Review Article

Role of Inflammatory Markers in Pathogenesis of Animal Brucellosis and Their Potential to be used as Diagnostic Tool

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ABSTRACT

Animal brucellosis is a contagious and zoonotic disease prevalent in many countries particularly in developing nations where unhygienic practices are more common. It is caused by an intracellular bacterium; hence the incubation period is prolonged. Most animals show clinical symptoms in last trimester which leads to economic losses. Trade restrictions are another problem for the endemic regions. In terms of public health, carrier animals are the source of the diseases either through consumption of animal products (milk, meat etc.) and also through direct contact with animals. The clinical diseases can be diagnosed through various methods including culture, serology, ELISAs, PCR, qPCR etc. However, the sub-clinical cases are difficult to diagnose and remains a threat to other animals and humans. So in this article the literature has been thoroughly reviewed regarding inflammatory markers that have potential to be used as diagnostic tools in carrier/ sub-clinical cases. Among these TNF-α, Interleukins, IFN-γ have been reported to be most widely used markers.

INTRODUCTION

Almost 60% of the infectious diseases are zoonotic, and they can be defined as the pathogens that inflammatory markers that have the potential Many food- borne zoonotic diseases are directly or indirectly transferred from animal products or they are playing their role as vector for transmission. The list is too long and among major diseases brucellosis is one of the neglected diseases which is prevalent around the globe and has equal importance for human and most of the animal species. Morphologically, causative organism is gram negative, intracellular, motile, non- spore forming, and coccobacillus [1-4]. Among the different members of Genus *Brucella* there is much DNA similarity present (>90%). The members of genus *Brucella* includes *B. abortus* (which mostly causes disease in large animals including cows. buffaloes, camel, equines and human), *B. melitensis* (small ruminants and human), *B. suis* (pigs and humans), *B. canis* (canines, human and cat), *B. ovis* (primarily sheep), *B. neotomae* (desert wood rat) and have many other species which includes *B. ceti*, *B. pinnipedialis*, *B. microti*, *B. inopinata* are very much importance in marine animals [5-6].

Brucellosis furthermore has major economic impact and causes the great economic loses (2.8 to 4.2 billion$) because of the reduced production, late term abortion, stillbirth, debilitating calves, in males' permanent infertility and low milk production in females and increased inter-calving interval along with trade allegation [6-7]. The 95.6% economic losses are shared by cattle in terms of livestock. Per animal cost (US$) in cattle is 6.8, buffalo 18.2, sheep 0.7 and 0.5 in goat indicating a major share of bovine brucellosis [7]. The disease is a huge threat to different species of mammals including domestic animals, sea mammals, food animals, fish of clean water origin [8] and wild life mammals [5]. Brucellosis in rodents is primarily caused by the *B. microti*, *B. neotomae* and certain biovars of *B. suis* they are considered as pathogenic species of genus *Brucella* in rodents [7-8].

Immune response of *Brucella* in most of cases is associated with murine infection. The activity and response of immune system in the host against *Brucella* infection is well explained by the murine model. However, the drawbacks of the immune system of host in response to *Brucella* can be well explained by the study of human and animal brucellosis. Very few studies have been conducted to understand the exact mechanism of infection of brucellosis and its pathophysiology in the host i.e. humans and animals. *Brucella* have the ability to replicate in wide variety of cells, however the main tropism is towards the endothelial cells, epithelial cells, fibroblasts and microglia. It replicates in the macrophages, dendritic cells and placental trophoblasts that limits its exposure to the host immune system and antibiotics as shown in Figure 1. Biomarkers are the measurable indicators in any biological condition that leads to alterations in cellular physiology or pathogenicity. In brucellosis these biomarkers can help in the diagnosis of diseases at early phase where this pathogen is just invading the reticuloendothelial organs [8-9].

METHODS

Literature search and selection criteria:

For this review, information was generated from various sources including Web of Science, Scopus, Google scholar, PubMed and google web etc. Keywords including animal brucellosis, bovine brucellosis, biomarkers for brucellosis etc. were used for the literature search. And only the published material with sufficient publication details and authentic sources were included. The flow diagram (Figure 2) presents the data about the selection of research articles reviewed in the present review.

DISCUSSION

Innate Immunity in Brucellosis

The invading pathogen is initially trapped by innate immunity, the first line of defense. Innate immune system produces the strong host immune response against the invading bacteria as well as stops the multiplication of pathogen, physical barrier, special kind of proteins like chemokines and cytokines, complement systems and proteins along with the phagocytes like monocytes and macrophages and certain kind of innate lymphocytes i.e., natural killer (NK) cells and dendritic cells all these are the key components of innate immune system [10].

Bacterial cell structures such as the flagellin, LPS and are identified by the innate immune

system by the non-specialized fashion and DNA components of bacteria is detected by the TLRs receptors the major TLRs involved in the detection and killing of bacteria are TLR2, TLR3, TLR4 , TLR5 and TLR9, the mechanism of TLR signaling helps to secretes out some kind of cytokines which enhance the release of certain kind of specialized cells and aid in the killing of bacteria, increases the phagocytic activity of macrophages and increases the capabilities of APCs to initiate the adaptive immune response [11]. Based upon the presence of LPS on the cell of the bacterium the *Brucella* is characterized into the smooth and rough kind of *Brucella* and the LPS is frail stimulator of TLR4. BtpA/TcpB and BtpB proteins potentiate the bacteria to interfere with the TLR signaling [10, 12]. These specialized proteins also interference the activities and signaling of other TLRs like TLR2 and TLR5 but the Signaling of the TLR9 is not so much influenced by these proteins so it is estimated that the TLR9 plays a vital role in the resistance of host mechanism against the *Brucella* [13].

The cytokines release plays a key role to protect against *Brucella*. The main cytokines involved in the protection against the *Brucella* includes TNF-α, IFN-γ and IL-12. However, some other kind of cytokine like IL-6, IL-1 and IL-8 are commonly released against the gram-negative bacteria. In experimentally infected mice with *Brucella* there was no release of such kind of cytokines and it might not be due to the fact that the mice are resistance but because of the poor signaling of TLR by *Brucella* [10-11].

In contrast to the salmonella infection in mice the concentration of pro-inflammatory cytokines increased 10 folds in *Brucella* infection. It was also reported that the heat killed bacteria enhance the secretion of TNF-α. While the production of TNF-α is associated with the live *Brucella* in human. Almost all similar reports have been published from the ruminants which supports the same evidence of TNF-α. However, in an experimental study, in a young heifer the ileum loop is ligated and it was noted that the release and concentration of the TNF-α was not affected by all these procedures, protocols and model and also not influenced by the attack of *B. melitensis* in young animals. In initial attack of *Brucella* on the trophoblast of the host. Omp31 is an outer membrane protein of *Brucella* which is responsible for the virulence of this pathogen for the different host range [10-11].

The core function of TNF-α is to activate and train the macrophages and their functions through both autocrine and paracrine signaling. Significance of TNF-α can be estimated from the fact that the production of IL-12 and IFN-γ is stimulated by the TNF-α and it is helpful in controlling the *Brucella* replication inside of mice by the joint activity of TNF-α and $CD8^+$ in IFN-γ knocked out mice and IFN-γ activated macrophages activity is stopped by the addition of anti TNF-α antibodies in mice from the murine model of controlling of *Brucella* [10,12].

In cattle, role of TNF-α and IL-12 in protection against the pathogen especially intracellular pathogen have been supported by many recent reports. In the local herds of cattle, the cows are naturally infected with the *Brucella* and the control and inhibition of replication inside of macrophages is totally different to that of the exotic European breed of cattle. In naturally infected cows with *Brucella*, the TNF-α and IL-12 are stimulated, however, in some animals the level of TNF-α and IL-12 are quite low and below the threshold level so not much significant for diagnosis [13].

However, on the basis of previous documented literature and reports by the scientist it was concluded that the IFN-γ has great role in the protection and defense against the *Brucella* infection [14], and this mediator is secreted by the $CD4^+$ and $CD8^+$ T-lymphocytes inside of macrophages mediated by the innate immune response in addition to this certain amount of IFN-γ is also produced inside the NK cells. The release of TNF-α is also responsible for the secretion of IFN-γ during the course of infection in cattle and the TNF-α is released just after the infection or future bacterial infection. In conclusion the IFN-γ plays vital role in the defense against *Brucella* in natural infected animals along with the *Brucella* immunology in host and TNF-α also have the same role because it just released after the infection and stimulate the macrophages to release IFN-γ which is helpful in combating the *Brucella* [11].

Adaptive Immunity in Brucellosis

For clearing of brucellosis infection, the innate immunity plays an important role to limit the bacterial replication and stimulation of adaptive immune system. The purpose of adaptive immune system is to limit pathogens with the help of B-lymphocytes and T-lymphocytes and

secreted antibodies. In case of *Brucella* there are three mechanisms are reported to control the *Brucella* by adaptive immune system [15]. Firstly, the activated macrophages kill the bacteria and the macrophages are activated by the IFN- γ that is activated by T- lymphocytes and produced by $CD4^+$ and $CD8^+$ this mechanism activate the macrophages, enhance their activity to kill the bacteria and secondly, this mechanism also involves in the killing of infected macrophages by cytotoxic $CD8^+$ T- lymphocytes thirdly this mechanism involves in the production of antibodies by the B- lymphocytes although this is not helpful in the killing of bacteria but helpful in the diagnosis of the disease [11,15].

IFN-γ and IL-2 have great role in the bactericidal activity of macrophages and these are released from the $CD4^+$ T- lymphocytes. The major agonist for the immune response mediated by the $CD4^+$ T-lymphocytes which involved in the production of IL-4, IL-5, IL-10 and IL-13 and the major functions of this immune system is to active the B- lymphocytes which in turn produces the antibodies. The significance of Th1 system was first understood from the mice model and from that time to till date it has been used to understand the pathophysiology of *Brucella* in the host cells. IFN- γ is first cytokine which has been secreted by the culture of splenocytes, T-lymphocytes, and several blood mononuclear cells inside the hos which is particularly the humans and animals this cytokine is secreted particularly in response to the *B. abortus* antigen [11, 16].

It has been observed that in murine model if the there is no production and lack of secretion of IFN-γ then the chances of the infection inside of host is several times more as compared to the animals in which IFN-γ is secreted by the T-lymphocytes. The lack of secretion of the IFN-γ has been reported in mouse model by injecting anti *Brucella* antibodies it was also observed that the IFN-γ neutralization in *Brucella* infected mouse the spleen serves as the bacterial colonization place [11-13, 16-17].

In cattle it has been also observed that after the vaccination against the *B. abortus* in there is deficit in production and secretion of the IFN- γ in addition to delayed or absence of Th1 response against this vaccination n strain this is due to lack of efficacy of vaccine in ruminants. It was also explained earlier that the release of IFN-γ is totally dependent upon the TNF-α and IL-12 any defects in these two cytokines may leads to the decrease or no production of IFN-γ it was first described in BALB/c mouse as compared to the C57Bl/6 in which increase production of IFN- γ so it is concluded that the susceptibility of infection is totally depended upon the secretion of IFN-γ in the host [16-17].

Polymorphism of Cytokines genes in brucellosis

Cytokines are basically the glycoprotein particles and are key players in controlling the hematopoiesis, inflammation and immune response particularly humoral [18-19]. Different cell types are responsible for the production of cytokines and they are very specific to act locally in paracrine fashion. Additionally, a huge number of polymorphisms pointed at the areas of cytokine genes that are significant in controlling expression i.e., promotor region. Due to some regular location in these important areas, it is theorized that they have some crucial role in the gene expression. Microsatellites are basically the short sequences of DNA that are composed of repeats of 1-5 base pair units. Polymorphism results because of the allelic variations. Microsatellites are randomly distributed over the genome, their biologically functions are still unknown but their abundance and regular positions close to the gene may be the hint that they are very much significant biologically. Cytokine SNPs are present in many cytokine genes [20]. Some selected cytokines and their polymorphism are discussed below.

Tumor Necrosis Factor (TNF) genes in brucellosis

In cattle the gene encoded for TNF-α is located on the chromosome 16 and has a length of approximately 33.5 kbp and splited into 10 exons and 9 introns. TNF- α forms the complex with those of other surface glycoproteins i.e., TNF-RII and then they play an important role in immune response and huge number of mutations have been recorded in different regions of this gene (dbSNP, NCBI, and Gene ID 338033. The gene also contains cAMP responsive element which is important for the gene regulations. The promotor region of -1 to -99 is responsible for the maximum secretion of TNF in the T-cells in addition to this an element with the repressor region is also situated in between -230 to -254 position. The UTR (Untranslated region) of 3' end also contains AU- rich element which is responsible for regulation of gene beyond transcription by

destabilizing the mRNA and also interfering the translation process. The TNF-α plays a potent role in inflammation irrespective of its kind of stimuli, however its upregulation has been observed in many chronic inflammatory conditions like brucellosis, tuberculosis, cancer, septicemia and AIDS (acquired immunodeficiency syndrome etc. In brucellosis, it has been reported that TNF- α production promotes the macrophages responses to clear the infection, however it has been reported that TNF- α in combination to IL-2 contributes towards immunopathology of *Brucella* [21-22].

Interferon-γ (IFN- γ) and its significance in brucellosis

The gene encoding IFN-γ is located on chromosome 5 in cattle and distributed with 4 exons and 3 introns with a length of 2.7kbp. Transcriptional mechanisms of IFN-γ are thoroughly explained previously. IFN- γ gene expression is strongly co- related with the methylation of CG region located at first intron and promotor region of gene. The promotor region is located at the position of 500 bp on the gene [22].

A microsatellite located at the first intron of the IFN-γ gives rise to the further 6 alleles and they have mutable CA repeats. The presence of allele 2 of microsatellite of this gene is significantly co related with in *vitro* high production of IFN-γ on the same side this microsatellite at the 5' end has been associated with the SNPs of CA repeats of first intron of IFN-γ gene (+874 T/A) that is positioned at putative NF-kB region, so, there is direct co relation between T-allele and allele 2 of the microsatellite of IFN-γ gene. Although one study also indicated that there is no co relation between IFN-γ microsatellite and IFN-γ levels [22].

Interleukin-6 (IL-6) gene and its potential significance in brucellosis

In bovine gene encoding IL-6 is present on the chromosome number 5 and it consists of 5 exons which are separated by 4 introns. The length of this is 5kbp. There are several potential binding sites have been pointed out so far, such GRE on AP-1 region and CRE an NF-kB on the promotor region of the gene. In addition to this a multiple responsive element (MRE), a NF-IL6 binding site and an IL-1 responsive element have been identified. IL-6 is one of the major acute phase players and regulates the acute phase response in the hepatocytes of human, and acute phase response is defined as the major homeostasis mechanism of the body to restore the normal state of the cell in any type of cell injury, infections and immunological disorders of the body. The regulation of major acute phase proteins, i.e., C-reactive protein (CRP) and serum amyloid A (SAA) is directly and indirectly linked to the IL-6 secretion. The regulation and activation of immune system is also depending upon the IL-6. IL-6 acts on B cells activated by IL-4 and IL-5 to induce the production of *IgM*, *IgG* and *IgA* and causes their terminal differentiation into plasma cells [23].

Acute Phase Proteins Response in Brucellosis

Homeostasis is defined as the state of steady internal body condition [24]. While the metabolism inside the body is under the influence of feedback mechanism and this mechanism in turns controlled by the different kinds of hormones and enzymes. Different kinds of infections (viral and bacterial), neoplasm and any physical and chemical trauma can change this homeostatic condition of body and results in cell injury which inflicts a local inflammatory response [25], which ultimately leads to the infiltration of inflammatory cells like monocytes, lymphocytes and increase migration of neutrophils in addition this vasodilation and coagulation occurs. Due to local inflammatory response vascular permeability of the blood vessels is increased which facilitates the leukocytes and leads to the development of inflammation with all five cardinal signs, [26]. Immune cells including macrophages, dendritic and mast cells recognize the condition and initiate the cytokines secretion which ultimately leads to a cascade of changes that is known as acute phase response comprised of acute phase proteins (APPs) synthesis, primarily from liver [27].

APPs are produced within hour of the infection or when the immune system initially recognizes the local infection or trauma. The acute phase response being the part of innate immune system initiate series of cascades i.e., complement system. When this complement system initiated this further activates the adaptive immune system this offers very precise defense mechanism against invading pathogen. The main purpose of all this acute phase response is to restore the normal homeostatic condition of the body but when the inflammation still persists it

may lead to the chronic inflammation [27-29]. The effects of different kinds of cytokines on the regulation of different acute phase proteins (APPs) are thoroughly explained by the different researchers at different times among them interleukin 6 (IL-6) is thought to be the principal acute phase mediator because it involves in the regulations of a number of plasma proteins further its concentration is directly linked to the concentration of these APPs in the blood [30-34].

The main bovine APPs includes c-reactive protein (CRP), Haptoglobin (hp), serum amyloid A (SAA), Hepcidin (Hepc), albumin, fibrinogen, transferrin, α-1 acid glycoprotein (AGP) and ceruloplasmin [31, 35]. APPs are mainly classified into two categories i.e., positive APPs and negative APPs the positive APPs are those proteins whose concentrations are increased by 10-100 times during the onset of infection while the concentration of negative APPs remains unchanged or decreases instead in any acute phase response [31, 36]. The major positive APPs in cattle are Haptoglobin, serum amyloid A, fibrinogen, α-1 acid glycoprotein and lipopolysaccharides binding protein, while the albumin, transferrin and ceruloplasmin are considered as the negative APPs in bovines [35-36].

a) Haptoglobin (Hp)

Bovine haptoglobin is a colorless protein synthesized in the liver and it has great affinity to bind with the hemoglobin of the red blood cells [37]. Structurally the haptoglobin is made up of 2 chains α and β the sizes of these chains are 20 kDa and 35 kDa, respectively and these two chains are linked together by disulfide bridge [38]. During the local inflammatory response, the Hp binds to the hemoglobin prior to the degradation by the bacteria, limiting the pathogen to gaining access to the free iron molecule that is main source of bacterial propagation [37-38], due to this fact the level of Hp starts increasing within hours of the inflammatory response and just after 24 hours the Hp level is manifolds higher in the blood, which attributes to be a good indicator for diagnosis.

b) Serum Amyloid A (SAA)

Serum amyloid A (SAA) protein was isolated named in 1970s. Basically, SAA is an apolipoprotein that has strong association with the HDL (High density lipoproteins). This protein has the striking role in the APR. the size of SAA is 20 kDa and this protein has common structural similarities in different species. As far as the bovine serum amyloid A is concerned, it has many isoforms, even the forms of SAA are variable between healthy and diseased animals [35, 38].

c) α-1 acid glycoprotein (AGP)

α-1 acid glycoprotein (AGP) is previously known as seromucoid or orosomucoid and the size of AGP is about 42 kDa [39]. AGP is a glycoprotein which is highly glycosylated. The striking role of AGP in acute phase response is still unclear but it belongs to the class immunocalins which have diverse immunomodulating functions [35, 40]. As mentioned above that the cytokines have great role in the acute phase response and these cytokines are released from the monocytes, AGP plays a crucial role in the release and suppression of these pro-inflammatory cytokines from the monocytes in addition to inhibition and aggregation of leucocytes. On the other hand, S/ C injection of turpentine to calves inflicts low increase in concentration of this APP, the difference in both conditions is this that the turpentine injection causes the local tissue damage which in turns results in the local inflammatory response while the LPS stimulation is just similar to bacterial infection. Ceciliani *et al.* [31, 35] has described a comprehensive detail about the functions of A more extensive and detailed description of the functions of APPs including AGP.

d) Hepcidin (*Hepc*)

Hepcidin (Hepc) is one of the positive acute phase proteins and is encoded by the HAMP gene [41]. In cattle it is a main regulator of the entry of iron in the body. This protein exists in the form of preprohormone and total number of amino acids present in this protein are 84. Biochemically Hepc is made up of the 4-beta plated sheets and these chains are linked together by disulfide bridge [42]. Hepc is iron regulator and plays an important role in the regulation of iron during the

acute phase response particularly in local inflammatory response [35, 43]. It inhibits the iron transport by binding to the iron transport channels located at the enterocytes and reticuloendothelial cells of plasma membrane. During the chronic disease the level of this protein is upregulated and the level of the iron decreases because of the high absorption of iron by macrophages [35, 44].

e) Fibrinogen (Fb)

Fibrinogen (Fb) is largest acute phase proteins with the size of 340 kDa and its main role is in the blood coagulation and fibrin formation [39], but in bovines it is considered to be the positive co-relation with the chronic diseases. Fb is already present in high concentration in the plasma, so the concentration of Fb is not dramatically changes during the acute phase response, only the 2-3 folds increase in concentration of Fb any infection is recorded its concentrations still remained elevated even after the 3-4 days post infection and if the disease continues its level remains elevated [42].

Potential use of APPs as biomarkers in brucellosis

Huge research has been carried out discovering the diverse role and association of APPs in chronic diseases more precisely the main role in the severity of diseases. In bovines, tuberculosis along with the respiratory tract infection in calves are the most widely diagnosed conditions globally and possess economic stress on the farmers [44-45].

The massive increase in the concentrations of the APPs, directly depends on the extent of the inflammation i.e., On the other hand, S/C injection of turpentine to calves inflicts low increase in concentration of this APP, the difference in both conditions is this that the turpentine injection causes the local tissue damage which in turns results in the local inflammatory response while the LPS stimulation is just similar to bacterial infection [31]. Difference in the APPs concentrations and changes also depends upon the nature of the pathogen and host immune response against the pathogen like huge changes in the concentrations of these APPs are recorded in bacterial infection as compared to viruses in addition to this the clinical signs also different in both type of infection [46-47]. Acute and chronic inflammatory infections can be differentiated on the basis of correlation of APPs including AGP, Hp and SAA [48], although the Hp and AGP values sometime intersect during inflammatory conditions as the Hp values are more pronounced in chronic conditions, while SAA is good indicator for acute reactions in cattle [49-50]. APPs values can be used for diseases tracking as the higher Hp concentration alongwith lower Alb concentration is an indicator of uterine infections in cattle at postpartum [51-52]. However, the younger calves show different responses and some time these APPs values can't be used as sensitive marker for the identification of diseases [53]. In experimental respiratory tract infections Hp concentrations were meaningfully raised already after two days post infection in calves that finally died from the infection. In freshly diseased calves, higher concentrations of Hp predicted that more antimicrobial treatments were required for the recovery from BRD [54]. A blend of early Hp and Fb measurements offers a more specific prediction for the severity of BRD, when sensitivity and specificity of Hp, Fb or Hp+Fb for recognizing BRD is considered [55].

Clinical diagnostic tests for brucellosis and their Limitations

Previously the diagnosis of *Brucella* was done through classical assays like oxidase and urease activity, H_2S production, CO_2 requirements etc. Later on, many diagnostic assays were developed [4, 56]. Here is an overview of most widely used diagnostic test for the animal brucellosis along with their potential limitations for the control of outbreaks:

Limitations of Rose Bengal Plate Test (RBPT)

Rose Bengal Plate test (RBPT) is used to screen the whole herd for brucellosis in ruminants [57-60]. This test is based on antigen-antibody reaction which shows agglutination as a positive result. To perform this test, equal volume of the serum and smooth *Brucella* culture stained with Rose Bengal dye in a mixed acidic buffered suspension is used. To avoid the unwanted or non-specific agglutination acidic buffer medium is used. High temperature has great influence on the specificity and sensitivity of test that's why laboratory is an ideal place to run the test

[59-60]. However, because of the easiness and high sensitivity this test is most frequently used at field level to screen the cattle and buffaloes herd as a pen side test. Although the sensitivity and specificity of RBPT is high but like other serological tests it has some limitations like it cannot differentiates between infected animals and vaccinated animals and some time it gives false positive results because of the cross reactivity with other serotypes like *E. coli O157* strains and some salmonella and *Yersinia* strains [57-58]. Furthermore, the specificity of this test is far low compared to other more superior serological and molecular Assay. Sometimes RBPT get interfered with the other bacterial infections like *E. Coli* , *Salmonella* , and *Y. enterolitica* . Different researchers have reported huge variation in terms of sensitivity and specificity of RBPT, so always positive results need to be verified by some most specific test [16, 57-58, 60]. The RBPT mainly detects the immunoglobulin G (IgG), but it can also detect Immunoglobulins M (IgM) [61]. The false positive results mostly observed during early phase (first week) of infection. Due to the cost effectiveness, highly sensitive and easy to perform Because of cost effective, easily performed and high sensitivity The RBPT is still a significant test to screen the bovine herds at field level [60].

Limitations of Enzyme linked Immunosorbent Assays (ELISA)

The main principle of ELISA is to identify antibody-antigen complex and antibodies isotypes in the serum of infected and vaccinated animals. In addition to other serological tests like RBPT, CFT and SAT ELISA is other more frequently used test for the detection of *B. abortus* [62]. In this assay complete cell or antigen or purified or semi purified crude lipopolysaccharides (LPS) are mostly used for detection of *Brucella*. Many kinds of ELSA have been developed for detection of antibodies from milk and serum including direct, indirect, and competitive ELISAs [63].

In spite of huge advantages of ELISA, it has some disadvantages as well, like it is not a cheaper/ cost effective for small farm holders, requires well develop and sophisticated laboratory in addition to trained staff, however it is easier to perform having same specificity to the CFT [65]. Gul *et al.* [57] also reported that ELISA is most suitable diagnostic test for confirmatory diagnosis of brucellosis for individual animals although the area where the vaccination is routinely used at early age [66].

Limitations of Molecular techniques

Polymerase chain reaction (PCR) is most important test to identify and differentiate the *Brucella* at specie level. For the diagnosis of brucellosis, Bricker and Haling [67] developed for the first time PCR assay named AMOS-PCR to differentiate the prevalent species, however identification of biovars through this assay is still not possible. During last few years Garcia- Yoldi and coworkers [68] developed another novel technique which is Bruce-ladder multiplex PCR that is one step assay which utilized primer pairs for the differentiation of all species of *Brucella* and its biovars. To reduce the chances of false results, Multilocus Sequence Typing (MLST) and Single Nucleotide Polymorphism (SNP), Multilocus sequence analysis (MLST) can be used for the construction of phylogenetic tree of different species of *Brucella* [69]. Another technique known as Multilocus variable number tandem repeats (MLVR) is used for the detection and differentiation of different isolates of *Brucella* and it considered as important molecular epidemiological tool. The whole genome sequencing is used but now it is being replaced with single nucleotide polymorphism (SNP) typing because it provides most suitable data at unbiased approach and cost effective in terms of other molecular techniques [70-71].

Isolation of *Brucella,* its bio-typing and associated limitations

Various methods used for growing *Brucella* have already been devised, ordinary basal medium is regarded as the best environment for this infection [90-91]. Vizcaíno *et al*. [72] discovered that adding 5-10% regular serum to the solution speed up the initial extraction of *Brucella*. [73]. On sera dextrose agar, *Brucella* colonies look translucent or yellowish caramel- colored Clusters have a clean, glossy appearance and are excessively high and rounded. In liquid media, *Brucella* grew slowly, while culture on a stationary liquid culture expedited the transition from smooth to rough types. Additionally, it has been noted that growing in liquid medium necessitates a prolonged incubation duration. For the detection and characterization of *Brucella* using densely mixed infected samples, extra growing media such as Farrell's bilayer medium and a water phase of Bordie Sinton's medium are typically utilized [71, 74].

Phage identifying, which relies on bacteriophage lysing the bacteria, and a measurement of redox metabolism patterns on specified proteins and glucose sources are the two main prevalent methods for biotyping. This last technique is risky, time- taking, and demands sophisticated equipment. [71].

GLOBAL DISPERSAL AND CONTROL OF ANIMAL BRUCELLOSIS

More than a half of million cases of human and animal brucellosis have been reported each year globally [66]. From the previous documented literature, it is concluded that the brucellosis is prevalent in almost every part of the world, however some developed countries like USA, NZ, Germany, Australia and Canada have declared brucellosis free countries [66]. Higher incidences have been recorded in poor or developing countries due poor husbandry practices along with the poor management and lack of abortion data at farm level, poor disposal of dead and aborted fetuses and calving at milking places etc. are core problems which are directly related to the high prevalence of disease [6].

Among the livestock species the highest prevalence of brucellosis has been observed in bovines and it was reported by various researchers at different times from the various part of the world. The reported prevalence in bovines is in range of 0-24% in different regions however the prevalence is highest in Asian pacific regions and in middle east countries [5, 75]. In central America and related countries almost 10-20% population of the cows and buffaloes are affected with the brucellosis, while in Mexico the incidence of brucellosis ranges in between 10-30% in bovines [66], posing serious threat to the human populations because of the zoonotic importance. In many European and most of the developed and Asian countries the small ruminants are potential source of disease as compared to the bovines [76-78].

In African countries the incidence of brucellosis is high, there is no proper legislation for the control of brucellosis exist. Different members of the genus *Brucella* like *B. melitensis, B. suis, B. canis*, *B. neotomae* and *B. pinnipedialis* are not specific to cause the disease in their host, in addition to cause the disease in primary host there is also the cross reactivity to different host species exist [6, 77]. Brucellosis is prevalent worldwide, however the exact figure of prevalence is missing in different parts of the world or a few reports are available which correctly elaborates the prevalence of brucellosis in different regions of the world, because of the tricky pathogenesis and attack of the causative agents and lack of possessions to track the disease in different locales to know the exact prevalence of disease in the respective regions. As this is chronic disease, antibiotic therapy due to antimicrobial resistance like many other poultry and livestock diseases does not work to cure, hence some alternative options like early diagnosis and culling remain a choice for eradication of disease from dairy herds [78-80].

Conclusion

Animal brucellosis has been endemic to many parts of the world and its prevalence rate is much higher in developing countries and being zoonotic, posing a serious threat to public health. The diagnostic strategies being used mostly work for the clinical cases, however, sub-clinical and carrier animals are misdiagnosed or declared false negative due to the limitations of these diagnostic assays. But they are continuously spreading the infections among the bovine herds as well as to the humans. So in this perspective, these inflammatory markers can be helpful for the early diagnosis of the latent infections in bovines. Similarly, they have much potential to be used as a diagnostic tool for the control and eradication of bovine brucellosis. Vaccination has its own drawbacks like, if animals are immunized during gestation, the vaccine may lead to miscarriages. Extremely small amounts of Rev-1 are risky and may not provide sufficient protection. Nevertheless, with brucellosis vaccinations, the level of protective immune response generated, tolerability difficulties for both males and females, interaction with serological assays, variable protection period and vaccine uniformity are all possible issues needs to be focused.

CONFLICT OF INTEREST

We have read and understood ALS policy on declaration of interests and declare that we have no competing interests.”

AUTHOR CONTRIBUTIONS

All authors have contributed equally for the draft preparation, editing and proof reading before the submission to the journal.

Figures

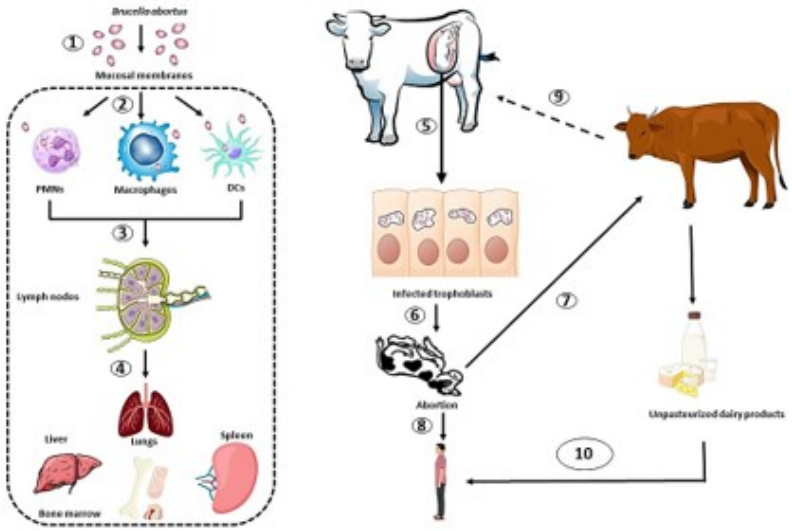

Figure 1: Transmission and Pathogenesis of brucellosis in humans and animals. 1) B. abortus entered in the body, 2) engulfed by the macrophages, polymorphnuclear cells or dendritic cells, 3) gets entry into lymphatic system, 4) spread towards visceral organs including lungs, liver, kidney and bone marrow, 5) via hematopoietic route reaches to gravid uterus having special affinity for trophoblasts, 6) leads to abortion, 7) animals may get infection from the infected fetus, fetal fluids and membranes, 8) Humans exposed directly to aborted fetus can be infected, 9) Carrier animal excrete the pathogen in milk, and 10) humans get infection through consumption of milk and its products.

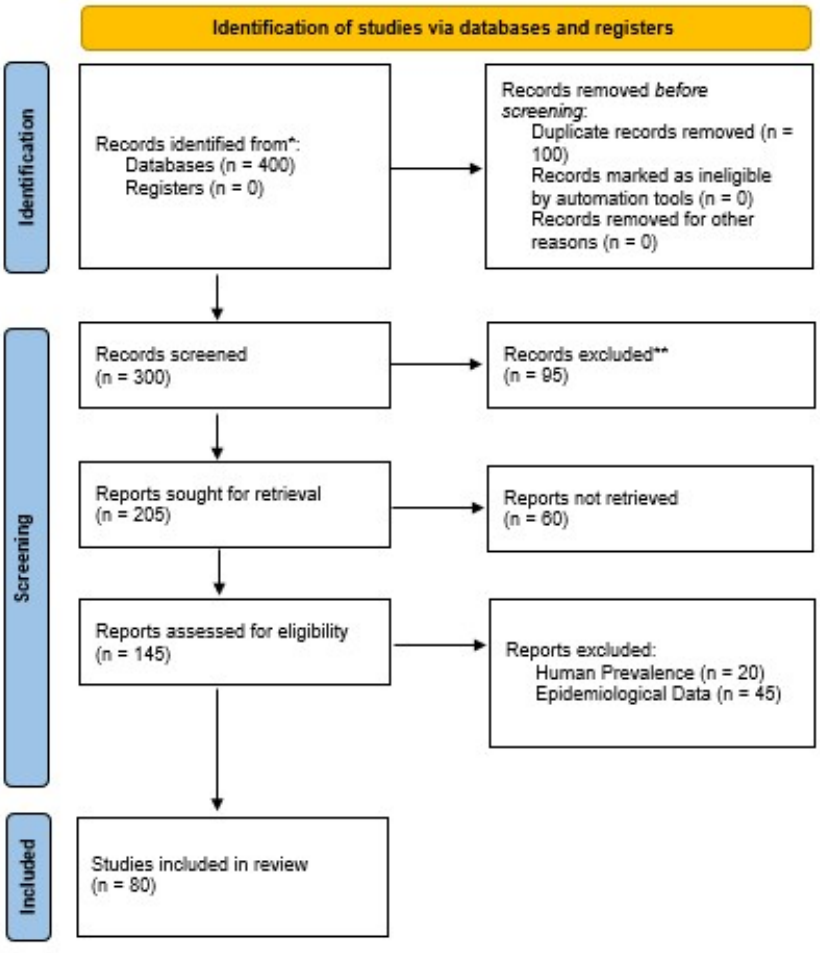

Figure 2: PRISMA 2020: Flow diagram of the study selection for this review.

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