Brucella: Potential Biothreat Agent

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Mehmet Doganay, Gizem Dinler-Doganay, Aysegul Ulu-Kilic, and Rebecca J. Ingram

6.1 Introduction

Brucellosis is an ancient disease, the etiologic agent being bacteria of the *Brucella* genus. While the disease has a global distribution in humans and animals, the majority of cases are reported in Mediterranean, Middle East, Central Asia, Africa, Central America, and Latin America [1, 2]. Due to the potential for misuse of these organisms, *Brucella* spp. are categorized as group B priority agents by both the Centers for Disease Control and Prevention (CDC) and National Institute of Allergy and Infectious Disease (NIAID) in the USA. The agent is also included on the lists of potential biological agents of weapons by the World Health Organization (WHO), the Biological and Toxin Weapons Convention (BTWC), and the North Atlantic Treaty Organization (NATO) [3–5].

The perceived threat of biological agents altered drastically after the deliberate release of anthrax in the USA through postal service in 2001 for both the public and scientific communities. Trepidation surrounding biothreats has been heightening for a number of reasons. There are numerous ongoing conflicts around the world, such as those in North Africa, the Middle East, and Afghanistan, and many illegal rebel organizations (including ethnic, separatists, leftist, and religious terrorist groups) are currently very active worldwide. A huge number of people have been displaced from

M. Doganay (✉) · A. Ulu-Kilic
Department of Infectious Diseases, Faculty of Medicine, Erciyes University, Kayseri, Turkey
e-mail: mdoganay@erciyes.edu.tr

G. Dinler-Doganay
Department of Molecular Biology and Genetics, Istanbul Technical University, Istanbul, Turkey
e-mail: gddoganay@itu.edu.tr

R. J. Ingram
Wellcome Wolfston Institute of Experimental Medicine, Queens University, Belfast, UK
e-mail: b.ingram@qub.ac.uk

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their homes, forced to migrate to neighboring countries, or become refugees because of ongoing civil war, conflict, and/or terrorist activities. The Syrian civil war is widely accepted as one of the worst humanitarian disasters since World War II [6]. On August 21, 2013, it was reported that sarin gas was dispersed in Syria, with more than 1400 civilians killed and thousands more affected [7]. International media reports suggest that chemical agents such as sarin gas or mustard gas have been used four times since the outbreak of civil war in 2011. It has been rumored that the biological agents *Bacillus anthracis* or variola (smallpox) virus could be used as a biological agent by the terrorist groups in the Middle East.

Brucella bacteria are easily obtainable from all routine diagnostic hospital laboratories. The agent is moderately easy to disseminate and results in moderate morbidity and low mortality rates. However, *Brucella* infections lead to huge economic losses in endemic countries, and there is still no available licensed human *Brucella* vaccine. Our commercial food chain is a particular area of vulnerability. The commercial food chain is highly complex involving a wide range of global producer and distributers. The intentional contamination of food supplies with *Brucella* is likely to result in major public anxiety and fear. Furthermore, *Brucella* spp. are highly infectious via the aerosol route; thus it could easily be misused as an agent for biological warfare. The global risk of biological attack increases annually due to migration, growing numbers of refugees, global travel and trade, terrorist interest in weapons of mass destruction, and advances in technology that have reduced the skill and technological resources required to manipulated pathogens [4, 8–10].

6.2 Microbiological Characteristics

Brucella species are aerobic, gram-negative intracellular coccobacilli or short rods (0.5–0.7 μm in diameter and 0.6–1.5 μm in length). The genus *Brucella* is a member of the family *Brucellaceae*. Currently, 11 recognized species have been reported: 6 terrestrial, 3 marine, and 2 proposed species. Up to 1985, the genus of *Brucella* was classified into six species: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae*. These are referred to as the as the six classical species and are all genetically related. *B. melitensis* and *B. suis* are generally more pathogenic in humans than *B. abortus* and *B. canis*. The species are further subdivided into biotypes, three have been defined for *B. melitensis*, seven for *B. abortus*, and five for *B. suis* [11–14]. In addition to the six classical species, five new species of *Brucella* have been identified: *B. ceti*, *B. pinnipedialis*, *B. microti*, *B. inopinata*, and *B. papionis*.

Brucella spp. are non-encapsulated and do not produce spores or flagella. They are readily grown on the common media used in microbiology laboratories. Optimal culture occurs on trypticase soy agar, *Brucella* agar, and serum dextrose agar using the classic biphasic culture (solid and liquid), blood culture technique, at a temperature of 35–37 °C with a pH of 6.6–7.4. While conventional culture requires a prolonged incubation of between 6 and 27 days, this is shortened by the use of

automated blood culture systems (up to 5 days incubation). Some biovars of *B. abortus* and *B. suis* require supplementary carbon dioxide, especially for primary isolation. When the bacteria are grown on blood agar, colonies are usually seen 0.5–1.0 mm in diameter, raised, and convex [1, 11].

The bacteria are able to persist in the environment for a long time (weeks or months) depending on the number of bacteria, sunlight, temperature, humidity, pH, nutrients, and presence of bacterial contents (Table 6.1). It is also known that the bacteria maintain their virulence in dry preparations for years [1, 11, 15, 16]. They are, however, sensitive to most commonly used disinfectants, pasteurization, heating, and ionizing radiation.

6.3 Brucella as an Agent of Biothreat

Biological agents have already been used for the purpose of "biological warfare," "bioterrorism," or "biocrime." Although no accepted definition exists, we propose the following definition: Biological warfare is the use of weaponized biological agents by a government against military targets. Bioterrorism is the threat or use of biological agents/toxins by individuals or groups to further their aims (political, religious, ethnical, or other ideological objectives). The use of biological agents for the purpose of murder, revenge, or exaction is called as "biocrime." A terrorist attack can be distinguished from criminal attack by their main objectives [3, 17]. The organisms listed by the WHO or CDC as potential biological agents are subdivided into three categories: antipersonnel, anti-animals, and anti-plants. As *Brucella* spp. are capable of causing disease in both humans and animals, there is the potential for them to be used to target both human populations and livestock [3, 5, 10].

During World War I, between 1932 and 1945, Germany initiated a biological warfare program with the intention to infect livestock and contaminate animal feed predominantly with *Bacillus anthracis* and *Burkholderia mallei.* During World War II, German scientists conducted biological weapons research on prisoners in Nazi concentration camps, testing live preparations of *Rickettsia*, hepatitis A virus, and *Plasmodium* spp. In published reports, it is not clear whether or not *Brucella* species were used [4, 17, 18]. Japan also conducted an extensive biological weapon program during World War II in Manchuria (unit 731 and 100). Experiments with various agents were carried out on prisoners of war, at least 10,000 died due to infections as part of this program [3, 18]. During the attack on Changteh in 1941, there were 1700 deaths and approximately 10,000 casualties due to biologicals among the Japanese troops; most cases were due to cholera. Although the Japanese biowarfare research program continued until the end of the World War II, field trials were terminated in 1942 [18].

In 1942, the USA had initiated an offensive biological program which was expanded during the Korean War (between 1950 and 1953). During the 1960s, the US military developed range of biological weapons, including various bacterial agents and the *Brucella* spp. During this period, *B. suis* was weaponized and formulated to maintain long-term stability and viability. Between 1944 and 1945,

Table 6.1 Survival periods of *Brucella* bacteria in various environments and substrates

Substrates or environment	Temperature and environment	Surviving time
Brucella melitensis		
Broth	pH > 5.5	>4 weeks
Broth	pH 5	<3 weeks
Broth	pH 4	1 day
Broth	pH < 4	<1 day
Soft cheese	37 °C	48–72 h
Yogurt	37 °C	48–72 h
Yogurt	5 °C, fat rate; 10%, 1.5%, and 3.5% pH 4.2–4.3	2, 3, and 5 days
Buffalo's yogurt	4 °C	20 days
Cream	4 °C	>4–17 weeks
Milk	37 °C	7–24 h
UHT milk	20 °C	>12 weeks
Dust	Depends on ambient humidity	15–40 days
B. abortus		
Solid surfaces	<31 °C, sunlight	4–5 h
Tap water	−4 °C	114 days
Lake water	37 °C, pH 7.5	>1 day
Lake water	8 °C, pH 6.5	>57 days
Soil dried	≈20 °C	<4 days
Soil wet	<10 °C	66 days
Manure	Summer	1 day
Manure	Winter	53 days
Farm slurry animal waste	Ambient temperature tank	7 weeks
Farm slurry animal waste	12 °C	>8 months
Cream	2–4 °C	>6–16 weeks
UHT milk	20 °C	>87 days
Sterilized milk	Room temperature	10 months
Buffalo's yogurt	4 °C	30 days
Yogurt	5 °C, fat rate; 10%, 1.5%, and 3.5% pH 4.2–4.3	2, 3, and 5 days
Brucella spp.[a]		
Water	20 °C	2.5 months
Still mineral water	20 °C	63 days
Raw milk	8 °C	2 days
Ice cream	4 °C	30 days
Cheese	Room temperature	3–12 weeks
Butter	8 °C	142 days
Meat	Frozen meat	<3 weeks

Summarized from the references [1, 13, 15, 16]
[a]Not given the species

B. suis was loaded into bombs, and field trials were carried out to test its efficacy against animal targets. Approximately 10 years later, human experimentation on military and civilian volunteers was conducted, using spherical aerosolization chambers in volunteers who were exposed to microorganisms when biological munition was exploded. By 1969, the USA announced that the offensive *Brucella* program had been terminated and all biological munitions were destroyed. They also state that the munitions developed were never used in conflict [17, 18].

The former Soviet Union had an extensive offensive biological weapons program. *Brucella* was one of the agents which they were working on. Ken Alibek, former deputy director who moved to the USA in 1992, stated that antibiotic-resistant strains of *Brucella* were developed and weaponized both in dry and liquid forms with production capability ranging up to hundred tons [4]. He also described a sophisticated system that had been constructed for bacterial delivery which had been extensive field tested in the Aral Sea [4]. By the end of twentieth century, interest in *Brucella* had waned, and the organism was replaced by *Burkholderia pseudomallei* in the biological weapon program [4, 18].

Several microbiological characteristics of the *Brucella* species make it tractable as a potential agent in bioterrorism or bio-war. These bacteria, particularly *B. melitensis* and *B. suis*, are highly infectious through the aerosol route, and the infectious dose for humans is relatively low, approximately ten to a hundred microorganisms. The organisms can enter the body through the respiratory mucosa and gastrointestinal tract, genital mucosa, conjunctivae, minor skin lesions, or abraded skin. The incubation period ranges from up to 1 week to several months. The infection may mimic infectious or noninfectious diseases. In humans, brucellosis is a debilitating and prolonged disease with acute, subacute, or chronic forms. The disease requires long-term antibiotic therapy, and there are only a limited number of antibiotic currently being used for treatment [4, 9].

Computer modeling suggests that following an aerosol attack with *B. melitensis*, the epidemic curve, by days after exposure, shows that 4% of cases would occur within 0–7 days, 6% in 8–14 days, 14% in 15–28 days, 40% in 29–56 days, 26% in 57–112 days, and 10% in more than 113 days. It was calculated that the economic cost of such an attack would be $477.7 million per 100,000 exposed people [19]. It is estimated that the release of 50 kg of *B. suis* from a plane along a 2 km line at a distance of 10 km upwind of a city of 500,000 people would result in the infection of 125,000 people and 500 deaths [18].

Another route of biological attack using *Brucella* is the deliberate contamination of commercial food products or animal feeds. This contamination could potentially occur during production, packing, storage, transportation, or delivery. Records of intentional or malicious contamination in the food supply chain between 1950 and 2008 were collected and analyzed; during this period, 464 events were recorded resulting in a total of 4187 deaths and 19,545 injuries [20]. It was reported that 12 of these events and 190 deaths are attributable to biological agents [20]. According to a report entitled "Chronology of Chemical and Biological Incidents Targeting the Food Industry," more than $100 million in lost income had been recorded between 1946 and 2006 [21].

To date, *Brucella* species have not been used against either civilians or military targets. However, this does not mitigate the potential threat of the intentional use of *Brucella* spp. in both endemic and non-endemic countries (particularly in Western countries). Given its zoonotic nature, an attack with *Brucella* spp. could lead to severe disease outbreaks in either human population or farm animals. There is currently rising concern around the danger of agroterrorism, targeting farm animals such as sheep, cattle, swine, and fish, processed food, and food storage facilities.

6.4 Brucellosis as a Zoonotic Disease

Brucellosis is a zoonotic disease, with the source of natural human infection being infected animals. Natural reservoirs of *Brucella* spp. are sheep and goats (*B. melitensis*), cattle (*B. abortus*), swine (*B. suis*), and dogs (*B. canis*). In Table 6.2, the reservoir host and potential human pathogenicity of *Brucella* spp. is outlined. The more recently described species of *Brucella* spp. were identified from wildlife hosts which include rodents, marine mammals, and baboons. Some of these species have not been widely identified in human infection; thus their infectivity and virulence is not completely understood [11, 12].

Generally, the animal reservoirs of *Brucella* spp. (Table 6.2) are asymptomatic carriers. Although subacute or chronic presentation of the disease may also be seen in infected animals, within the host, the bacteria target organs and tissues, in particular the reproductive system which includes the placenta, mammary glands, testis, and epididymis. *Brucella* infection results in placentitis and miscarriage during the last trimester of pregnancy. Epididymitis and orchitis are seen in the male. There are no specific clinical indications of brucellosis in animals, and

Table 6.2 The host preference of *Brucella* species and pathogenicity for humans

Species	Reservoir	Pathogenicity for humans	Human cases (worldwide)
B. melitensis	Sheep, goat, camel	High	++++
B. abortus	Cattle, buffalo, yaks, bison	High	+++
B. suis	Swine	High	++
B. canis	Dog	Moderate	Rare
B. ovis	Ram	No	No reported cases
B. neotomae	Desert and wood rats	No	No reported cases
B. ceti	Dolphin, porpoise, whale	Mild	Few cases
B. pinnipedialis	Seal	Mild	Few cases
B. microti	Vole, fox, soil	Unknown	No reported cases
B. inopinata	Unknown	Mild	Few cases
Brucella papionis sp. *nov*	Baboons	Unknown	No reported cases

Summarized from the references [11, 12, 14, 22]

diagnosis is based on the isolation of bacteria, the detection of bacterial antigens in clinical samples, or the demonstration of a specific antibody response. Transmission can occur directly between animals, which can result in miscarriage, or alternatively during mating from genital secretions or semen during or to offspring via milk. In domestic animals, infection can occur if the barn, pasture, animal feed, and/or water sources have been contaminated. *Brucella* infection leads to abortion, stillbirths, decreased fertility, and low milk production in livestock. Therefore, infection results in economic losses and can pose a serious public health threat in endemic countries [12, 14, 22].

Transmission to humans occurs through direct contact with infected animals and/or their excretions (urine, semen, and mammary fluid, genital secretions), contaminated blood or carcasses, or dairy products (milk, fresh cheese, cream, butter). Naturally occurring brucellosis is regarded as a food-borne disease, an occupational infection, or rarely laboratory-acquired infection. The disease is predominantly acquired from the consumption of raw/unpasteurized milk or other unpasteurized dairy products, particularly fresh cheese. Another common source of infection is occupational contact with infected animals. Farm workers, shepherds, butchers, veterinarians, and meat-packing employees are considered to be at high risk in endemic regions [1, 12, 22]. Laboratory workers (particularly those working in hospital diagnostic laboratories in endemic countries or in reference laboratories for zoonotic disease) are also at risk of *Brucella* infection [23, 24], and accidental laboratory-acquired infection has been reported worldwide. While human-to-human transmission is rare, brucellosis resulting from sexual transmission or blood transfusions has been reported [25, 26].

Brucellosis remains one of the most common bacterial zoonotic diseases worldwide. The WHO estimates that 500,000 new human cases of *Brucella* infection occur annually [2], and infection of livestock leads to significant economic losses, particularly in developing countries [22].

6.5 Clinical Presentation of Human Brucellosis

Brucella infection in humans can occur through ingestion or inhalation, via contact of broken skin with infected animal tissue or body fluids through broken skin or eyes. After infecting the host, the bacterium penetrates mucosal barriers and enters the bloodstream, facilitating dissemination throughout the body [12]. *Brucella* spp. are intracellular bacteria which reside and multiply within mononuclear phagocytes (monocytes, macrophages, and dendritic cells); they are able to avoid the host's intracellular killing [27]. The bacteria spread within the phagocytic cells to the reticuloendothelial system (RES) (localizing mainly at the joints), the central nervous system (CNS), the cardiovascular system (CVS), the respiratory system, and the genitourinary tract. The incubation period of disease varies depending up the virulence of strain, the route of entry, and the infectious dose. It is often difficult to determine precisely when infection has occurred as the incubation period while using 1–4 weeks can be up to several months (Table 6.3).

Table 6.3 Clinical manifestations of patients reported from the studies published after 2000

Clinical manifestations	Interval for the percentages
Fever	55–100
Malaise	68–90
Arthralgia	66–87
Sweating	19–96
Myalgia	36–49
Back pain	6–58
Nausea/Vomiting	21–30
Abdominal pain	6–28
Hepatomegaly	6–50
Splenomegaly	7–60
Osteoarticular involvement	19–54
Cardiovascular involvement	0.4–1.8

Summarized from the references [28–35]

Brucellosis is a systemic disease which affects various organs or body systems. The disease generally presents with intermittent fever, chills, arthralgia, myalgia, and malaise. Although most commonly a systemic infection, brucellosis may also cause a localized infection involving specific organ systems such as the skeleton system, central nerve system, heart, liver, and lungs. It is also associated with focal abscess formation particularly in RES and the skeleton system. The localized form of brucellosis occurs with untreated acute or chronic disease. Focal infection occurs in approximately 30% of cases.

The symptoms of brucellosis are similar, regardless of the bacterial species involved. However, the severity of these symptoms varies; *B. melitensis* and *B. suis* cause severe infection, while *B. abortus* is associated with a greater proportion of subclinical cases, and *B. canis* infection usually causes only a mild disease [15].

Although there is no clinical experience with intentional released brucellosis, both naturally occurring and intentional released brucellosis are likely to have a similar presentation. Thus, the clinical symptoms and laboratory findings of naturally acquired brucellosis may be considered to be representative for brucellosis due to intentional release.

Cases of brucellosis are arbitrarily classified into clinical types based on the duration of symptoms. The disease is classified as “acute” when there has been less than an 8-week duration. The disease is deemed “subacute” from 8 to 52 weeks and “chronic” beyond 52 weeks [16].

Approximately 50% of patients develop acute illness; they present with a range of non-specific symptoms which include fever (over 38.5 °C in 85% of patients, with an intermittent pattern), night sweats, weakness, fatigue, malaise, headache, nausea, vomiting, arthralgia, and myalgia [28]. Upon physical examination, the clinical findings are also variable and non-specific; most commonly they will include hepatomegaly, splenomegaly, and osteoarticular involvement [28]. Patient

symptoms typically resolve within 2–4 weeks, but a limited number of patients will develop chronic disease or have relapses.

If there is a reoccurrence of disease, 3–6 months after completion of therapy, this is termed relapsing disease, and this occurs in 5–30% of patients. Relapsing brucellosis tends to be a milder form of disease than the initial attack [36]. While antibiotic resistance is currently not a significant issue in the treatment of brucellosis, relapsing diseases is often associated with the use of inappropriate antibiotic treatment of the initial disease [37, 38].

Subclinical cases of brucellosis are usually asymptomatic; they are characterized by positive, low titer, serology, and negative bacterial cultures. The subclinical form of the disease frequently occurs in abattoir workers, farmers, and veterinarians in endemic areas [16].

The chronic form of brucellosis is usually associated with undiagnosed and untreated disease. It typically has a febrile pattern and is mainly characterized by fatigue, depression, myalgia, and arthralgia. This clinic form resembles “chronic fatigue syndrome.” It generally occurs in older individuals (over 30 years old) and rarely occurs in children. In chronic brucellosis, localized disease usually manifests as spondylitis, hepatitis, epididymitis, or endocarditis [39].

A meta-analysis of clinical manifestations of brucellosis provides a comprehensive evaluation of scientific literature published between 1990 and 2010 [40]. Fever was identified as the most common symptom, observed in 80% of patients regardless of age. Given this high proportion, brucellosis should be considered as a differential diagnosis for fever of unknown origin. The most common presentation of disease is musculoskeletal system involvement. Arthralgia affects 65% of patients; in contrast arthritis was reported in only 26% of patients. Arthritis generally involves large joints, with those most commonly effected, in descending order, the sacroiliac, knee, hip, vertebra, and ankle. While bursitis, tenosynovitis, and osteomyelitis have also been described, they are rarely seen. Spinal involvement is the foremost cause of the debilitating and disabling complications and is seen in 6–12% of cases. Musculoskeletal involvement is more frequent in young patients, whereas older patients are more prone to spinal involvement and complications such as paravertebral, epidural, and psoas abscess formation. The lumbar region was the most frequently involved, but it is known that the disease can affect the entire vertebral column [41]. Prosthetic joint infection due to *Brucella* spp. is extremely rare but should be considered in endemic countries.

Involvement of the genitourinary system can present with epididymo-orchitis, cystitis, pyelonephritis, interstitial nephritis, glomerulonephritis, prostatitis, and renal abscesses. These complications occur in 2–20% of cases. Epididymo-orchitis was observed in one in ten men and thus appears to be the most affected organ [40].

Neurobrucellosis is seen in 2–7% of the cases, the manifestations range from headache, alterations in behavior, and confusion to nerve deficits, acute/chronic meningitis, encephalitis, radiculitis, and myelitis. While it is not uncommon for patients to report depression, psychosis, and mental fatigue, these symptoms are greatly underestimated in the diagnosis of neurobrucellosis [42].

Pulmonary involvement in brucellosis can occur either as a result of inhalation of infectious aerosol or hematogenous spread. This presentation is rare, occurring in only 7% of patients with brucellosis [40]. Signs and symptoms of pulmonary involvement can range from mild, non-specific such as cough, mucopurulent sputum, and flu-like symptoms to sever bronchitis, interstitial pneumonitis, lobar pneumonia, lung nodules, pleural effusion, hilar lymphadenopathy, and empyema.

Gastrointestinal complaints such as dyspepsia, anorexia, and abdominal pain are frequent, occurring in up to 50% of patients with brucellosis. However, severe complications including hepatic or splenic abscess, cholecystitis, pancreatitis, ileitis, colitis, and spontaneous peritonitis are relatively uncommon. A mild to moderate increase in transaminases may be observed; 38–53% of patients have elevated baseline values of aspartate and alanine aminotransferase [43]. Mild jaundice may be observed; however, deep jaundice is seen rarely in patients with brucellosis.

Brucellosis causes hematological abnormalities; it is particularly associated with anemia and leucopenia. The disease may also cause thrombocytopenia, pancytopenia, and/or disseminated intravascular coagulation. Occasionally, brucellosis has been reported to induce severe autoimmune hemolytic anemia which is refractory to traditional corticosteroid therapy [44].

The eyes and ears can be affected with brucellosis. Ocular manifestations are most frequently seen during the chronic phase of disease, with the most common presentation being uveitis. More serious complications can also occur; these include corneal ulcers, iridocyclitis, nummular keratitis, choroiditis, optic neuritis, papilledema, and endophthalmitis. The auditory system is affected during acute brucellosis, and all diagnosed patients should be evaluated for hearing loss [45].

Cutaneous manifestations of disease are usually non-specific and only occur in 1–14% of patients with brucellosis. These can include macular or maculopapular rash, scarlatiniform, papulonodular, and erythema nodosum-like eruptions, ulcerations, petechiae, purpura, granulomatous vasculitis, and abscesses [46].

Although brucellosis is not, in itself, a fatal disease, some of the complications associated with the disease may be lethal. The leading cause of brucellosis-related deaths is cardiac and CNS complications. The incidence of endocarditis, myocarditis, pericarditis, endarteritis, thrombophlebitis, and/or mycotic aneurysm of the aorta or ventricles is low; they have been reported to occur in only 1% of cases. Recent advances in surgery, combined with effective medical treatments, have proven successful in preventing death due to endocarditis [47].

Brucellosis can be a severe, debilitating, and sometimes chronic disease with the potential to affect a variety of systems within the body. The mortality rate associated with brucellosis is as low as 2%, as appropriate treatment generally results in complete recovery without complications. Due to the non-specificity of the clinical features of brucellosis, the disease can imitate a number of syndromes and, thus, has been labeled “mimicking disease.” Infectious disease such as tuberculosis, malaria, typhoid fever, and infectious mononucleosis or other noninfectious diseases such as chronic fatigue syndrome, collagen vascular diseases, autoimmune diseases, and tumors should all be considered in differential diagnosis of brucellosis.

6.6 Diagnosis and Treatment of Brucellosis

As the clinical picture of brucellosis is non-specific in humans, diagnosis needs to be supported by medical history, physical examination, and appropriate laboratory tests. Inquiries should be made about potential occupational exposure, travel to an enzootic region, and consumption of unpasteurized/raw milk and dairy products while taking the medical history.

The gold standard for diagnosis of brucellosis is isolation of the bacteria from either blood cultures or other tissues. *Brucella* spp. can be isolated from the bone marrow, tissues (liver, spleen), cerebrospinal fluid (CSF), synovial fluid, etc. A prolonged incubation is required as *Brucella* spp. are slow-growing bacteria; however, automatized blood culture systems, which are now routinely used in most clinical laboratories, allow for the detection of bacteria within 1 week [48]. The sensitivity of the detection in blood cultures ranges from 50 to 90%; this is dependent on several factors including the stage of the disease, the culture medium utilized, and previous antibiotic usage. Identification of *Brucella* spp. and antibiotic susceptibility testing requires the use of biosafety level-3 (BSL-3) protocols due to the high risk of laboratory-acquired infections. Species-level identification, which requires detailed phenotypic or molecular assays, while essential for epidemiological studies, is not required for the initiation of therapy [49].

Brucellosis diagnosis is predominantly based on serology due to the low sensitivity of the culture. A variety of serological tests have been devised over the past 100 years beginning with a simple agglutination test. A range of tests are routinely used for the diagnosis of the disease; these include Rose Bengal plate tests (RBPT), serum agglutination tests (SAT), complement fixation tests (CFT), and an enzyme-linked immunosorbent assays (ELISA) [1, 42].

The RBPT is performed using a suspension of *B. abortus* colored with Rose Bengal stain. It is a simple and rapid slide-type agglutination test based on the reactivity of antibodies against smooth lipopolysaccharide (S-LPS). This is the preferred screening test as it has a high sensitivity of 93%. The limitation of this test is that there is a much lower sensitivity in chronic cases and reduced specificity in endemic regions. As a result of these limitations, the WHO guidelines recommend the confirmation of positive samples using SAT [1, 42].

The gold standard for serological diagnosis of brucellosis is SAT; this assay is also based on the detection of antibodies against S-LPS. The test is performed in tubes, by serially (doubling) diluting sera which reacts with a constant amount of *Brucella* antigen. The visible agglutination titers reflect the concentration of antibodies in the serum, usually ranging from 1:20 to 1:1280. Either an elevated SAT titer of $\geq$1:160 or demonstration of a fourfold increase from acute to convalescent titers is considered diagnostic. In order to reduce the incidence of false positives, in endemic areas, the recommended cutoff is $\geq$1:320. The presence of high non-agglutinating IgG defined as "blocking antibodies" may result in false-negative results in SAT. It is important to note that active brucellosis cannot be excluded in patients with SAT titers lower than 1:160. During the early stages of infection, the titer may be below the cutoff; therefore repeat testing may be required

[1, 50]. Lower SAT titers may also be seen in chronic and relapsing cases, and therefore antiglobulin (Coombs) test may be more appropriate for diagnostic confirmation of chronic and relapsing cases. In addition to these technical limitations, SAT is time and labor intensive.

The use of ELISA allows rapid, sensitive, and reliable diagnosis of brucellosis [51]. Both IgM- and IgG-specific antibody detection by ELISA have been shown to have a good concordance with SAT and Coombs tests and are more sensitive in chronic cases [52, 53]. In endemic areas ELISA is a recommended over conventional agglutination [52]. There are however conflicting views, with some studies suggesting that ELISA is less sensitive in the detection of anti-*Brucella* antibodies than more conventional serological tests [54].

A newer serological test is Brucellacapt, which is based on the immunocapture-agglutination of total anti-*Brucella* antibodies [55]. This assay shows a high sensitivity and specificity in the diagnosis of human brucellosis, not only in the first stages of the disease but also in cases with long evolution and in relapses and reinfections. A decrease in specific antibody titers following successful treatment and clinical cure is more pronounced and rapid in Brucellacapt than SAT and Coombs test. Therefore, Brucellacapt titers can be considered to be a good marker of infection, particularly when used during patient follow-up [50].

Brucella DNA can be detected using polymerase chain reaction (PCR) assays in either cultures or clinical specimens. PCR has been proven to be more sensitive than blood culture and more specific than serologic tests in both acute and chronic brucelloses. Working with highly infectious live cultures carries a risk of laboratory infection which is greatly reduced when working with DNA [56].

While complete blood count, erythrocyte sedimentation rate, C-reactive protein, and liver function tests are not specific for the diagnosis, they are useful in the diagnosis and monitoring of the disease. The sensitivity and specificity of brucellosis diagnosis are improved using a combination of two or more diagnostic tests and compatible clinical symptoms [1, 16].

The objective of the antimicrobial therapy in brucellosis is to reduce disease symptoms, shorten the duration of the symptomatic period, and reduce or prevent complications or relapses. Given that *Brucella* spp. are intracellular microorganism, antibiotics capable of reaching a high intracellular concentration must be used. Prolonged treatment with a combination of two or more drugs is recommended in order to prevent relapse [1, 11, 16].

The World Health Organization (WHO) recommends an antibiotic regimen of oral doxycycline 100 mg twice a day for 6 weeks plus oral rifampicin 600–900 mg daily for 6 weeks or streptomycin 1 g intramuscularly daily for 2–3 weeks for the treatment of uncomplicated brucellosis [1]. A meta-analysis of clinical trials published between 1985 and 2012 found that this is the most widely used treatment regimen [36]. There are, however, alternative treatment options. A doxycycline-rifampicin regimen has the advantage of oral administration, while a regimen which combines doxycycline and streptomycin has been shown to be superior, both in terms of treatment failure and relapse rates [57]. Several studies have reported the

efficacy of other alternative combinations: quinolones and rifampicin, co-trimoxazole and rifampicin, and triple regimens with doxycycline, rifampicin, and aminoglycoside [58]. The combination of co-trimoxazole and rifampicin is particularly recommended for children and pregnant women where tetracyclines are contraindicated. Monotherapy and short course of therapies (<6 weeks) are not acceptable treatment strategies for brucellosis [1].

There is no recommended treatment regimen for complicated brucellosis. For endocarditis, spondylitis, or meningitis, the agents of choice are similar. Triple therapy regimens including the combination of aminoglycosides plus doxycycline and rifampicin are considered as the first line as they offer good efficacy and low rates of treatment failure and relapse. The duration of therapy for complicated cases should be prolonged to more than 8 weeks [58].

Brucellar endocarditis is a rare complication with high mortality. The optimal antibiotic regimen and duration of therapy remain unsolved. Many authors have reported satisfactory results with perioperative antibiotic therapy and surgical treatment (prosthetic valve replacement) [47, 59].

Spinal brucellosis is the leading cause of debilitating and disabling complications. Spondylitis may extend to neighboring vertebrae. The paravertebral and epidural spaces present with abscess formation which requires a longer duration of antibiotics, occasionally combined with surgery. Surgical interventions are recommended as the last resort when there are persistent systemic symptoms despite adequate antimicrobial therapy, vertebral collapse, or septal abscess [60].

The WHO-recommended treatment of neurobrucellosis is the standard regimen of doxycycline plus streptomycin, with the addition of rifampin or co-trimoxazole. A prolonged duration of the treatment is also suggested, with a minimum duration of 6–8 weeks, with possible further extension depending on the clinical response [1]. In a multicenter study, which included 215 adult patients with neurobrucellosis, the average duration of treatment was about 4.5–6.5 months. This study also presents data supporting the use a month of parenteral ceftriaxone treatment in combination with doxycycline and rifampin. They found that ceftriaxone-based regimens provided significantly shorter duration of therapy than oral treatment [61].

Even with the use of recommended antimicrobial regimens, therapeutic failure and relapse occur in 5–30% of patients with brucellosis; this is usually associated with shorter duration of treatment or ineffective antibiotic regimens [37]. Resistance to antimicrobial drugs particularly for first-line regimens is unusual. To date, only increases in the MICs of ceftriaxone and streptomycin have been reported in Turkey [62]. Relapsing cases of brucellosis have not been shown to be related to drug resistance.

Brucellosis has a widespread geographic distribution; however it mainly affects developing countries. In order to prevent disease, it is crucial to identify simple, inexpensive, efficacious treatments and design effective control programs.

6.7 Biotechnology Applications to Detect and Identify *Brucella* Species

Biological weapons are a serious global concern [9, 63, 64]. Biotechnological advancements can be misused for the development of antibiotic- and vaccine-resistant, undetectable, more stable, easier-to-handle, and lethal biological agents which could be used in a bioterrorist attack. If a bioterrorism outbreak were to occur, clinicians, pathologists, and microbiologist's first aim would be to identify the causative agent. It may, however, not be an easy to accurately detect the microorganism due to applications of intricate genetic engineering strategies. Therefore rapid and sensitive detection is likely to require multiple methods from a variety of specimen types to facilitate the correct identification of bacteria causing the epidemic. Currently, each bacterial detection method has its own pitfalls and usually requires additional tests to confirm the results.

Various biotechnological tools can be used to detect and identify *Brucella* spp. Diagnosis of *Brucella* in samples generally relies on culture-based methods and serologic tests. Sensitive culturing of bacteria is dependent on there being sufficient numbers of viable *Brucella* in the sample. After a positive isolation of *Brucella* spp. is achieved, biotyping, serotyping, phage typing, nuclear sequencing, restriction endonuclease fragmenting, and hybridization can be used for detailed characterization of the *Brucella* species. Failure to isolate *Brucella* does not necessarily rule it out as the causative agent. Another frequently used diagnostic relies on serologic tests, which are mainly based on the detection of antibodies which are produced following infection with *Brucella* spp. Both validated and in-house agglutination assays, precipitation tests, and Western blotting tests are used for serologic detection of *Brucella* spp. in centers worldwide. Antigens from S-LPS obtained from *B. melitensis* and *B. abortus* are generally for the serological diagnosis of *Brucella* spp. Due to the existence of *B. canis* and *B. ovis* as rough colony forms, detection of antibodies for these species is only achievable using major outer membrane protein antigens. The requirement for multiple testing for accurate assignment of *Brucella* species is a limitation of serology tests. There is a need for identification of novel target antigens to be used in these tests, for example, there are currently no specific serologic for the detection of *B. melitensis* infection in small ruminants [65]. Another limitation of serologic testing is the lack of standardized reference antigen, resulting in variations in the test results [42]. To accurately differentiate species and biovars, serologic testing is used in combination with PCR-based techniques, such as enterobacterial repetitive intergenic consensus sequence PCR, repetitive intergenic palindromic sequence PCR, amplified fragment length polymorphism analysis, monolocus sequence analysis, and multi-locus variable-number tandem repeat analysis [9, 42]. The sensitivity and specificity of these techniques for accurate detection of *Brucella* spp. are dependent on the laboratory conditions and a highly skilled technical personnel existence; there is a requirement for the development of robust, standardized, and validated methods.

While isolation of *Brucella* bacteria is considered the decisive method of diagnosis for brucellosis, due to the difficulties associated with this technique and

serological testing, new efforts to standardize and validate PCR-based diagnosis techniques are underway. PCR-based technologies offer sensitive and reliable detection of the genus. The development and validation of these tools for routine diagnosis will also eliminate issues associated with contamination with other bacteria, most commonly *Yersinia*. Hundreds of PCR-based methods have been developed for the detection and typing of *Brucella* spp. directly from milk, whole blood, serum, semen, body fluids, and tissues from neonates of aborted fetus [9, 42, 56]. They all involve the extraction of DNA using available commercial kits. Depending on the source of the sample, the efficiency of the kits' DNA isolation capacity will vary [56]. The extraction of DNA from blood can be problematic due to the presence of inhibitors; this necessitates repeat washing of the blood with either water or lysis buffer, removing contaminating hemoglobin. Single pairs of PCR primers to identify *Brucella* spp. at the genus-specific level are used for testing human blood samples; however higher sensitivity is achieved when targeting multiple genes (especially the combinations of primers targeting *bcsp31*, *omp2a*, *omp2b* genes) in a single PCR reaction [56, 66]. Additional improvements have facilitated the use of multiplex and real-time PCR assays [66], both of which have been shown to be highly effective for the detect *Brucella* spp. at a biovar level [56]. There can however be some misleading results, for example, discerning *B. suis* biotype 4 from *B. canis* at the biovar level, due to similarities observed in their PCR patterns [67]. Molecular methods are faster and more sensitive than traditional methods; despite this, the routine application of these tests for the diagnosis of *Brucella* spp. is currently limited. Validation of these tests is necessary in order to meet the quality control and assurance criteria for diagnose of *Brucella* infection in clinical samples, before they are used in routine laboratories. Additionally, since these PCR-based methods rely solely on the current genomic knowledge of *Brucella*, these methods should be updated as variations arise in *Brucella* genome.

While improvement in both serologic and PCR-based methods is underway, there are efforts to find alternative routes to diagnose a quantitative brucellosis using biosensors or *Brucella*-specific nanobodies. These assays have the potential to offer rapid, inexpensive, and easy-to-use methodologies for the detection of *Brucella* bacteria in the environment or clinical samples [9, 68–70]. Biosensor-based detection technologies of bacteria quantify the signal produced after a biological response is converted to electrical signal. Most of the biosensors are based on labeling techniques, where the target molecules get labeled either before interaction or after binding of the target on the sensing surface. Due to the long time scales and high costs associated with the development of labeling-based, optical, label-free, biosensors are being investigated. Optical biosensors also offer the potential for real-time detection. Various types of biosensors have been designed for *Brucella* spp. detection, some of which allowed very specific recognition [9, 68–70]. Recently, two nanoscale biosensors were designed which utilize gold nanoparticles and oligonucleotide probes to directly visualize *Brucella* spp.; these allow detection of *Brucella* spp. at pg/μL concentrations [69]. The same researchers have also designed a label-free DNA hybridization-based electrochemical geno-sensor on palladium nanoparticles which acts as a transducer allowing for the sensitive quantification

and detection of *Brucella* species [68]. A surface plasmon resonance immuno-biosensor has been developed which targets DNA fragments of *B. melitensis* using two different probes covalently attached to different 4-MBA/Au SPR chips [70]. This SPR-based biosensor allows label-free nanomolar range detection of *B. melitensis*; this holds promise as a rapid and sensitive detection technique in pathology laboratories. Another new detection strategy is based on nanobodies, which are single-domain camelid-derived antibody fragments that have been genetically engineered and are highly soluble and stable. Nanobodies are retrieved from *Brucella*-immunized camelid (NbBruc02 and 03 constructs) using phage display; this is followed by re-cloning the genes in a protein expression plasmid and subsequent purification of the nanobodies [71]. These nanobodies can detect *B. abortus* and *B. melitensis* antigens, offering the ability to differentiate the two main but highly similar species [71].

6.8 Control and Prevention

Public health preparedness, early stage responses, and counter measurements are very important for the prevention of intentional released biological agents. Biological threat analysis and public preparedness require a multidisciplinary approach which should include law enforcement, governmental organization, and medical and scientific preparedness. Public health preparedness includes medical awareness, surveillance, laboratory skills, and diagnostic capabilities in order to strengthen our ability for identification of the potential biological agents in developing and developed countries. An effective system is required to allow the intelligence and security services, law enforcement, and health authorities to work together. Both civilians and the majority of healthcare workers have little or no knowledge of the potential illnesses caused by biological agents including *Brucella* species. They may not, therefore, suspect a deliberate released disease during the early phase of an incident. There is a need to train healthcare workers (HCWs) in the recognition and initial management of biological incidents. Education and training program must cover the characteristics of biological agents, clinical presentations, diagnosis, treatment and prophylaxis of the disease, infection control procedures for HCWs, suspected sample collection, and contaminated sample handling, as well as decontamination procedures. Rapid communication systems between governmental organizations are also required to allow the immediate sharing of information when an unusual incident is suspected [1, 5, 9, 10].

An intentional release of *Brucella* species would not cause a sudden outbreak of disease. The outbreak could induce a smooth curve, gradually increasing followed by a decrease over a period of 2–3 months [19]. Local governors and security personnel must therefore be aware of the suspected incidents in their regions. Primary care and family physicians, public health workers, emergency service physicians, infectious disease physicians, and hospital epidemiologists should be aware of clustered human cases of brucellosis. Indications of the deliberate release of *Brucella* would include large-scale outbreaks or unusual setting clusters of

brucellosis, especially where *Brucella* infection is in not endemic cases with no previous travel history to endemic regions or suspected food consumption and no history of occupational or laboratory exposure. Veterinarians and veterinarian health workers should also be aware of increasing animal cases in their regions. The source of unexpected *Brucella* infection and clustered human and animal cases must be analyzed epidemiologically. In endemic countries, it will be very difficult to differentiate naturally occurring *Brucella* infection from intentional released infection. Many physicians working in industrial countries are not familiar with clinical presentation of brucellosis. For this reason, the diagnosis of human cases may be delayed [4, 8, 9].

If an attack were to occur, appropriate environmental sampling and rapid identification of the agent released is essential to allow the appropriate preventive and medical measures to be rapidly instigated. Planned intervention should include triage of suspected or known exposed victims, protection of HCWs and other responders, prevention of public fear and panic, initiation of decontamination procedures, prophylaxis, and monitoring the outbreak. For the early detection of biothreat agents including *Brucella* spp., molecular techniques such as genetic probe assay, nucleic acid amplification, immunoassay, and enzyme inhibition using a silicon-based biosensor are now available. Some of the biosensors which have been recently developed to detect the *Brucella* spp. in the environment may also be employed [1, 4, 9, 14].

In the event of a biological attack, HCWs, technicians, and other responders should wear the N95 masks, goggles, impermeable clothing, gloves, and shoes to protect them from airborne *Brucella* infection. All victims should be evacuated from the attack area. Although *Brucella* spp. are unable to penetrate intact skin, the biological agent from human skin should be removed using water or soap and water; the clothes from victims should be disposed of in order to minimize the risk of infection by accidental conjunctiva and other mucosal inoculation or ingestion of viable bacteria. All contaminated victim clothes should be burned or decontaminated by effective disinfectants [1, 5, 9, 72]. For hospitalized patients, patient isolation is not required because of the low risk of human-to-human transmission [1].

Contaminated foods should be destroyed, by the trained individuals, in protected areas. The *Brucella* bacteria can survive in the environment for varying periods (Table 6.1). Buildings can be decontaminated using chlorine-based liquid sprays, formaldehyde steam produced by heating paraformaldehyde, or other disinfecting fumigants. In limited areas, 3% phenol or 10% hypochlorite solution may be applied by a trained person wearing a protective mask, goggles, gloves, and gown. Currently, it is extremely difficult to certify that a building is clean after decontamination due to an intentional release of a biological agent [1, 5].

Vaccination is very important components for the prevention of infection in individuals' exposure to released *Brucella*. Although there is no licensed human *Brucella* vaccine, the live human vaccines *B. abortus* strain 19-BA and *B. melitensis* strain 104 M have been used in the former Soviet Union and China, respectively [22, 73]. Human vaccine studies are under development; however they have only shown limited efficacy and induce serious medical reactions. Subunit vaccine studies

Table 6.4 Recommendations for post exposure prophylaxis of brucellosis

	Regimen (administration route and daily dose)
Adults	Doxycycline: 100 mg bid per os and rifampicin: 600–900 mg per os once daily
Pregnant women for breastfeeding women: cessation of breastfeeding is recommended	Doxycycline: 100 mg bid per oral and rifampicin: 600–900 mg per oral once daily
Children >8 years	>45 kg: adult dose <45 kg: 2.2 mg/kg per oral twice daily and rifampicin: 10–15 mg/kg per oral in 1 or 2 doses daily
Children <8 years	Trimethoprim (6–8 mg/kg/day) and sulfamethoxazole (30–40 mg/kg/day) per oral in 1 or 2 divided doses and rifampicin: 10–15 mg/kg per os in 1 or 2 doses daily
Recommended duration of prophylaxis	3–6 weeks

Summarized from the references [1, 4, 8, 9, 72]

are, however, showing promise for successful future vaccine development [73, 74]. Most veterinary vaccines are based on live-attenuated strains; they have been successful in the control of livestock infections. The most commonly used veterinary vaccines against *Brucella* infection are *B. abortus* strain 19 and *B. abortus* strain RB51 for cattle, *B. melitensis* strain Rev 1 for sheep and goats, and *B. suis* 2 for swine. Although the Rev 1 vaccine is highly infectious for humans, it is considered to be the best vaccine for the control of brucellosis in sheep and goats [22]. Currently, antibiotic prophylaxis would be the only option to prevent infection following the deliberate released brucellosis. There is no experience with antibiotic prophylaxis in cases exposed to *Brucella* bacteria. The current recommendations are based on the derived data from accidental laboratory exposure. Table 6.4 summarizes the current recommended antibiotic prophylaxis [1, 4, 8, 9, 72].

In conclusion, *Brucella* spp. is highly infectious via the aerosol route making them an attractive pathogen for those with nefarious intentions. The global biologic risk of biological attack is increasing for a variety of reasons. Scientists need to focus their efforts on the development of a new safe and effective human *Brucella* vaccine and new drugs for the treatment of *Brucella* infection. When preparing biodefense systems, countries should consider countermeasures against *Brucella* spp. along with other priority biological agents.

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