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Current Methods of Human and Animal Brucellosis Diagnostics

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ABSTRACT

Brucellosis is an urgent infectious disease of livestock and wild animals and the commonest human zoonosis. Diagnosis of brucellosis is rather complicated and it has to be obligatorily confirmed by laboratory testing. Direct bacteriological and molecular methods and indirect serological tests are used for brucellosis diagnostics. The choice of the diagnostic tools depends on the overall epidemiological situation in the region and the objectives of the study: validation of the diagnosis, screening (monitoring), cross-sectional studies or confirmation of brucellosis-free status of the region. The review describes current bacteriological, serological and molecular methods, routinely used for the diagnosis of brucellosis in humans and animals. The perspectives of brucellosis diagnostics are also discussed.

Keywords: Brucellosis; Diagnostics; Bacteriology; Polymerase Chain Reaction; Serology

1. Introduction

Brucellosis is a widespread infectious disease of livestock and wild animals and the commonest human zoonosis. The etiological agents of brucellosis are *Brucella* species—small, Gram-negative, aerobic, facultative intracellular, coccobacilli bacteria. About 10 *Brucella* species are currently known. They infect sheep (*Brucella ovis*), goats (*Brucella melitensis*), cattle (*Brucella abortus*), pigs (*Brucella suis*), dogs (*Brucella canis*), rodents (*Brucella neotomae*, *Brucella microti*) and some marine mammals, mostly cetacean (*Brucella ceti*) and seals *Brucella pinnipedialis*) [1]. In animals, brucellosis predominantly affects the reproductive system inducing abortions and reduced fertility. Human brucellosis is characterized by a long-term progression and the disorders of the nervous and cardiovascular systems and osteoarticular apparatus. The disease often leads to a partial or complete loss of the physical efficiency [2]. *Brucella melitensis*, *Brucella suis*, *Brucella abortus* and *Brucella canis* are the most frequently occurred *Brucella* species transmitted from animals to humans [3].

In spite of a significant progress in the brucellosis control, there still remain regions where the infection persists in domestic animals and, therefore, transmission to the human population frequently occurs. According to the World Health Organization data, more than half of a million human brucellosis cases are officially registered each year [4]. The traditional regions of human brucellosis spread are the countries of Mediterranean basin, the Near East, South America, and possibly sub-Saharan Africa [5]. However, the global epidemiology of brucellosis has greatly evolved over the past 15 years and new foci of human brucellosis have arrived, particularly in the Central Asia. The Middle East has traditionally been considered as an endemic area. Indeed, five of the ten countries with the highest incidence for human brucellosis are in this area, including Syria that has the highest annual incidence of brucellosis worldwide [5]. Seven republics of the former Soviet Union (Kyrgyzstan, Tajikistan, Kazakhstan, Azerbaijan, Turkmenistan, Armenia and Uzbekistan) are included in the 25 countries with the highest incidence of the disease worldwide, while another country of this region, Mongolia, is ranked the second [5]. These countries have emerged as the most important loci of human brucellosis worldwide in recent years. Since brucellosis is a serious public-health problem, the disease control has become a national priority in

these countries.

Besides a threat to human healthware brucellosis spread in livestock foci is also causing serious problems to the national economies. According to the International agreements on the veterinary regulation [6] if brucellosis is detected in at least one herd, the resettlement and sale of animals from the whole foci region should be prohibited. Such strict limitations lead to the significant brucellosis mediated economic losses. International studies confirm that the incidence of human brucellosis is significantly reduced in the regions where the brucellosis foci among domestic and wild animals are completely eliminated [5]. A crucial step for the detection and elimination of brucellosis is a timely and accurate diagnosis.

Brucellosis diagnosis is rather complicated because the disease may have an incubation period varying from 5 days to 5 months and can progress in various forms: acute, chronic or asymptomatic [7]. Symptoms of the acute phase of brucellosis—fever and weakness in humans and abortion in animals—are common to a wide range of different diseases [1]. Therefore the final diagnosis of brucellosis has to be obligatorily confirmed by laboratory testing.

Brucellosis diagnostics is based on bacteriological and molecular methods (direct tests), and serological *in vitro* and allergic *in vivo* methods (indirect tests) [2]. The choice of the diagnostic method depends on the overall epidemiological situation in the region and the objectives of the study: validation of the diagnosis, screening (monitoring), cross-sectional studies or confirmation of brucellosis-free status of the region [1].

2. Bacteriological Methods

The "gold standard" of the brucellosis diagnosis is the direct bacteriological testing: cultivation of *Brucella*, isolated from body fluids (blood, cerebrospinal fluid, urine and others) or tissues [8]. Identification of bacteria is based on their morphology, staining and metabolic profile (tests for catalase, oxidase and urease activities) [1]. For blood and other body fluids culturing the biphasic method of Castaneda is recommended [2]. Bacteriological diagnosis of brucellosis is severely limited by the fact that *Brucella* is a hazardous bacterium, and its isolation has to be done in specially equipped level 3 laboratories. Moreover, it is a very labor-intensive and time-consuming procedure. However, the isolation and cultivation of bacteria are also necessary preliminary steps for staining and biotyping of *Brucella* species.

The staining of bacterial isolates using Stamp method is the classical direct method of brucellosis diagnosis. It consists of the basic fuchsine staining followed by de-colorization with diluted acetic acid. Despite the fact that this method is non-specific and some other pathogenic bacteria such as *Chlamydophila abortus* and *Coxiella burnrtii* will be colored in a similar way, it is often used to obtain the preliminary results [9].

Biotyping of *Brucella* species, isolated from the biological samples, provides significant epidemiological data that allow tracing the focus of infection and the ways of its spread. Classical biotyping of *Brucella* species is made on the base of phenotypic differences of surface lipopolysaccharide (LPS) antigens, sensitivity to staining, CO_2 dependence, H_2S production and other metabolic properties, phage lysis, as well as the ability to grow in the presence of alkaline fuchsine or thionine [10]. Until recently, the methods of agglutination with antibodies against rough or smooth LPS, such as agglutination with antibodies against the A and M epitopes of O-polysaccharide chain, were widely used for biotyping. However, there is a cross-reaction with the epitopes of the surface LPSs from bacteria of some other genera, such as type of *Yersinia*. In addition, *Brucella* is a highly homomorphic genus and classic typing methods do not allow to differentiate isolates of the same species and biovars [11]. Moreover the classic methods of biotyping require standardized methods of analysis and highly qualified personnel to perform them and therefore are held almost exclusively in reference laboratories.

3. Molecular Methods

In order to avoid difficulties of bacteriological testing the molecular biological techniques, often based on the polymerase chain reaction (PCR) amplification, are successfully used for *Brucella* identification and typing [12]. The first crucial step of PCR based methods is DNA isolation from biological samples, since its quality has a significant impact on the sensitivity of the method [13,14]. Initially, PCR based identification has been developed for the determination of bacterial isolates [15], but now these methods are also used for detection of *Brucella* species in clinical samples of human and animals [13]. The most simple and reliable method of *Brucella* identification is PCR with a single pair of primers, specific to the bacterial DNA sequences, such as 16S - 23S rRNA operon, *IS*711 or *BCSP*31 genes [1,13]. Using a combination of several primer pairs for amplification of *BCSP*31, *OMP*2*B*, *OMP*2*A*, *OMP*31 genes, encoding the external membrane proteins, it is possible to identify the four *Brucella* species: *Brucella melitensis*, *Brucella suis*, *Brucella abortus* and *Brucella canis* [16]. Another method, based on the combination of seven PCR reactions, allows discrimination between six *Brucella* species [17]. There are PCR methods for identification of some *Brucella abortus* biovars [18] and distinguishing between S19 and RB51 strains of *Brucella abortus*, used for vaccination against pathogenic strains [19,20].

More effective method of diagnosis and identification

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of *Brucella* is multiplex PCR. It provides identification of all known *Brucella* species, including pathogens of marine mammals, at the species or even biovars level by using certain combinations of primer pairs. The first multiplex PCR based test for *Brucella* detection was developed in 1994 [21]. It allowed identification of the four *Brucella* species (*Brucella abortus*, *Brucella melitensis*, *Brucella ovis* and *Brucella suis*) and was named AMOS PCR for the first letters of species names. AMOS PCR identifies only a few biovars of each of the four species and can't distinguish individual biovars of the same species. Later on this method has been improved to detect more biovars and identify *Brucella* S19 and RB51 vaccine strains [22,23].

Recently a number of real-time PCR methods for *Brucella* detection in clinical samples were developed. The advantages of real-time PCR are speed (since there is no need to analyze the PCR products by agarose gel electrophoresis) [24], high sensitivity in comparison to the conventional PCR [25], and reduced samples contamination. Various samples can be analyzed by this method, including cell culture, blood, serum, and tissues [26].

Other methods of PCR based identification of *Brucella* include a multilocus analysis of genome regions with a variable number of tandem repeats (MLVA) [27] and multi locus sequencing of genome regions of the bacterial isolate (MLSA) [28]. These methods are based on the quantifying the number of tandem repeats in a particular locus of bacterial genome and are used for *Brucella* genotyping not only at the level of genus and species, but also biovars.

Although up-to-date PCR-based methods of *Brucella* identification and genotyping have several advantages in comparison with classical bacteriological methods, they also have some significant problems. The sensitivity and accuracy of PCR based methods strongly depend on the methods of DNA isolation and the quality of the isolated DNA (especially for multiplex PCR). There still remains the problem of false negative results, because the PCR is inhibited in the presence of some admixtures, such as EDTA, RNAases, DNAases, gems, heparin, phenols, urea, and many others, from the clinical samples or DNA isolation and purification procedures. False positive results may also occur as a result of sample contamination. It is further necessary to develop the positive and negative controls and standardize the conditions for PCR reactions with clinical samples [24].

4. Classical Serological Methods

The indirect methods of brucellosis diagnostics are based on the detection of the immune response to a bacterial infection. Most of these methods have been initially developed for testing of cattle and then were used to test the domestic goats and sheep (except for the analysis of milk), and later were adapted for the monitoring of certain species of wild animals [1].

The most commonly used serological tests are based on the detection of antibodies against the smooth surface LPS, since they are immunodominant antigens of *Brucella.* For the specific detection of *Brucella ovis* and *Brucella canis* infection antibodies against rough LPSs of *Brucella* are used.

The indirect methods of brucellosis diagnostics include agglutination tests, complement fixation tests, precipitation tests and primary binding immunoassays [2, 29-34].

The first serological test for brucellosis—slow agglutination test (SAT)—was described in 1897, it is based on the sedimentation of the complexes of IgM antibodies with *Brucella* cell antigens [29]. The reaction is slow since it requires an overnight incubation at 37˚C. SAT lacks specificity and sensitivity, although it is inexpensive and easy to perform.

Another broadly used simple method of brucellosis diagnostics is the Rose Bengal test (RBT) [29]. It is a simple spot agglutination test where drops of stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction. The results are received in several minutes. The test is an excellent screening test but may be oversensitive for diagnosis in individual animals, particularly vaccinated ones.

One of the approaches to remove none specific reactivity is the precipitation of high molecular weight serum glycoproteins. It is commonly done by addition of rivanol to serum followed by removal of the precipitate by centrifugation and either a rapid plate type agglutination test with undiluted serum or a tube test using serum dilutions starting at 1:25 [2].

Complement fixation tests provide the detection of anti-*Brucella* antibodies that are able to activate complement [31]. The complement system consists of a complex series of proteins which, if triggered by ananti-gen-antibody complex, react in a sequential manner to cause cell lysis. Since this test is difficult to standardize, it is progressively being replaced by primary enzyme-linked immunosorbent assays (ELISA) [2].

Indirect ELISA (iELISA) method is based on the specific binding of antibodies present in the test sample with immobilized antigen. The binding event is visualized using chemically or enzymatically derived fluorescent, luminescent or colorimetric reaction. Many iELISA tests are available on the market [2].

Antibodies against smooth LPS are used in all the above mentioned tests. They have a common significant disadvantage: O-polysaccharides of *Brucella* are similar to that of *Yersinia enterocolitica* and other bacteria. It leads to the false positive results and thus reduces the

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specificity of the test [35-38]. Partly this problem is solved in the competitive ELISA (cELISA), where the specific epitopes of *Brucella* O-polysaccharides are used as antigens, but the sensitivity of cELISA is significantly lower than the iELISA [39,40].

Another interesting method is the fluorescence polarization assay (FPA) [34]. It is based on the physical principle of the mass-dependent change of the molecules rotation speed in a liquid medium. The smaller the molecule, the faster it rotates and the depolarization of a polarized beam of light occurs. In FPA the serum sample is incubated with a specific *Brucella* antigen, conjugated with a fluorescent label. In case there are anti-*Brucella* antibodies in the serum, large fluorescently labeled antigen-antibody complex is formed, which can easily be distinguished from the unbound antigen negative control. FPA method has a high specificity but less sensitivity than iELISA [41]. In Europe and the USA FPA method is used in programs to monitor and control the spread of brucellosis, but it requires special equipment and it is not suitable for rapid and easy testing.

The *Brucella* specific seropositive response is the confirmation of the infection, but it doesn't provide any information about the type of *Brucella* species, the time of infection, the phase of the disease, or even that the animal actually has the disease at the time of sample collection, since antibody titer can be quite high for a long time after the acute phase of the disease. It is known that in the acute phase of brucellosis the IgM antibodies are primary produced, and then, after a short period of time, IgG antibodies are produced [2]. Amount of IgG antibodies may be reduced after treatment, however, a high level of IgG antibodies circulating in the blood, can persist in the absence of the acute phase of the disease. At the chronic form of brucellosis IgG antibodies dominate in blood samples, while IgM antibodies are not detected or found only in small amounts. Most iELISA methods predominantly detect IgG and its subclasses, and Wright reaction mainly detects IgM [1]. Thus, using the combination of these methods it is possible to obtain the kinetics of the immune response and to distinguish between acute and chronic phases of the disease.

Another group of tests are allergic or skin-allergic tests for brucellosis [2]. It identifies specific cellular immune response to the under the skin administration of *Brucella* antigen. This test clearly confirms the actual cases of brucellosis and allows distinguishing them from the false-positive results of other tests.

5. Recent Advances in Proteomic Methods of *Brucella* Diagnostics

Both classical microbiological and serological methods of brucellosis diagnosis, as well as PCR based methods have some significant disadvantages despite their intensive development. For serological methods the main disadvantage is the lack of specificity. As it was already mentioned, *Brucella* smooth LPS are very immunogenic, but they have a cross-reactivity with LPS of other genera of bacteria. This fact significantly reduces the specificity of the diagnostics. Moreover, some species of *Brucella* (e.g. from *B. canis* and *B. ovis)* have rough surface LPSs and cannot be detected using standard tests for the presence of antibodies against smooth LPSs. So the problems of recognition of stages and forms of brucellosis, as well as the disjunction of brucellosis infection and vaccination events in cattle are still remain unsolved.

An alternative immunological method of brucellosis diagnostics, which is able to solve the above mentioned problems, is the detection of antibodies to *Brucella* species-specific proteins in serum or in other animal tissues and body fluids. Over the last twenty years there have been many efforts to identify the immunologically active *Brucella* proteins to which antibodies are produces in the organism of an infected animal or individual in a quantity sufficient for their detection. For example, 31-kDa *Brucella* cell-surface protein (BCSP31) was isolated, cloned and characterized from the *Brucella abortus* cell extract [42,43]. It is an immunogenic surface protein, which is highly conserved (100% homology) among *B. melitensis*, *B. abortus* and *B. Suis*. Moreover, the recombinant BCSP31 is efficiently expressed and purified from *E.coli* cells, as well as specific monoclonal antibodies against BCSP31 have been obtained [44]. The combination of these properties makes BCSP31 a potential candidate for use in brucellosis diagnostic tests.

Besides BCSP31, 26 kDa periplasmic protein (bp 26), isolated from *B. abortus* [45] and *B. melitensis* [46,47], has also been cloned and characterized as an immunodominant antigen. This protein was detected in human and sheep blood in the acute phase of the disease, but not for chronic or asymptomatic form of the disease. Therefore bp26 can be used as a marker of acute brucellosis. Another 60 kDa protein HtrA/DegP, which demonstrated high activity in the reaction with serum from brucellosis infected animals, was isolated from a collection of *Brucella* recombinant proteins, cloned and expressed in E.coli. HtrA/DegP specific antibodies were detected in the serum of animals experimentally infected with *B. abortus*, *B. melitensis* and *B. canis* [48]. High immunogenicity was also observed for *Brucella* proteins Omp16, Omp10, chaperone GroEL, VirB operon surface proteins, the chaperone protein trigger factor TF [48-54]. In spite of the intensive search for *Brucella* proteins suitable for vaccination and diagnostic purposes over the years, the protein, which suits all the requirements and is able to replace LPS as a diagnostic antigen, is still to be found [55].

Recently the results of *Brucella* study using system

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biology methods of genomics and proteomics were published. Through the sequencing of the complete genomes of 8 bacterial isolates from five *Brucella* species (*Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella ovis* and *Brucella canis*) [56], it became possible to create a library of the predicted protein-coding open reading frames (ORFs) in *Brucella* genome. Such a ORFeome library was made for *B. melitensis* [57], but since the high degree of homology between the genomes of *Brucella* species [56], *B. melitensis* ORFeome library can be used to analyze any type of *Brucella.* The protein coding DNA fragments were amplified using PCR and cloned into the DNA vector, which has been transformed into *E. coli* cells. The resulting library consisted of 3091 clones, each containing a single ORF. It was 96.7 percent of the total number of found ORF [57]. *B. melitensis* ORFeome library was immobilized on DNA microarray and used for the analysis of the expression of *Brucellaabortus* genes [58].

System biology methods also provide possibility to identify the antibodies produced by the organism in response to *Brucella* infection and also to predict the serodiagnostic properties of bacterial antigens. *Li Liang* with coauthors analyzed antibodies produced by the organism of human and goats in response to the acute form of brucellosis caused by *B. melitensis* using proteomics methods [55,59]. The protein microarray, containing 1406 *B. melitensis* proteins, was designed. It was used for the analysis of human serum samples from the patients with acute brucellosis among the population of Peru, where brucellosis is endemic. As a negative control serum samples of clinically healthy patients from Peru and USA, where brucellosis cases are rare, were used. The immune response to brucellosis in goats, experimentally infected with *B. melitensis*, was also studied. 13 *Brucella* proteins (serodiagnostic antigens) were identified, for which antibodies were detected in the serum of *Brucella* infected patients, but not patients from the control group. 18 serodiagnostic antigens, recognized by the immune system, in experimentally infected goats, but not healthy animals, were also detected. Antibodies only against two common serodiagnostic antigens were detected in serum of both humans and goats. The observed results made the authors think that the immune response of experimentally infected animals (natural hosts of *Brucella*), differs from that one in humans [55].

In another work, using a protein microarray containing complete proteome of *B. melitensis* (3046 proteins), authors identified 122 immunodominant and 33 serodiagnostic antigens and characterized them according to their possible function, structure and cellular localization, significant for immunogenicity [59]. This microarray was used to diagnose a patient with a focal form of skeletal muscle brucellosis with negative results in agglutination test. Moreover, for the first time the kinetics of antibody production in humans not only in the acute phase of the disease, but also in the chronic form was measured using this microarray. It was shown that immunopathogenicity mechanisms of acute and chronic brucellosis have fundamental differences [60].

6. Conclusions

Diagnosis of brucellosis in livestock and humans is not a simple task. The "gold standard" of *Brucella* detection is its recovery from the host, but it is a labor-intensive and time-consuming procedure, that has to be done in specially equipped laboratories. Since that molecular diagnostic tools are becoming more and more common for brucellosis diagnostics. They are rapid, safe and cost effective in comparison with direct bacteriological testing.

PCR-based methods for *Brucella* identification in biological samples are becoming very important tools for brucellosis diagnostics at the species level and biovar level. However, PCR analysis of the clinical samples must be fully validated before the routine use in laboratory testing for brucellosis. Multiplex real-time PCR seems to be the most promising method of *Brucella* DNA detection. The next-generation sequencing methods can also be used for diagnostics of brucellosis. They are still too expensive, but they are becoming more and more popular and accessible.

Serological tests for brucellosis have been invented more than a century ago, however the perfect test has still not been developed. Traditional serological methods of *Brucella* diagnostics are based on the detection of antibodies, specific to the surface LPS. It leads to a low specificity of these tests. An alternative approach, which is able to solve the above mentioned problem, is the detection of antibodies to *Brucella* species-specific proteins. It seems like that there is no single universal immunodominant protein, but up-to-date proteomic methods allow analysis of the whole *Brucella* proteome in order to identify a series of such proteins. The methods of system biology can not only be successfully used in the diagnosis of brucellosis, but they can expand the understanding of fundamental biological processes in the *Brucella* infected organism, including those leading to the great variety in the immune response [61].

The technological advances should be associated with the development of new immunoassay multiplex technologies with fluorescent or chemiluminescent detection systems, *i.e.* protein microarrays. Recently, the mass spectrometry approach was suggested for identification and genotyping of *Brucella* [62]. This method provides fast and reliable identification of bacteria at the species level, but it requires special complex equipment, which is available only in big laboratories.

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All the above mentioned methods can be extremely sensitive and accurate, but they can't be used in field conditions, for example in farms, where laboratory testing is not available. Since that they are more suitable for *Brucella* detection in humans, but not in livestock. For the routing screening of animals simple and accurate point-of-care tests (such as lateral flow assays, immunochromatographic strips, portable microfluidic devices, cathodic electrochemiluminescence chips) are needed. So in our opinion the development of brucellosis diagnostic test is associated with easy-to-use point-of-care test for the preliminary diagnostics and high sensitive and specific methods for the further laboratory testing.

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