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Mini-Review Article

Different Methods for Diagnosis of *Brucella* and *Legionella* spp. in Various Samples

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Abstract

Community-acquired pneumonia and brucellosis are of the main agents causing mortality in the world. The most important limitations for the identification of these agents include their high resistance against environmental conditions, impossibility of rapid and on time diagnosis, low level of infective dose, and lack of existence of vaccine against many types of them. Hence, on time action and diagnosis of agent of infectious diseases has been regarded as a solution for preventing incidence of this type of disease. Because of the danger of *Brucella* and *Legionella* bacteria, studying isolation methods is one of the most important measures regarding detection of agent of this type of attacks. The present study is a review study, which is conducted using existing studies and also using library method in order to investigate the detection of two mentioned bacteria in various samples.

Keywords: *Brucella* spp, *Legionella* spp, Diagnosis

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Introduction

Brucellosis is a type of zoonotic disease, which can affect wide ranges of livestock and wild animals in addition to human. In human, brucellosis is a systemic disease, which can affect many tissues and organs and cause a series of nonspecific symptoms. *Brucella* species live in the body of many animals especially livestock, and human can be infected accidently through touching infected livestock or consuming their dairy products [1].

These bacteria are gram-negative coccobacilli, which can cause disease in human and animals [2]. Among 8 species of *Brucella*, only 4 species of *B. abortus*, *B. melitensis*, *B. canis* and *B. suis* are pathogenic for humans. *Brucella microti*, *B. inopinata*, *B. ceti* and *B. pinnipedialis* are isolated from animals but can occasionally cause disease in man [3]. After entering through skin or mucosa, *Brucella* bacterium would be phagocytosed by monocytes or multi-core leukocytes and because of the ability to survival inside phagocytosis and would be extended by blood flow to liver, spleen and other organs like heart, kidney, joints, central nerve system, and genital tract [4]. The latent period lasts for 1-3 weeks and sometimes 6-7 months in some cases.

In 30-50% of cases, outbreak of disease is acute yet it is gradual in other cases [4]. Brucellosis in human can be observed in three acute clinical, under acute, and chronic forms [5]. In the annul congress of American legioners in a hotel in Philadelphia in 1976, an acute pneumonia occurred, and as a result 34 legioners lost their life [6]. Since then, in different regions throughout the world, the disease has been reported and there have been some cases of isolating *Legionella* in community and hospital-acquired pneumonias. The prevalent disease exists in water channels, which is responsible for legioner disease, which is an acute form of pneumonia and can be fatal. In addition, it can be an agent of pontiac fever, which is also a self-limited disease [7]. On the other hand, simultaneous infections of *Legionella* have been reported with other respiratory pathogens like Streptococcus pneumoniae, *Haemophilus influenzae*, and *Mycobacterium tuberculosis*. *Legionella pneumophila* should be considered among creative agents of community and hospital-acquired pneumonia[8]. *Legionella pneumophila* is a gram-negative heterotrophic aerobic bacterium without spores and capsules with 0.5-0.7 µm in width and 2-20 µm in length. Types of this bacterium are polar, under-polar, or with lateral flagellum, needing cysteine and iron for their growth [8]. To date, over 49 species and 50 serotypes have been detected in this kind, in which at least 18 species are pathogenic for human [9]. About 85% of legioner disease can be caused by *L. pneumophila*, of which 50% can be created by serotype 1 and 10% can be created by serotype 6.

Water is the largest source for legionellas. The bacterium has been found in sweet water regions around the world. 1% to 3% of community-acquired pneumonia and over 1-30% of hospital-acquired pneumonias is resulted from Legionella. Mortality rate in different individuals has been from 5% to 30% and in old people and in individuals with immune system disorder has been reported even to 80% [10-14]. The mentioned bacterium can influence lungs through creating aerosol in water tracts and then can cause pneumonia. These systems have been the main agent of prevalence of infection resulted from the bacterium through creating micro-drops of infected water to *L. pneumophila*. Through inhaling these infected drops, the bacterium would enter lung and then would be swallowed by macrophages. During this process, *L. pneumophila* prevents connection of lysosome to infected phagosomes. Then, it would be augmented inside the vacuoles and then would be resulted in pulmonary edema and pneumonia, whereby this can be fatal [15].

Given that Legionnaires and brucellosis are of major causes of causing mortality in the world, these diseases have extremely debilitating side effects that can even cause death, so timely and accurate diagnosis is an important factor. To identify bacteria, in addition to clinical epidemiology and positive signs, we need related to the laboratory evidence (the positive results culture, serology and PCR methods) [16]. In this study with literature review various methods that have been used to identify the bacteria in clinical sample were collected.

Conventional methods

Culture

Although isolation and detection of *Brucella* have been considered as the most reliable and accurate method for detection to date, the method has also some limitations as follows: long-term culture incubation, positive response during 4-6 days, in some cases to 2 weeks and more than it, and in 2% of cases after 27 days. In addition, confirming identity of colony after passing required time for culture and need to biological safety cabinet (class 3) with specific laboratory conditions are other limitations of the mentioned method. To detect the bacteria, blood culture, lymphatic glands, and marrow cultures can be applied. To acquire better results, biopsy should be conducted in fever period. Some defects of this method include being time consuming, danger of infection of personnel, and gaining unreal negative results [17].

About *Legionella* which is in form of *cocobacilli* in clinical cases and tissue in gram stain. The difference between this bacterium and other bacteria is that it takes gram stain hardly. Hence, using fuchsin has been proposed instead of safranin in order to paint bacillus in a better manner. In order to cultivate and isolate this bacterium from specific environment of BCYE (buffered charcoal yeast extract) with incubation of 3-5 days is applied. Culture has a very high value as the golden standard method for detection of legionella, although long time of incubation for observation of colony (2-5 days) is required for selective and non-selective culture mediums, mentioned that effect of antibiotic treatment affected by culture is a disadvantage of this method [18].

Serology

Serological tests are generally divided into three areas: the classical or conventional tests, primary binding assays and developing technology.One of the serologic methods in detection of *Brucella* spp. is sero-agglutination or Wright test. Coombs Wright Test can be applied in cases that Wright test is negative and the result might be because of existence of blocking antibodies. Complementary stability test and florescent antibody test can be also applied for purpose of detection [17].

ELISA is one of the methods for measuring immune system response, which can be conducted in solid phase, and thus many hedges of immune evaluation methods in liquid phase such as long time of test, primary preparation and high unspecific joints would not be observed in the present method [19]. In indirect ELISA test, serum would be added to coated antigens in solid phase, and at the next stage antibody against the first antibody would be added.

If there are specific reactions among antigen, antibody, and substrate isolation of chromogen, colorful reaction would be created. Sensitivity of the method is more than direct ELISA method [19]. *Burcella abortus* S99 (Weybridge) is common strain applied in producing *Brucella* antigens for detection purposes, which its reason is genetic stability and lack of change in phase of colony of the strain[20]. Produced LPS from B. abortus S99 can be applied as an antigen in producing detection kits for infections resulted from melitensis and Switzerland *Brucella* [21].

In order to detect legionella infection, serological methods can be generally applied as follows: direct florescent antibody test (DFA) and indirect florescent antibody (IFA). Detection of urine antigen of LPS by enzyme immunoassay (EIA) is an adequate detection method with specification of 100% and sensitivity of 70-100% [22]. ELISA is one of the methods for detecting of *Legionella*. Studies have shown to use LP protein FLA and PILE as coating antigen for ELISA [23]. The serology methods are less sensitive compare with molecular methods, specifically in early stage of disease which antibodies are low. Also the serology methods can not known different species of bacteria. Serologic methods are not specific and antibody titers remain positive mostly for a long time after treatment, even in cases of complete coverage of the disease, so molecular methods cover these disadvantages. Additionally molecular methods have more sensitivity and specificity compare to serology methods [24].

Molecular Method

PCR

PCR is a fast and simple procedure which is not dangerous for executer and is an appropriate way to segregate the different kind of Brucella of sample. To recognize the Brucella types and brucellosis in human, even in food contamination, PCR is a good way to do on BCP31 or 16S-23S rRNA which are unique in *Brucella* spp.[25].

Application of PCR in infectious diseases has been summarized as follows:

- Detecting microorganisms that cannot be cultivated commonly
- Detecting microorganisms that have slow growth
- Detecting microorganisms that have been discovered recently and there is no method for detecting them so far, like Tropheryma Whippelii
- Scanning, detecting, and following response to treatment regarding virus viral infections.

The studies investigated that duplex PCR assay was done to simultaneous and rapid detection. Also multiplex PCR was accomplished to distinguish *B. pinnipedialis*, *B. neotomae*, *B. melitensis* and *B. abortus*. Technically PCR spread to detection the different types of *Brucella* and vaccine species but generally the distinction of bacteria including *Brucella* by PCR is costly and time-consuming because of preparation and primary process. Actually PCR need the certain proportion of some subjects including concentration of $MgCl_2$, dNTP and Taq polymerase to gain the best quality and high sensitivity and specificity. Also reach the denaturation and annealing appropriate time to get the yields and better result is time – consuming and costly and even need high qualified of executer [26].

LAMP (Loop-mediated isothermal DNA amplification)

Lamp is one of the isothermal reproduction that temperature is constant in all of steps. The reaction without any need to denaturation of DNA with assistant of polymerase is done by the ability of succession in DNA and also it would be used from 6 special primers.That is done about *Legionella* which is used from the method for detecting of *Legionella* species from each other, that has high specificity of 92%, but because of using of 6 primers is costly [27]. There are not studies that show that this method has been used to identify *Brucella* spp.

Real time PCR

Real time PCR is an assay with high specificity. Study shows that different species of *Brucella* recognized by using real time PCR which used primer and probe from bcsp31 and about 42% of cases was recognized by real- time PCR find negative with conventional PCR.This method has advantages over the conventional PCR. For example, absence of gel associated analyze at the end of PCR assay, leads to decrease in risk of contaminate and increase in the rate of analysis [28]. The technique of real time PCR for identification of Legionella spp. in clinical and environmental samples is used. In most studies, the genes of 16S rRNA and mip have been used [29, 30].

Microarray

The modern technology, which was innovated for the first time in 1996 and was named as DNA array, genetic chips, DNA chips, and biologic chips, is achievement of scientists of genetic science in order to gain an instrument with parallelizing, minimizing, and automating features for rapid study of genes. As traditional methods have not the required efficiency for studying genes, it is possible to study tens of thousands genes at the same time using microarray method. Microarray is an instrument for measuring and gaining information on genes. Every known gene sequence would be printed on a glass or nylon array as a prob. mRNA of tissue or blood sample would be marked by florescent colors and probs would become hybrid on an array.

In this method, stains containing DNA, RNA, protein or antibody with thickness of 20-200 micron would be arranged on a specific glass slide and make microarray or biological chip. These chips would become hybrid with desired samples and after conducting next steps by laser scanner device, obtained results would be analyzed and studied using relevant software. Using this technology, it would be possible to discover genes, to discover diseases, medicines, genetic scanning, to determine performance of new genes, function of recently known genes, how is interaction of genes, and to analyze genetic differentiation. Moreover, through this method, detection of pathogen viruses and bacteria, toxicology, classification of cancers such as leukemia and detection of genes would be possible for expression of tens of thousands genes at the same time. Two kinds of array have the most application, which are presented as follows:

i. DNA Complementary spotted

ii. Oligonucleotide Array, which is abbreviated to Oligo

In oligo method, every gene would be depicted in 16-20 forms, which every one can be short sequence of nucleotides and a perfect match (PM) is obtained from a piece of gene. Against these 20 nucleotides, there are other 20 oligonucleotides, which their central sequence is equal with each other and these nucleotides are known as mismatch (MM) nucleotides. A size of gene expression in average is intensity of differences in these 16-20 modes. In order to investigate information of DNA microarray, statistical software like SAS, S-plus, STATA, and R can be applied. R is more common than others because of its high capability in working with wide range of data [31].

Microarray is faster compare to culture based methods and the speed along with accuracy .The results of microarray cause to further using of microarray in food microbes. Microarray using increase in many countries in food industrial rapidly [32]. An extend range of human pathogens was identified in extract samples of dust from 19 region of Iraq including *Coxiella burenetii*, *Brucella* spp., *Mycobacterium* spp., *Clostridium perfringend* and *Bacillus* spp. which these microbes was distinct by some technique including microarray [33]. Some studies have done gene expression in Brucella ebortous by microarray assay which it accomplished to investigate the pathogenesis and biology of *Brucella* [34].

Conclusion

As resulted diseases from *Brucella* and *Legionella* bacteria have no specific and clear factor for detection and detection methods based on culture and isolation are mostly time consuming and have low accuracy, rapid detection of these pathogens is considered in important subjects for study. At the present study, recent advanced techniques that have high speed and accuracy in detection of pathogens have been studied. Advantages and limitations of using molecular methods have been also studied. Even in regard with using unit detection method such as real-time PCR approach, applied different target genes may limit detective sensitivity. It seems that using microarray method is suitable for detection of this type of pathogens. Thus, limitations of specific detection methods, along with other technologies, can be applied in order to confirm required sensitivity and specialty for results of detection. Combining it with other methods can enhance efficiency of specific evaluation.

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