloves and clothing and respiratory protection are necessary for personnel working in a *Brucella* vaccine-manufacturing plant. Animals are considered lifelong *Brucella* carriers providing a large continuous source of human infection. A lower incidence of human brucellosis is likely to result by developing an animal brucellosis control programme. Reporting brucellosis in both humans and animals has been considered essential [77] so that the need for an animal brucellosis control programme could be evaluated, whereas international involvement may be necessary at every level, that is planning, implementation, and so on in endemic developing countries. Mass livestock or young animal vaccination along with animal slaughter after testing, whether voluntary or compulsory, can be applied depending on the epidemiology of each zone and the

progress during the course of the programme, as in the more recent control programme applied in the Azores [78]. Currently, vaccine strains 19 and RB 51 are used to prevent *B. abortus* infection and Rev 1 is used to prevent *B. melitensis* infection. Furthermore, animal movement should be controlled [77]. Test and slaughter (with compensation) alone can be applied when only a few cases occur. Surveillance is critical for sustaining control or eradication. Identification of potential new brucellosis cases in herds or flocks of animals not vaccinated for *Brucella* is made so that all animals could be tested and infected animals could be slaughtered. *B. melitensis* eradication from small ruminants has proven a difficult task for technical and financial reasons [77]. Only if surveillance is constant will human cases be reduced to an acceptable level. Human cases are considered to be an index of animal brucellosis control programme in addition to animal abortions [77]. When indicated, recent nonimported exposed cases, representing control programme failure to prevent brucellosis, should be investigated and distinguished from past exposure cases (Table 3). Another obstacle to a successful control programme could be contact between domesticated animals and animals in the wild, as in case of cervids in contact with cattle that endangers the brucellosis-free status of some parts of the United States [6]. As mentioned above, in addition to domestic transmission cycle of the disease, there is also a sylvatic component and *Brucella* has the potential to survive in nature; hence, brucellosis eradication *sensu stricto* might not be feasible.

Conclusion

One of the reasons why brucellosis still remains an elusive disease in humans concerns weaknesses in our understanding of the host–pathogen interaction. Recent methodological advances might possibly contribute to the identification of potential new targets for diagnosis, intervention and prevention of brucellosis. Despite being a significant food-borne [44] and occupational hazard, brucellosis may be overlooked; thus, physicians should remain alert, particularly in the case of patients living/residing in or returning from endemic zones. In clinically suspected cases, collaboration between microbiologists and clinicians is essential. An international reference laboratory should be expected to ensure harmonization and standardization of tests and procedures worldwide [79]. However, integrating new molecular techniques with conventional methods would perhaps provide a rapid and accurate diagnosis of human brucellosis as may be necessary in a biowarfare setting [61], or in difficult-to-diagnose cases. The value of doxycycline in postexposure prophylaxis of brucellosis has just been validated [14,80]. Nevertheless, in the future, therapeutic approaches designed to facilitate antibiotic action (by neutralizing the acidic intracellular environment) or delivery (through systems as liposomes or

polylactide-coglycolide microspheres) should be incorporated [74]. Alternatively novel agents as tigecycline, an antibiotic similar to the tetracyclines, may be used as monotherapy [74]. Research efforts to develop a well tolerated and effective human vaccine should be further supported, whereas integrated public health activities should be promoted in order to have a beneficial effect on the morbidity (and mortality) of brucellosis in humans.

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