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The Prospect of Molecular Epidemiology of Brucella Species in Iran

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ABSTRACT

Human brucellosis and its prevalence are directly correlated with the presence of animal infections in various regions. The infections associated with Brucella species have been reported in numerous animals. The incidence of these infections has recently increased in the endemic regions in Iran, including Zagros areas and the northeast of the country. Therefore, there have been difficulties in the diagnosis of the infectious cases since there is the possibility of encountering resistant strains. Furthermore, the spread of immerging strains is among the challenges that rapidly affect animals, even vaccinated livestock. Antibiotic-resistant strains are important in livestock since drug resistance may rapidly spread to humans. Therefore, continuous investigation is required in the case of drug resistance or immerging strains. Conventional typing procedures are no longer used due to several difficulties. Identifying the type of Brucella could provide adequate data on epidemiological surveillance, investigation of the infection outbreaks, tracking the diseases, identifying the immerging types, reviewing the success rate of eradicating the infections, and examining the outbreaks in the endemic areas. The reports regarding the application of molecular typing methods are still under development. Extensive research has been focused on the typing of brucellosis, proposing controversial results and aiming to improve the applied procedures. This review aimed to assess the ability of the introduced molecular methods and their status for identification and typing procedures. In addition, the frequency and distribution of Brucella species and subspecies have been investigated.

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Introduction

Human brucellosis is associated with numerous clinical presentations and diagnostic errors (1). According to reports, Brucella strain was first isolated in Iran from a bovine fetus in 1944 (*B. abortus* biovar three). In 1950, the isolation of *B. melitensis* (biovar one) was later reported in a sheep in Isfahan. Both types were also identified in human infections, as well as in various livestock across Iran. *B. melitensis* biovar two and three play a key role in human infections and are isolated from animal infections as well (2,3).

Currently, several Brucella species have been documented and confirmed based on their particular host, pathogenicity, and genomic and phenotypic characteristics. These species include *B. abortus, B. canis, B. ovis, B. melitensis, B. suis, B. neotomae, B. pinnipedialis, B. ceti, B. microti*, and *B. inopinata*. In some cases, the similarity of these species has been estimated at 100% in some parts of the genomes (4). At the biovar level, 8, 3, and 5 biovars has been identified for *B.melitensis, B.abortus* and *B.suis* respectively (5-7).

Conventional typing procedures include biotyping and biochemical tests, such as growth on media containing dye, hydrogen sulfide production tests, agglutination with monospecific antisera,

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and phage susceptibility assessments. However, these methods are no longer used since they are time-consuming and associated with the risk of handling live cultures, while requiring high expertise for interpretation. Moreover, the yield and speed of the recovery rate of organisms from blood specimens are less than 5% (8,9). Therefore, the present study aimed to evaluate the status of the disease in Iran using advanced molecular techniques for identification and typing. In addition, we attempted to provide a brief description of the previous studies regarding the frequency and distribution of Brucella species and subspecies in Iran.

Literature Review

The Status of Brucellosis in Iran

Brucellosis is a commonly neglected bacterial disease in the world. Brucella species have been reported to cause economic loss due to the severe morbidity in livestock. Several published papers have emphasized on the effects of environmental, social, and economical factors on the incidence of brucellosis. Currently, brucellosis has been reported in various provinces in Iran (10-12). In total, there were 68,493 registered cases of brucellosis during 2011-2014 in Iran.

Brucellosis infections are among the foremost emerging and reemerging diseases. Unprotected borders have led to the smuggling of livestock from the neighboring countries to Iran. Consequently, emerging brucellosis could be easily transmitted to the population by the infected livestock considering that animal husbandry is mostly traditional in the majority of the regions in Iran. As a result, it is expected that new types of these infections be observed with delayed diagnosis. Therefore, the identification of the Brucella types that are particularly involved in human infections could help researchers determine the status of the diseases.

Clinical forms of brucellosis may be acute, subacute, chronic, and even asymptomatic (1). Chronic brucellosis may be sero-positive, yield negative outcomes or not be isolated on culture media (13). However, this type of brucellosis may not be easily identified or diagnosed (6,14). The patients most probably receive ineffective treatments, and delayed diagnosis may lead to the progression of the infection to more complicated types. The progression of chronic brucellosis is defined as the interval between the manifestations of the initial symptoms and definitive diagnosis (14,15). Ineffective treatment or possible drug resistance may also cause relapse during the course of the infection. Relapse in the patients with chronic brucellosis is considered to be among the other consequences of the inadequate or improper treatment of the disease. Relapse often occurs within six months after the treatment, while the symptoms may be milder compared to the initial symptoms in approximately 5-40% of the patients (16). Routine methods cannot be used to differentiate these infections from reinfection. On the other hand, conventional techniques and polymerase chain reaction (PCR) only identify Brucella up to the species level (8).

The sero-negative cases of brucellosis may progress to complicated types, which are often observed in the hospitalized patients with the history of misdiagnosis or improper treatment. Misdiagnosis may be due to the presence of other species or subspecies as well, such as Brucella canis, which is not diagnosed by routine serological procedures (6,17). Unfortunately, this concern has not received the attention of healthcare professionals in order to upgrade the routine procedures in diagnostic laboratories (5). Applying reliable methods has been frequently emphasized and could be helpful with the use of an in-vitro diagnostic kit with high sensitivity and specificity.

The Frequency of Brucella Species and Subspecies in Iran

Several domestic studies in this regard have been carried out in various areas in Iran, which may not be considered proper investigations in terms of the epidemiological aspects. These studies have mostly reported the prevalence of various Brucella species in Iran, while the majority of these studies have basic defects in terms of the research design since they have used available samples instead of the random selection of the specimens. As a result, these specimens have not been able to thoroughly represent brucellosis even in the studied regions.

The majority of the available reports in this regard have been focused on determining the effectiveness of the applied methods rather than verifying the frequency of various Brucella species. This issue should be addressed properly, especially in the case of the reported molecular procedures (18-24). In some cases, the reported findings in this regard have been extracted from short student projects without addressing the requests of health services and their requirements. Nevertheless, these reports have only represented existence of specific species and subspecies rather than their prevalence. On the other hand, some of the recent, highly cited reports in this regard have clarified the frequency of Brucella species and subspecies in the endemic regions in Iran. Older references may not have properly represented Brucella species due to the emergence of new types, especially in the regions in the vicinity of

borders.

A recent report in Isfahan (Iran) has indicated that 62% of the isolates belong to *B. melitensis* type I although types II and III have also been identified. Furthermore, they have reported the detection of *B. abortus* types II, II, III, IV, and V (25). However, Bahmani has reported that *B. melitensis* was predominant in 20,236 strains based on gene sequencing analysis (69.3%) in human and animal isolates in Hamadan (Iran) (26). Similarly, Alamian collected specimens from humans and livestock, reporting that *B. melitensis* type I and *B. abortus* type III were the only detected types (27). Similar findings have been proposed in a research conducted in Fars province (Iran). In another study, Sharifiyazdi et al. (2012) identified Brucella species in milk samples (28). Moreover, they detected *B. melitensis* types I and II and *B. abortus* type III in Fars province (Iran). Other reports have shown the detection of *B. canis* in Fars province in various breeds of dogs (29).

Several studies have been performed in this regard in Sharkord (Iran). According to the findings, the incidence of brucellosis is significantly higher in this region compared to other areas. Furthermore, Saeedzadeh investigated the aborted fetuses of sheep and goats, and the obtained results confirmed that the isolates mostly belonged to B. melitensis type I, while a few cases belonged to B. melitensis type II.

The isolation of *B. melitensis* has also been confirmed in another report in this region (30), while Doosti et al. (2011) examined bovine blood samples and reported that the majority of Brucella spp. belonged to the *B. abortus* species (31). Investigation in Zanjan province has proved that B.abortus and *B. melitensis* are both existed (32,33). However, some investigations on dairy product in Kerman (Iran) have revealed that all the isolated organisms belonged to *B. melitensis*, and only one organism (*B. ovis*) differed (34).

Apparently, the variety of Brucella species is more than expected. Reports have only highlighted the presence of specific species and their subspecies without determining their prevalence due to the applied methods. As a result, the findings of these studies may be conflicting. Some of the recent, highly cited published papers have been able to clarify the frequency of Brucella species and subspecies in a few endemic regions in Iran.

Briefly, older reports have not presented proper indications for the spread of Brucella species due to the possibility of the emergence of new types, particularly in the regions in the vicinity of borders. All the applied procedures in these studies have been performed using home-brewed molecular methods in unapproved testing conditions, thereby leading to negative results due to low sensitivity.

Recent Improvements in the Molecular Diagnostic Methods of Brucellosis

The identification of the exact species is essential to the interpretation of the epidemiological data on animals and humans for the accurate diagnosis and controlling measures of brucellosis. Therefore, the typing of various strains is the main task of referral centers, which should be carried out continuously. Use of molecular techniques has proven successful for the initial diagnosis and specific identification of the involved Brucella species in the infection. Meticulous evaluation and optimization should also be considered in order to obtain the most advantageous uniformity and reliable results in setting up the selected protocol prior to the diagnosis of the specimens in the patients. The required parameters for the standardized diagnosis of brucellosis are specificity, analytical sensitivity, and optimization of the clinical samples and their volume (5).

Various target genes have been used to detect the Brucella genomes. The reported sensitivity and specificity vary in different studies. Furthermore, the criteria are also substantially different in various pairs of primers. The most commonly used primer pairs for the amplification of these genes include BCSP 31(B4/B5) (35), 16SrRNA(F4/R2) (36), 16s-23S 16S–23S intergenic transcribed spacers (ITS) (37,38), 16S-23S rRNA interspace (39,40) , IS711 (bruc1/bruc5) (41,42), omp2 (43), outer membrane proteins (omp 2b, omp2a, and omp31), proteins of the omp25/omp31 family of Brucella species (44), and arbitrary primers (45). The most simultaneously applied detection methods are based on the differentiation of *B. melitensis* and *B. abortus* (32,33). These protocols have been designed as a duplex or multiplex PCR, along with genus-specific regions.

One of the mentioned protocols is real-time PCR, which has been described by Redkar et al. (46). Real-time PCR uses IS711 elements as a target genome for the detection of three Brucella species, including *B. abortus, B. melitensis*, and *B. suis* (biovar I). However, Nagalingam et al. (2012) have recently reported a specific multiplex PCR for the detection of four Brucella species, including *B. abortus, B. melitensis, B. canis*, and *B. suis* (7). This recent format of multiplex PCR is known as Bruce-ladder PCR, which is a modification of the previously reported format AMOS PCR (47). The success of this tetraplex PCR has been frequently reported recently since its introduction in various studies. In addition to the identification of clinical specimens, all Brucella-specific primers could be

used to confirm the isolated organism in the necessary cases.

Brucella infections may occur in various parts of the body, including the nervous system, where the number of the detectable causative agents is relatively low. PCR protocols, especially real-time PCR, are associated with great advantage although their sensitivity and specificity have been reported variably (50% and 60%, respectively). This discrepancy might be due to various influential parameters, such as extraction methods, limited detection protocols, and type of the applied specimens (5,48,49).

Molecular Typing Methods

Epidemiological studies require rapid and accurate typing procedures (26,50). Several molecular methods have been applied for the typing of Brucella species, while they have not generally been accepted since they are not standardized or easily applicable as routine procedures. The most commonly reported procedures include multilocus variable number of tandem repeat analysis (MLVA), multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and PCR-restriction fragment length polymorphism (RFLP). These typing procedures are able to differentiate Brucella species, while their ability and outcomes are in conflict. Their outcomes are not completely identical in terms of the serotyping results at the subspecies level. Therefore, they are under constant evaluation and upgrading.

Typing methods (e.g., MLST) have enabled the confirmation of classical taxonomy and could represent a clue for the notions regarding single nucleotide polymorphism. However, MLST has been applied less frequently compared to the other typing methods and has been gradually replaced by other methods in numerous studies.

MLST has been reported to have several important limitations, including costly equipment, being time-consuming, and insufficiently discrimination in routine use (51). Variable number of tandem repeats (VNTR) loci exist in bacterial genome. Within the past few years, MLVA has become a promising differential technique, fulfilling most demands since it is simple and accurate and enables the possibility of typing. In addition, its outcomes are usable in epidemiology (52).

MLVA has improved remarkably within the past few years and is still being upgraded for better discriminatory results (53-55). There has been extensive global research on the typing of Brucella subspecies, while only few studies have been performed in this regard in Iran. MLST and MLVA have recently been compared with each other in terms of the typing of *B. melitensis, B. abortus*, and *B. suis*. It has been concluded that both these methods provide consistent conclusions despite minor disagreements (56).

PFGE is another method that has been considered a 'gold standard' typing procedure for pathogens. In this technique, intact chromosomes are digested using restriction enzymes in order to generate series of DNA fragments with various sizes to form different patterns. Since DNA fragments are substantially large, they need to be separated with special electrophoresis fields. The outcomes of this technique have been compared with PCR-RFLP in some studies, and these methods have been used for 27 isolated *B. melitensis* strains. However, Bahmani et al. (2017) have concluded that PCR-RFLP has been unable to separate human and animal *B. melitensis* biovar from each other and from vaccine strains (26). On the other hand, the PCR-RFLP analysis of omp genes has been reported to be useful for the differentiation of biovar in some other studies although it has revealed additional polymorphism within some biovars (57,58).

A comparative study in this regard has been performed on the *B. suis* strain using the PFGE and MLVA methods. Furthermore, it has been reported that both these methods are highly relevant to each other and to biological typing. Previous findings have also denoted that MLVA could be clustered into 23 genotypes, whereas PFGE could be clustered into nine PFGE genotypes. As such, it has been concluded that MLVA has a higher discriminatory power compared to PFGE fingerprinting for *B. melitensis* (59).

In another study, Chen et al. compared MLVA and PFGE in 63 isolated strains of *B. suis* (50). The obtained results have confirmed that these procedures could not effectively distinguish the type in the same species although MLVA is more effective than PFGE owing to its relatively higher discriminating ability. These conflicting results show that these techniques require further improvement.

Other applied typing methods are single-nucleotide polymorphism and whole-genome sequencing. These techniques need the development of databases to add all the identified genomes in the case of lacking such metadata, which may cause tracing difficulties, while providing more regulation compared to common typing methods.

Briefly, MLVA as a format of MLVA16 seems to be the most reliable method, which is widely used for the investigation of outbreaks (60,61). However, it should be standardized based on reference strains in order to adjust the results in the reference laboratories. The whole procedure of MLVA could be performed with cost-efficient and reliable results in laboratories without sophisticated electropho-

resis equipment. Moreover, this method is simple, cost-efficient, fast, and easy to use although the high-resolution agarose gels need to be used on electrophoresis, and the method lacks convenient, unambiguous calculations. Another disadvantage of MLVA is that the data cannot be compared directly in the laboratories applying this technique. Also, the occurring point mutation at any given time could influence the outcomes.

Numerous studies and non-identical results have confirmed that the mentioned protocols are still to be enhanced to become 'gold standard' typing procedures. In this regard, external quality assessment could be performed for the accurate, detailed definition of these procedures (48,62,63).

Conclusion

At the present time, these limited studies are not able to achieve a gold standard typing method, although the "MLVA" method has many advantages. Setting up a few regional reference laboratories with sufficient scientific capabilities and technical facilities at the endemic regions will help our knowledge to be upgrated more rapidly, with applying the effective implementation of the quality assurance program by independent body

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Conflict of Interest

The authors declare no conflict of interest.

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