# 1. BRUCELLOSIS - AN UNKNOWN AND UNDERDIAGNOSED INFECTION IN BULGARIA

***R. Nenova, I. Tomova***

*NRL High Medical Risk Infections*
*National Centre of Infectious and Parasitic Diseases*

**ABSTRACT**
Brucellosis is still the most common zoonosis worldwide. Despite this, it is unknown and underdiagnosed infection in non-endemic areas. For some parts of the world it became a re-emerging infection. After several decades brucellosis re-emerged in Bulgaria. In 2005 an outbreak of imported human cases was detected and soon after, two autochthonous outbreaks occurred (2006 and 2015) with a total of 161 persons diagnosed.

***Key words:*** brucellosis, re-emergence, outbreak, autochthonous, microbiological diagnosis.

Brucellosis, also known as Maltese fever, "undulant fever", Gibraltar fever, Bang's disease, is a zoonotic infection caused by microorganisms from the genus Brucella. It is a chronic relapsing disease known for millennia. Nowadays it still remains an important economic and medical problem, especially in endemic areas, where significant losses of livestock and high morbidity rate among human population are reported. In some other regions the disease remains unknown and underdiagnosed. After several decades brucellosis became a re-emerging infection in Bulgaria with the occurrence of two autochthonous outbreaks in 2006 and 2015 with a total of 161 diagnosed persons.

**Dr. RUMIANA NENOVA**
**National Reference lab on high threat bacterial infections**
**NCIPD**
**blvd. Stoletov 44**
**Sofia, BULGARIA**
**Tel: + 3592 8319125**

**THE CAUSATIVE AGENT**
Genus Brucella is named in honour of David Bruce, who isolated *Brucella melitensis* (Table 1) in 1887 from a British soldier in Malta (1). A second species , *B. abortus* was isolated from cases of epizootic abortion in cows (2), and both agents were put together in the genus Brucella due to their similarity (3). Later Huddlestone described *B. suis*, which causes infectious abortions in pigs (4). In the 50s of the last century two other species have been identified: *B. ovis*, causing reproductive problems in sheep and *B. neotomae*, isolated from rats in the United States (5, 6). Further, the causative agent of abortion in dogs, *B. canis* was described in 1968 (7). Due to the very close genetic similarity (>90%) between the members of genus *Brucella* it is considered as monospecific genus, but different species are classified as biovars of *B. melitensis* (8). From a practical point of view (main reservoir) it is adopted that the traditional names of the species could be used for non-taxonomic purposes (9). In the 1990s some newly isolated strains, pathogenic for marine mammals and phenotypically different from the first six species were categorised as *Brucella* spp. (10, 11). They were named depending on the animals they affect: *Brucella ceti* sp. nov. (cetaceans as preferred hosts) and *B. pinnipedialis* sp. nov. (seals as preferred hosts) (12). The ninth described species, *B. microti*, was isolated from wild common voles and red foxes during epizootics in Central Europe, but was also recognized as a soil contaminant (13). Based on phenotypic and genotypic tests it was incorporated in

genus *Brucella* and so far, there is no evidence for its pathogenicity for humans. Furthermore, another two strains (BO1 and BO2), similar to *B. ovis* were isolated from human clinical samples. Based on phenotypic and molecular analyses they were considered as a novel species, named *B. inopinata* (14, 15). The last described species, *B. vulpis* sp. nov., was isolated from Austrian red foxes and carries the *Brucella specific* IS711 and bcsp31, but 5% of its genome was acquired from different soil bacteria (16,17).

**Table 1**. *Brucella* species according to their natural host and pathogenicity for humans.

| ***Brucella*** | | | |
|---|---|---|---|
| *B. melitensis* | 1-3 | goats, sheep, camels | high |
| *B. abortus* | 1-7, 9 | cattle | variable |
| *B. suis* | 1, 3 | pigs | high |
| | 2 | pigs, hares | low |
| | 4 | Canadian deer and reindeer | variable |
| | 5 | rodents | high |
| *B. canis* | - | dogs | low |
| *B. ovis* | - | sheep | not reported |
| *B. neotome* | - | redents | not reported |
| *B. ceti* | - | cetaceans, dolphins, whales | human cases are reported |
| *B. pinnipedialis* | - | seals | human cases are reported |
| *B. microti* | - | common voles, red foxes | not reported |
| *B inopinata* | - | unknown | human cases are reported |
| *B. vulpis* | | red foxes | not reported |

Brucellae are small Gram-negative coccobacilli or short rods, which are facultative intracellular parasites. They are non-motile, non-spore forming, without true capsules, pili and natural plasmids. Although brucellae are aerobes, some types require additionally CO 2 , especially for primary isolation. All members are fastidious and need rich peptone media supplemented with blood and/or serum. The lipopolysaccharide (LPS) of the cell wall is the immunodominant antigen. It cross-reacts with other Gram-negative bacteria such as *Yersinia enterocolitica* O9, *Escherichia coli* O157, *E. hermannii*, *Salmonella enterica* O:30, *Vibrio cholerae* O1 and *Stenotrophomonas maltophilia* (18). The protein antigens (outer membrane and cytoplasmic) have a protective effect against brucella by stimulating the cellular and humoral immune response in infected individuals. They are common for both - smooth and rough types and do not cross-react with other Gram-negative bacteria (19).

## SOURCES AND ROUTES OF INFECTION

The main source of infection for humans are domestic animals. Their significance is determined by the most common host species (Table 1). Different types of *Brucella* spp. have different geographical distribution. The most widespread, in the Mediterranean region and the Arabian Peninsula is *B. melitensis*, which causes the majority of outbreaks. *B.abortus* is usually causative agent of sporadic cases or small outbreaks. *B. canis* is less pathogenic, mainly for immunodeficient persons, while *B. ovis* and *B. neotome* are non-pathogenic for humans. (19, 20, 21). There are reports that marine representatives can cause serious

infections in humans, including laboratory acquired (22, 23, 24). Brucellae are released in large quantity with the fetus, placenta, and amniotic fluid during abortion and stillbirth, as well as with milk, urine, and vaginal secretions. Brucellosis is mainly an occupational disease. Infection can occur via skin lesions and healthy mucosa (incl. conjunctiva) during breeding of infected animals. Quite often another way of human infection is consumption of unpasteurised dairy products and poorly heat-treated meat. Contamination of hands is also important for the oral route. Air-powder mode of infection is performed by inhalation of dust containing brucellae while working with leather, wool, and soil. Laboratory acquired infections occur via several mechanisms: production of infectious aerosols, accidental inoculation through skin and mucous membranes, or ingestion of infectious materials. (25). Other, less common routes are: blood transfusion (26), via placenta (27), breast feeding (28), or possible sexual transition (29). In endemic areas the infection rates are the same in children and adults and most often the disease occurred after consumption of contaminated food. In non-endemic areas, such as Bulgaria, mainly adults are infected (occupational or imported cases) (20, 30).

**PATHOGENESIS**

Brucellae tend to invade and survive in the host. They manage to avoid intracellular killing in phagocytic cells and multiply in macrophages. Through the circulatory system they reach the regional lymph nodes and afterwards - various organs, mainly the reticuloendothelial system. These processes determine the diverse clinical manifestations of the disease (31). The intracellular survival of bacteria is facilitated by virulence factors such as LPS components, superoxide dismutase and some outer membrane proteins (19, 32). Protection of the macroorganism is carried out by anti-LPS antibodies and T cell-mediated activation of macrophages by the protein antigens (19). The antibody production by B lymphocytes is of small importance for the immune protection, but has a great diagnostic value.

**CLINICAL PRESENTATION**

Brucellosis has a variety of nonspecific symptoms and can mimic other infectious and non-infectious diseases. The clinical presentation depends mainly on the stage of the disease. The early symptoms include: fever, sweats, malaise, anorexia, headache, joint and muscle pains, depression. The so called “undulant fever” could appear. Physical abnormalities are generally few and include lymphadenopathy (10-20%), splenomegaly, and/or hepatomegaly (20-30%). Because of the untypical presentation of the disease, it often remains unrecognised or misdiagnosed, especially in non-endemic areas (33, 34). Chronic brucellosis (duration more than 12 months) is presented with recurrent relapses of the above-mentioned symptoms or focal infections. Antibiotic therapy in this phase is less effective. The most common complications (92%) are musculoskeletal: sacroiliitis, peripheral arthritis, spondylitis, bursitis, incl. in Bulgarian patients (34), followed by affection of reproductive, nervous and cardiovascular systems, and more rarely lung, kidney, eye and skin manifestations (33). Serious complications include meningitis, endocarditis, osteomyelitis (35, 36, 37). Relapses are very typical for brucellosis, especially after inadequate and/or delayed treatment (38). The rate of clinical relapses among cluster of imported cases in Bulgaria was also high (38%), because of the aforementioned reasons (34). Mortality is generally low (1-2%) and is due to life-threatening complications.

**TREATMENT AND CONTROL**

Antimicrobial susceptibility testing for *Brucella* spp. is not routinely performed, because of: rare antimicrobial resistance; discrepancy between *in vitro* and *in vivo* results; high risk of laboratory acquired infections and lack of clear interpretive criteria (39). In special circumstances minimal inhibitory concentration (MIC) by serial dilutions in Cation-Adjusted

Mueller-Hinton Broth and E-test on enriched Mueller-Hinton agar are performed (40, 41). Treatment is difficult because of the intracellular location of the bacteria. Prolonged therapy and multiple antibiotics are imperative for achieving a cure (42, 43, 44). Several regiments are recommended: doxycycline 6 weeks + rifampicin 6 weeks or doxycycline 6 weeks + streptomycin for 2-3 weeks (gentamicin 1 week). The use of ciprofloxacin and co-trimoxazole is optional (esp. pregnant women and children younger than 8 years), but without any priority over the above-mentioned antimicrobial agents. Therapeutic failure or relapses are generally not caused by resistance, but by premature discontinuation of the treatment. Patients with relapse are usually retreated with the same antimicrobial agents, but often the result is not satisfactory. Recurrence of brucellosis may occur from a persistent focus of infection that requires additional treatment, for example surgical drainage. Due to serious side effects human vaccines are not applicable. Decrease of the incidence is achieved through control of the infection in animals and the contamination of dairy and meat products. Because of the low infectious dose in aerosols (10 to 10,000 cells depending on the species) *B. melitensis*, *B. abortus*, and *B. suis* are listed as category B potential bioterrorism agents (45, 46).

## DIAGNOSIS

Brucellosis is a disease with wide range of symptoms and multiple clinical forms. Diagnosis is difficult and complex. Confirmed diagnosis is based on laboratory data interpreted along with the clinical and epidemiological ones.

## LABORATORY EXAMINATION

Culturing is performed mainly on blood samples. Other more rarely used clinical materials are bone marrow, cerebrospinal fluid (CSF), pleural and synovial fluids, urine and tissue or abscess materials. It is extremely important to avoid contamination of the samples, because of the prolonged time for cultivation. Serum or less commonly CSF, are used for serological testing. PCR is performed mainly on whole blood or serum. Clinical samples should be handled very carefully, although human tissues do not contain high numbers of brucellae (47). Serological testing should be performed without using special precautions, other than personal protective equipment (PPE). In a case of positive culture, strict precautions are required due to the dangerous numbers of organisms presented. Subculturing and other manipulations with living brucellae must be performed by using practices and procedures required for Biosafety level 3.

**Direct detection of *brucellae*.** It could be performed both with direct immunofluorescent microscopy (DIFM) and molecular methods. DIFM could be used for examination of clinical samples, as well as for identification of bacterial isolates. It is also a valuable method for preliminary diagnosis in case of suspected bioterrorism. PCR tests for direct detection of *Brucella* DNA use inactivated samples, which is a great advantage considering the low infectious dose. However, the specificity and sensitivity of PCR vary between laboratories due to the lack of standardisation regarding the sample type and processing, the target genes, the visualisation of products and others (48). Blood and serum are most commonly tested with PCR, but various other clinical materials also could be used (49). Some authors propose serum as a more suitable for PCR, because of the lower quantity of inhibitors in it, as well as its easier processing (50). A number of genus specific primers targeting *bscp31*, omp-2, 16S rRNA, IS711, and other genetic elements are used (Table 2).

Species-specific primers are applied rarely, mainly for epidemiological and scientific purposes, since in general the particular type of *Brucella* is irrelevant for the therapy (48). Real-time PCR techniques for detection of *Brucella* spp. are also developed (51, 52, 53, 54). These methods have a high sensitivity (<10 cells/reaction) and specificity (99-100%), due to the usage of more than one marker for detection of *Brucella* DNA in clinical specimens. In recent years a number of multiplex methods based on real-time PCR are designed (55).

**Table 2**. PCR methods for detection of *Brucella spp*.[a)]

| Primers | Target | Amplicon length | Sensitivity | References |
|---|---|---|---|---|
| Not specified | 43κDa OMP [b)] | 635 bp [c)] | 0.1 pg [d)] | Fekete et al. 1990 |
| B4/B5 | *bscp31* | 223 bp | 10-100 fg [e)] | Bailey et al. 1992 |
| JPF/JPR | omp-2 | 193 bp | 0.025 fg | Leal-Klevezas et al. 1995 |
| Ba148-167F/ Ba928-948R | 16S rRNA | 800 bp | Not reported | Herman et al. 1992 |
| F4/R2 | 16S rRNA | 905 bp | 80 fg | Romero et al. 1995 |
| O1/O2; I1/I2 | IS711 | 325 bp<br>52 bp | 70 fg | Al Nakas et al. 2002 |

[a)] Ivanov, I. 2010. Molecular methods for detection, identification and typing of highly dangerous bacterial pathogens. PhD Thesis.; [b)] OMP-outer membrane protein.; [c)] bp- base pair.; [d)] pg- picogram.; [e)] fg- femtogram

**Cultivation.** Isolation of the causative agent remains "gold standard" for confirmed diagnosis. Blood culture is leading in the bacteriological examination for brucellosis, but some data indicates that the amount of bacteriais low (1.3 -1000 CFU/ml), even in the acute phase of the disease (56). Bone marrow is the most suitable material in sub-acute brucellosis, after a negative blood culture and/or previous antibiotic treatment. Other samples are tested rarely. Due to the possibility for contamination of the blood cultures during their prolonged cultivation, as well as the high risk for the laboratory personnel, biphasic culture media are recommended for isolation of *Brucella* spp. (57). The bottles are incubated at 35-37$^{o}$C, in humid atmosphere, with 5-10% CO2 (mainly for *B. abortus*). They are inspected daily until visible growth on the agar phase is observed. *Brucella* colonies appear not earlier than the fourth day, but the majority of positives occur between the 7th and 21st day. Therefore, conventional culture methods require 21 days to 6 weeks. Before discharging the bottle, a blind subculture is performed (57, 58). Lysis-centrifugation method is faster (2-4 days), but increases the risk for laboratory accidents, as well as for contamination of the blood culture (59). The automated systems for continuous monitoring of samples, such as BACTEC and BacT/Alert greatly increase the sensitivity of the method and reduce the time for detection of brucellae (57, 60). For other materials like bone marrow, CSF, synovial fluid, and various tissue homogenates biphasic media, as well as blood and chocolate agar could be used. Because *Brucella* spp. are fastidious, enriched media such as Trypticase soy agar, Heart infusion agar, Brucella agar, and Columbia agar should be used. Addition of blood in different concentrations and 5-10% horse serum enables the growth of the demanding species. The plates are incubated at the above-mentioned conditions for 10 days. For materials with small concentration of bacteria (urine, milk), enrichment could be achieved via inoculation of

guinea pigs. Recovering of brucellae from contaminated specimens requires media with different antibiotic supplements such as selective Brucella agar, Farrell medium and others (61).

**Identification.** Suspected colonies are subcultured on blood or chocolate agar. *Brucella* spp. are small Gram-negative coccobacilli that form non-haemolytic colonies on blood agar, and do not grow on MacConkey agar. The latter is useful for differentiation from other small

**Table 3.** Differentiation of *Brucella* species from other Gram-negative coccobacilli.

| **Characteristic** | ***Brucella* spp.** | ***Acinetobacter* spp.** | ***Bordetella bronchiseptica*** | ***Haemophilus* spp.** | ***Francisella tularensis*** |
|---|---|---|---|---|---|
| **Specimens** | blood; bone marrow | various | various | blood; CSF | wound secretion; blood; aspirates |
| **Gram stain** | faintly staining small coccobacilli | coccobacilli/ short rods | coccobacilli / small rods | small coccobacilli | very small coccobacilli |
| **Catalase** | +[a)] | + | + | **V** [b)] | + (weak) |
| **Oxidase** | + | - [c)] | + | **V** | - |
| **Urease** | + | - | + | **V** | - |
| **Motility** | - | - | + | - | - |
| **X and/or V factor requirement** | - | - | - | + | - |
| **Growth on blood agar** | + | + | haemolysis | satellite growth | poor requires cysteine |
| **Growth on MacConkey agar (48 h.)** | - | + | + | - | - |

[a)]+ positive; [b)] V - variable; [c)] – negative

Gram-negative coccobacilli that could be isolated from the same clinical materials (Table 3).

Brucellae are slow-growing and therefore visible growth of subcultures could be seen not earlier than 48 hours. Colonies are small, smooth, raised, transparent, with regular edge and shiny surface. Nonsmooth variants occur, especially after longer subcultivation. Only *B. ovis* and *B. canis* have stable nonsmooth (R) form. The presumptive identification on genus level is based on morphological, biochemical and serological criteria. Brucellae are oxidase, catalase and urease positive, and show positive slide agglutination reaction with specific *B. abortus* and *B. melitensis* antisera (19). The use of commercial identification systems is not reliable, if *Brucella* spp. is not included in the database. As mentioned above, identification to genus level is sufficient for the etiological treatment. PCR can be used for screening after therapy when *Brucella* DNA is not detectable if the treatment is successful (62, 63). In the cases of relapse PCR tests could become positive again, which is helpful for evaluation of the

patient, as specific IgG antibodies persist long after completion of therapy. However, the confirmation of relapse is bacteriological (64).

Typing is performed in highly specialised and well equipped laboratories. Phenotypic methods include: sensitivity to dyes (growth in the presence of methionine and basic fuchsin); speed of urea hydrolysis; production of H2S; phage sensitivity (Tb, Wb); reaction with monospecific (A and M) sera; determination of the S/R morphology. Based on these phenotypic characteristics *B. suis*, *B. abortus* and *B. melitensis* are divided into biovars (Table 1). For reasons mentioned above, molecular techniques have certain advantages over phenotypic methods. For outbreak investigations and phylogenetic studies molecular typing of *Brucella* isolates to subspecies and strain level could be made by Bruce-Ladder and multilocus variable-number tandem-repeat analysis of 21 loci (MLVA-21) and 16 loci (MLVA-16) (65, 66). For example, MLVA-16 performed on 162 human *Brucella* isolates from Turkey indicate that they are most closely related to the neighbouring countries' isolates included in the "East Mediterranean" group (67) Using MALDI-TOF MS for investigation of 131 *Brucella* human isolates (*B. abortus*, *B. melitensis*, *B. suis*) a 100% identification at genus level was obtained (68). The discrimination to the species level was not reliable. While comparing data obtained from MLVA on 152 *Brucella* isolates with those from MALDI-TOF MS, other authors concluded that the latter could indeed discriminate between different species and biovars(69).

**SEROLOGICAL TESTS**

Brucellae are fastidious and highly pathogenic bacteria. Therefore, serological methods are essential. Proper detection of all stages of the disease and differentiation between active infection and convalescent period demands the usage of several serologic tests. In general, two types of antigens are used - whole cells and antigen extracts. The first group demonstrates antibodies to cell surface antigens, mainly LPS which is responsible for the cross-reactivity with other Gram-negative bacteria. Such tests are: Rose Bengal slide test (RBST), serum agglutination test (SAT, Wright), complement fixation test (CFT), anti-globulin tests (Coombs' test, Brucellacapt) and indirect immunofluorescence microscopy (IFA). They are not suitable for *B. canis* and *B. ovis*, whose LPS is incomplete (R form). The second group of tests is based on purified LPS or protein extracts, used mainly for ELISA or different precipitation reactions (70, 71). RBST is fast and very sensitive. It is strongly positive in the initial acute phase of the disease, but cross reactions occur with sera from patients infected with *Y. enterocolitica* O9, so the result should be confirmed with the other tests. Positive SAT indicates active infection and together with 2-mercaptoethanol enables the monitoring of brucellosis with long duration. Single titre ³1:160 or seroconversion is indicative of brucellosis. Lower titres (1:80) should not be overlooked, especially in the onset of the disease (64) as well as in non-endemic are a s, like Bulgaria. Such results should be int e rpreted according to the clinical and epidemiological data. Retesting after 1-2 weeks is reasonable, as it was demonstrated in our experience during the Bulgarian outbreaks 2006-2008 and 2015 (unpublished data). Human anti-globulin test (C o ombs') detects blocking (non-agglutinating) a n tibodies in the chronic stage of the infection, but this method is laborious and requires 48 hours. In such cases Brucellacapt (Vircell, Spain), a o n e-step test detecting both agglutinating and non - agglutinating IgA and IgG antibodies, is mo r e suitable. It has good sensitivity and specificity comparable with those of the Coombs' test ( 72). ELISA could detect the different classes of immunoglobulins and is useful for patient s' follow-up. It was found that all three classe s immunoglobulins appear quickly after the ons et of the infection. With time Ig M levels tend to decrease, while IgG and IgA persist for l onger periods (64). Quick and easy immuno-chrom atographic tests for screening, especially for

field testing during outbreak have been developed and implemented (73). Monitoring of treatment in patients with brucellosis requires continuous tracking with serological tests. De crease in titres indicates good prognosis, while long lasting high values point out persistence of the disease and drift to chronification. Du ring relapse IgG and IgA antibodies but not I gM, could be detected in patients' serum. Ser ological results, especially single titres should be interpreted according to the clinical and epidemiological data.

**BRUCELLOSIS AROUND THE WORLD**

Brucellosis is one of the most common zoonosis in the world (74). It remains a major problem in the Middle East, especially in Syria (above 100‰ per year). Turkey has annual incidence of 8-50‰ (20, 75). In the rest of Asia, the incidence is still high in Mongolia (50-100‰), but the rate of brucellosis in some former Soviet republics (Kyrgyzstan, Kazakhstan, Tajikistan, Azerbaijan) significantly increases and new foci of the disease appear. The incidence in Australia, Canada, and the USA is low (<2 cases per million). According to data from the Centre for Disease Control and Prevention about 80 new cases are diagnosed annually in the USA (76). Mexico is the main source for importation of human brucellosis in USA. In Latin America, the incidence is generally low, with the exception of Mexico and Peru (10-50 cases per million) and to a lesser extent for Argentina(2-10 cases per million) (20). The Mediterranean basin is one of the major endemic regions for brucellosis (77, 18). In the European Mediterranean countries, the incidence has been reduced significantly through overall control of animal brucellosis. Annual numbers of the cases in Spain, Portugal, Italy and Greece have been dropping according to the latest reports from the European Centre for Disease Prevention and Control (78). Most of the EU Member States, especially in Western Europe have brucellosis-free status. They report annually a small number of imported cases. In 2011, a total of 332 confirmed cases of brucellosis were reported by 28 EU/EEA countries. The notification rate was 0.07‰. The majority of all confirmed cases (68%) are still reported from Greece, Spain and Portugal. On the Balkan Peninsula brucellosis is a main problem in Albania where the disease is underestimated. High incidence is observed also in the Former Yugoslav Republic of Macedonia, Kosovo, and Bosnia-Herzegovina. (20,79,80). Three outbreaks of bovine and 17 of ovine/carpine brucellosis were detected and 26 humans were infected in Serbia during 2014. According to the Public Health Institute of Republic of Macedonia 299 animals for 2014 and 36 (1.8‰) humans for 2013 were proved as positive for brucellosis. In Greece 8.64% of the sheep and goat flocks and 0.97% of the cattle herds were infected with brucellosis in 2012 (81). The highest incidence of human brucellosis in the EU (1.44‰) for 2013 was also registered in Greece.

**BRUCELLOSIS IN BULGARIA**

The first cases of human brucellosis in Bulgarian citizens were described by foreign authors (82). In 1903 Neusser demonstrated a resident of town Lom infected with *B. melitensis*, and later on Praussnitz described another patient with brucellosis caused by *B abortus*. Beiling reported several Bulgarian human isolates of *B. melitensis* , originating from Svishtov and Ihtiman regions, which were sent to him during 1914 and 1918. Mollov was the first Bulgarian author who diagnosed five cases of human brucellosis in our country. In 1913 Andreev, detected the disease in a 7-year-old boy from Plovdiv and published the case for the first time. Until 1948 more human cases were described by Detchev, Dobrev, and Ganov. The latter reported an infected veterinarian in the city of Silistra.

Ovine and carpine brucellosis, caused by *B. mellitensis* occurred naturally in Bulgaria in the past, but the country has been considered as free since 1941 (19). In the following more than filthy years few epizootics due to importation of infected animals were registered (83, 84).

Bovine brucellosis in Bulgaria was first reported by Acad. Stefan Angelov in 1924. He described 188 cases in cows for a period of ten years (1924-1933). Later on, Toshkov, Kuyumdjiev, Iliev, Ivanov and other authors reported brucellosis with different frequency in cattle, pigs, buffaloes, sheep, goats, and horses, mostly around Sofia and Samokov (82). Autochthonous animal cases of brucellosis caused by *B. abortus* were still reported during 1953-1954 (84). There was also evidence for sheep, horses, and dogs infected with *B. abortus* infection after contact with sick cows (84, 85). From 1922 to1980 bovine brucellosis was introduced several times in Bulgaria as a result of importation of infected cattle from Central and Western Europe (84, 86). During one of these outbreaks, caused by cows infected with *B. abortus*, 32 persons with brucellosis (breeders and veterinarians) were diagnosed by the National Diagnostic Research Veterinary Medical Institute. Cases of brucellosis in veterinary specialists during 1948-1950 were described by Angelov and Kuyumdjiev. Laboratory acquired infections were also reported, first by Ganov, in a 29-year-old technician and later (from 1966 to 1968) brucellosis was detected in 14 other laboratory workers in a Research Institute.

As a result of the strict measures undertaken by the National Veterinary Service (NVS) autochthonous cases of brucellosis in cattle, sheep, and goats were not registered after 1958(19). For the period 1958-2006 epizootics with non-pathogenic or low pathogenic for human species(*B. suis*, *B. ovis*, *B. canis*) were reported. Infections with the non-pathogenic for humans *B. suis*, v. Danika (biotype 2) occurred among pigs from the East Balkan breed (86, 87, 88). From 1987, when first reported officially, brucellosis in dogs caused by *B canis* is widely spread among domestic and free-ranging dogs (86, 89, 90).

Nowadays brucellosis is a re-emerging disease in Bulgaria (91). Thirty-seven persons with brucellosis were diagnosed by the National Reference Laboratory for High Medical Risk Infections (NRLHMRI) in 2005. Most of them were from the region of Sliven. All patients, except one who worked in Cyprus, were animal breeders in sheep farms or resided in Greece. Data from NVS for 2005 as well as for the previous years showed no evidence of *B. melitensis* and *B. abortus* positive animals in Bulgaria. Based on these and data obtained from the patients' epidemiological investigation, all 37 cases were classified as imported (81, 30).

In 2006, after a period of more than 40 years during which Bulgaria was brucellosis-free, the first autochthonous cases were detected by the NRL HMRI. This was the start of an outbreak, caused by *B.melitensis,* which occurred after illegal import of goats from Greece. By the end of 2008 several regions were affected (81). In four of them (Smolian, Haskovo, Yambol, and Stara Zagora) an epidemiological link between human and animal cases was established. The highest incidence of the disease was registered in 2007 when 58 new cases were detected ($0{,}74‰_{000}$). The majority of them occurred in Haskovo region. All infected persons have had contact with *Brucella*-positive animals and/or consumed dairy products from their milk. The epidemiological investigation showed that sale of infected animals without certificates led to the spread of the disease in 11 villages of three districts. By the end of 2008 a total of 88 autochthonous human cases were registered. All *Brucella*-positive animals (496 goats, 117 sheep and 7 cattle) were destroyed. The stringent measures undertaken by the medical and veterinary authorities led to a significant decrease in human case numbers during 2009-2014 with 0 to 4 reports per year without any epidemiological link.

In July 2015 a patient was diagnosed with brucellosis. He was resident of Kyustendil district and without any connection with the regions affected in 2006-2008. By the middle of August an outbreak focus was found with 31 newly registered cases (92). *B. melitensis* was identified

as the causative agent. A total of 36 patients were diagnosed until October 2015 (93). Based on data obtained during the investigation, breeding of animals and consumption of unpasteurised milk and homemade soft cheese appeared as the main risk factors for transmission of the disease. All infected animals were destroyed (94).

The investigation conducted by different governmental authorities pointed out that importation of infected animals from a neighbouring country, where the disease is endemic, was the reason for the occurrence of both recent brucellosis outbreaks in Bulgaria. As stated previously, free movement of goods and of people between EU Member States is a fundamental policy of the Community but has some negative epizootological and epidemiological effects, one of which is the re-emergence of brucellosis caused by *B. melitensis* in Bulgaria (81).

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