Natural Resistance Against Brucellosis: A Review

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Abstract: Natural resistance against brucellosis was reviewed from historical and biological perspectives with regard to animals, humans and *Brucella* spp. Unfortunately, brucellosis continues to be a serious worldwide bacterial zoonosis of major significance to animal and human populations. Host genetic, innate and adaptive immune factors significantly influence the outcome of brucellosis as does the enabling strategies of intracellular *Brucella* to evade host factors resulting in a delicate co-evolutionary balance for long term survival for both host and pathogen. Natural (innate) resistance mechanisms include the complex of host cell surface receptors for *Brucella* pathogen-associated molecular patterns, Toll-IL-1 receptor mediated pathways, factors mediating effective macrophage and dendritic cell maturation and activation, carbohydrate binding proteins, antimicrobial peptides, and inflammatory cytokines orchestrated and regulated by the host genome. Heritability of natural resistance has long been recognized as a complex multigenic trait, however new tools for understanding the genetic basis for innate resistance are now providing a deeper knowledge to identify genes and polymorphisms associated with resistance or susceptibility. Polymorphisms of the 3'UTR of the candidate solute carrier gene, *SLC11A1*, have been investigated extensively in numerous host species yielding contradictory variable degrees of association with natural resistance to brucellosis in ruminants, and indicating the need for international standardized phenotyping protocols. By coupling new genetic tools with rigorously controlled phenotyping protocols, it is anticipated that applying genetic selection as an additional approach to controlling infectious diseases, such as brucellosis, in domestic animals will become increasingly feasible in future.

Keywords: Natural resistance, genetic resistance, ruminants, Brucellosis, NRAMP1, SLC11A1.

INTRODUCTION

The Disease

Brucellosis is a major worldwide bacterial zoonosis. It has remained a disease of global importance since its discovery by Bruce in 1888 [1, 2]. The causative agent of brucellosis is a group of gram-negative facultative intracellular bacteria belonging to the genus *Brucella*. The members of this genus are subdivided into seven species categorized by antigenic variation and primary preferred host: *Brucella melitensis* (sheep and goats), *B. abortus* (cattle), *B. canis* (dogs), *B. suis* (hogs), *B. ovis* (sheep), *B. neotomae* (rats) and *B. maris* (marine mammals) [3]. Given that genetic factors and innate immune receptors significantly influence the outcome of infectious diseases, the primary host preference exhibited by the individual *Brucella* species reflects an extremely complicated battle of two genomes and the co-evolutionary balance between the pathogen's genome, which has evolved strategies enabling survival within the preferred host [4, 5], and the primary host genome, which has evolved strategies of innate and adaptive immune responses which suppress pathogen expansion usually through redundant host biological systems [6-10]. Why then is genetic resistance not being used more in modern livestock industries since it is not especially due to the lack of evidence of genetic control of disease resistance? Perhaps it is because regulatory officials, owners, producers and other industry managers do not recognize the potential of genetic resistance, not necessarily as the tool to replace traditional methods of disease control, but to add another approach to reduce the impact of bacterial pathogens on animal health and to play a role in system-based approaches, such as the pre-harvest pathogen reduction program. Significant losses caused by bacterial diseases continue to curtail livestock industries despite traditional control measures. These problems and concerns suggest that other measures to control infectious disease should be sought to enhance animal health management programs. Newer strategies to increase the overall level of resistance at herd and population levels by using selective breeding programs to enhance natural resistance would be expected to contribute significantly in this regard. The ability to effectively and economically apply genetic selection to the problem of controlling infectious diseases in domestic animals will become increasingly feasible in the future. Investigators in several branches of science are bringing us even closer to this aim by providing basic information on the genetic constitution of domestic animals, specific genes controlling mechanisms and standardized phenotyping protocols applied in innate and adaptive resistance to bacterial diseases. Utilization of such basic information to enhance herd health would be a significant adjunct to current

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and future bacterial disease control modalities for diseases of domestic animals such as brucellosis within the next decades.

Worldwide brucellosis continues to be a major economic and public health concern. Geographical regions listed as high risk includes the Mediterranean Basin, South and Central America, Eastern Europe, Asia, Africa, the Caribbean, and the Middle East [11]. Of even greater concern is the fact that brucellosis is considered a re-emerging problem for countries such as Israel, Kuwait, Saudi Arabia, Brazil and Colombia [12]. World Health Organization data documents approximately 500,000 human cases of brucellosis per year [11]. The absence of a human vaccine means that along with pasteurization and proper sanitation, the prevention of human infection requires control of the disease in the animal hosts. The epidemic potential of *Brucella* species, the efficiency of aerosol infection, and the lack of an efficient human vaccine also make this airborne pathogen a potential agent of bioterrorism [3]. In fact, successful attempts at weaponizing *B. suis* occurred as early as 1954 [13]. In 1999, a suspected case of human brucellosis with an atypical clinical presentation prompted an investigation of possible bioterrorism in New Hampshire and Massachusetts [14], thus with very few exceptions, virtually all members of the genus *Brucella* are now classified as biosafety level 3 pathogens and have been placed on the Select Agent list of Bioterrorism as part of the 2001 U.S. Patriot Act [15].

The Natural History of Brucellosis

In most hosts, *Brucella* exposure occurs orally, aerosol or transplacentally, except in dogs, sheep, and swine in which brucellae are also sexually transmitted. Like other intracellular pathogens, brucellae require four major steps for successful infection: adherence, invasion, establishment, and dissemination [16]. The exact mechanism of bacterial entry into the host cell is not known [3]; however, evidence supporting a role for pattern recognition receptors (PRRs), including leukocyte function-associated antigen 1 (LFA-1), complement receptor 3 (CR3), alpha-5 beta-1 and alpha-V beta-3 integrins, mannose-6-phosphate receptors (MPR), and Fc receptors, supports the existence for *B. abortus* pathogen-associated molecular patterns (PAMPS) binding macrophages mediating the binding and internalization of the pathogen [17]. More recently, Kim and Watarai *et al*. indentified lipid raft microdomain interactions between *Brucella* and class A scavenger receptors [18-21]. Additionally, Capparelli *et al.* found that the haplotype pair HYA/HYA at the MBL (mannose binding lectin) locus of water buffalo is associated with resistance to *B. abortus* infection and the haplotype pairs LYD/LYD with susceptibility. Inhibition of the antibacterial activity following heat treatment of the serum, addition of specific MBL inhibitors or anti-human MBL antiserum provided evidence that the antibacterial activity was related to the serum MBL [22].

The intracellular niche of infecting brucellae includes the early *Brucella* containing vacuoles (BCVs) and later the specialized acidified phagosome within the macrophages of the host, called brucellasomes [23-25]. Qin, Pei *et al.* succinctly reviewed these steps: *Brucella* are internalized from the host cell plasma membrane and orchestrate the biogenesis of early *Brucella*-containing vacuoles (BCVs); BCVs acidify but fail to accumulate mannose 6-phosphate receptors and cathepsin D as markers for late endosomes and lysosomes, respectively; rather than maturing, BCVs fuse with membranes that contain endoplasmic reticulum (ER) resident proteins, including calreticulin and calnexin; trafficking with a compartment that contains the autophagosomal marker monodansylcadaverin; *Brucella* spp. replicate in an ER-like compartment; and then presumably lyse the host cell to disseminate and metastasize [25]. Macedo *et al.* found that susceptibility of MyD88 knockout (KO) mice to *B. abortus* infection is due to impaired dendritic cell (DC) maturation and lack of IL-12 production. Since DC maturation is a critical link between innate and adaptive immunity, MyD88-dependent signaling appears to be required for the development of IFN-gamma-producing T cells and efficient control of *Brucella* infection, providing insights into the orchestration of innate and adaptive immunity for control of *B. abortus* infection [26]. Toll-like receptors (TLRs), including TLR2, TLR4 and especially TLR9, interact with *Brucella* spp. and bacterial components and initiate mononuclear phagocyte responses that influence both innate and adaptive immunity. MyD88-dependent and Toll-IL-1 receptor (TIR)-domain-containing adapter-inducing interferon-beta (TRIF)-independent signaling pathways are involved in *Brucella* activation of innate immune cells through TLRs [27].

Once inside the phagosome, the internalized *Brucella* surviving the initial oxidative burst and the nutrient/oxygen deprived environment subvert the host cellular machinery through the interaction of the pathogen's Type IV secretion system (*virB*) with the host's endoplasmic reticulum [24]. More recently, from the pathogen side of the interaction, Sengupta *et al.* have shown that *Brucella* spp. genomes encode a protein, named TcpB, bearing homology with mammalian Toll/IL-1 receptor domains and whose expression causes degradation of the phosphorylated signal competent form of the adapter MyD88-adapter-like (MAL). Interestingly, the presence of TcpB leads to enhanced polyubiquitination of MAL and may be responsible for its accelerated degradation. The authors conclude that TcpB represents a unique pathogen-derived molecule that suppresses host innate-immune responses by specifically targeting an individual adapter molecule in the TLR signaling pathway for degradation [4]. The outcome of this host-pathogen interaction is prevention of lysosomal fusion, neutralization of phagosomal pH, utilization of nitrate ions for anaerobic respiration, and regulated multiplication of the bacteria within the phagosome. The accumulated bacteria eventually escape the phagosome and are disseminated to other host cells. In female ruminant hosts, *B. abortus* interacts with the placental trophoblasts associated with the tissue tropism activity of erythritol produced by these cells. Within these cells, brucellae enter their acute replicative stage, with placental disruption resulting in fetal loss or the birth of weak, infected offspring [28]. The pathologic lesions of domestic and laboratory animals have been reviewed previously [9]. In humans, *Brucella* spread to the lymph nodes, spleen, liver and bone marrow with associated pathological manifestations of untreated infection often being meningitis, endocarditis, spondylitis, and arthritis [3].

The clinical presentation of human brucellosis can include fever, malaise, arthralgias, hepatomegaly, splenomegaly, lymphadenopathy, peripheral arthritis, sacroiliitis, epididymoorchitis, vomiting and diarrhea [29].

MECHANISMS OF NATURAL DISEASE RESISTANCE TO BRUCELLOSIS

Historical and Biological Perspectives

Natural (innate) resistance to bacterial diseases was observed over one hundred thirty years ago as familial tendencies in resistance or susceptibility to diphtheria in humans [30], but the genetic implications of this observation were not appreciated at that time, because it would be another twenty years until the rediscovery of Gregor Mendel's studies. In domestic livestock, it had been long observed that disease manifestations rarely occur in all members of the population exposed to bacterial pathogens, e.g. early studies of resistance to *Salmonella pullorum* in poultry [31] and *B. suis* in swine confirmed a major role for genetic control [32-34]. These early observations were largely ignored, because antibiotics were discovered in the late 1920's, vaccines were developed for several animal diseases, and lastly, the genetics of natural disease resistance seemed unduly complicated, and there was concern that planned breeding programs to increase natural resistance would be too slow to have an impact and would compromise productivity. The animal genome always influences and sometimes determines susceptibility to bacterial diseases; however, due to the huge variety of pathogens and the multitude of complex host defense mechanisms involved, rarely does a simple understanding of resistance emerge. Although some of the observed variation in natural resistance is related to environmental factors, a significant component of variation in natural disease resistance is heritable and, therefore, stably passed from parent to offspring. The ability of a naive host to resist primary infection by a pathogen is known as natural disease resistance. F. B. Hutt specifically defined natural disease resistance as "the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure or immunization, of which the major component is heritable and, therefore, stably passed from parent to offspring" [35, 36]. Given this series of complex host-pathogen interactions, it is obvious that control of natural bacterial infection and resulting disease would rarely be controlled by a single gene, although expression of an allele at one locus can significantly affect disease pathogenesis in individuals while at the herd and population levels, many genes would be operational in controlling the spectrum of disease expression. Obviously, selection for innate host resistance [37] should be considered as additive to one of several strategies, e.g. adaptive immunity through vaccination, hygiene and regulatory control measures, for integrated control of brucellosis. In attempt to balance host innate resistance and strategically poised *Brucella* escape mechanisms, we must also consider that once the selected innate resistance gene(s) spreads into the host population, *Brucella* variants expressing epitopes or other survival mechanisms that "innate resistant" animals fail to recognize or clear may be a consequence, as each strives for survival [38].

Overview of Innate Resistance Mechanisms

Natural disease resistance involves primarily the nonspecific immune response in vertebrates. Nonspecific immunity includes pattern-recognition receptors (PRRs) that bind to ancient and conserved bacterial PAMPs as well as single-stranded viral ribonucleic acid (ssRNA) [39], Toll-like receptors (TLRs) [40], nucleotide-binding oligomerization domain proteins (NODs) [41], lipopolysaccharide binding protein (LBP) [42], and mannose-binding protein (MBP) [43] are eukaryotic receptors that all recognize PAMPs. Binding of these PRRs with their target PAMPs results in opsonization, endocytosis or phagocytosis of the bacteria or virus, and initiates the transcription of nonspecific immune response genes. The products of these genes include antimicrobial peptides [44] and inflammatory cytokines [45]. This innate immune response, which occurs immediately after infection, serves to sequester the pathogen, limits its replication, and in some instances, clears the pathogen altogether. When the innate immune response is bypassed, evaded or overwhelmed, the adaptive immune response becomes paramount to host survival.

Phylogenetically the non-specific innate immune response predates the specific adaptive immune response. Innate immunity is found in some form or another in all multicellular organisms, while the adaptive immune response, appearing around 400 million years ago, is only found in vertebrates [46]. It was earlier thought that because the innate response was more primitive than the adaptive immune response, the innate response was also less advanced, and therefore not as important as the adaptive response. The innate immune response was thought to function merely as a stopgap measure during primary infection until the adaptive immune response could be activated. Additionally, because the innate immune response does not lead to immunological memory, it was considered less critical to overall immunity in higher organisms than was the adaptive immune response. However, there has been a paradigm shift in the scientific community with regards to the importance of the innate immune response [47], and the prevailing thought now is that innate immunity not only functions as the first line of defense against invading pathogens, but it also plays an instructive role in coordinating an effective adaptive immune response for short and long-term defense [48]. Thus, natural disease resistance, the nonspecific and specific immune responses both play significant roles in the outcome of *Brucella* infection.

Innate Immunity Interactions with Adaptive Immunity

In addition to the major role that BoLA plays in adaptive protective immunity, endogenous IL-12 produced during infection with *B. abortus* has been found to promote the production of IFN-gamma and the *in vivo* clearance of *Brucella* in mice by activating natural killer (NK) cells [49]. Human Vgamma9Vdelta2 T-cells play an important role in the early response to infection with intracellular pathogens, yet their number was dramatically increased in the peripheral blood of patients with acute brucellosis and specifically activated by non-peptidic low molecular weight

compound(s) from *B. suis* lysate or by soluble factors produced by *B. suis*-infected macrophages [50].

Host Cell Membrane Receptors

Rezazadeh *et al.* investigated the role of human Toll-like receptor-4 (TLR4) polymorphisms that have been reported to reduce immune responsiveness to microbial pathogens, especially *Brucella* spp. They investigated the polymorphism in TLR4 gene (Asp299Gly) in patients with brucellosis and healthy volunteers matched for sex, age and geographic area. The 896G allele was significantly much more prevalent in patients with brucellosis compared to healthy controls Also the frequency of the G allele of TLR4 gene was significantly higher in male patients with brucellosis compared to the same sex in control group. Multiple logistic regression analysis found that male patients heterozygous at allele G gene had a significantly higher risk for brucellosis with an odds ratio of 2.89, revealing an association between the genetic polymorphism in TLR4 gene and susceptibility to brucellosis [51]. Hashemi *et al.* evaluated Fc gamma RIIa polymorphisms in patients with brucellosis such that the frequency of the two alleles and three genotypes for Fc gamma RIIa were virtually the same for H131 and R131 alleles in healthy controls and brucellosis patients, respectively. They concluded that the Fc gamma RIIa polymorphism was not decisive for the acquisition of brucellosis [52].

The A/C polymorphism (Ser128Arg) of E-selectin has been described to alter ligand binding function. Rafiei *et al.* found that the frequency of the Arg/Arg genotype of the Ser128Arg polymorphism was significantly increased in human brucellosis patients compared with healthy controls. After stratification of the patients according to disease duration, the association between the Arg allele and brucellosis was investigated disclosing that only in a subgroup of the patients with disease onset less than 38 weeks was the odds ratio significant, suggesting that the Arg/Arg genotype of the E-selectin gene polymorphism in codon 128 may be a genetic factor influencing susceptibility to *Brucella* infection [53]. Haidari *et al.* evaluated the single-nucleotide polymorphism C-159T in the promoter region of the CD14 gene that has been implicated in susceptibility to infectious diseases, and determined the CD14 genotype in human patients with brucellosis and healthy volunteers from the same rural area. The prevalence of genotype TT was significantly higher in the patients with brucellosis while the healthy controls had a higher prevalence of genotype CC. Furthermore, patients who were homozygous for allele T of promoter of CD14 gene had a significantly higher risk for developing brucellosis with odds ratio of 3.03, thus their data provided evidence suggestive of an association of the CD14 gene polymorphism with susceptibility to brucellosis [54]. Since the host cellular prion protein (PrP(C)) may function as a cell surface receptor and/or portal protein for *B. abortus* in mice [19], Seabury *et al.* evaluated the nucleotide and amino acid variation within exon 3 of the prion protein gene (PRNP) in US bison populations, identifying a non-synonymous single nucleotide polymorphism (T50C), resulting in the predicted amino acid replacement M17T (Met --> Thr) while no variation (T50; Met) has yet been found in domestic cattle [55]. Interestingly, 80% of bison possessing the C/C genotype were *Brucella* spp. seropositive and suggesting a potential association between nucleotide variation within PRNP exon 3 and the presence of *Brucella* spp. antibodies in bison, potentially implicating PrP(C) in the natural resistance of bison to brucellosis infection [55].

Cytokines

Rafiei *et al.* investigated the association between transforming growth factor (TGF)-beta1 polymorphisms at codons 10 and 25 and brucellosis in human brucellosis patients and healthy volunteers. The frequencies of TGF-beta1 codons 10 C and 25 G were significantly higher among brucellosis patients than among healthy controls, as was that of TGF-beta1 codon 10 C/C, suggesting that genetic polymorphism in codons 10 and 25 of the TGF-beta1 gene may contribute to the susceptibility of human brucellosis [56]. Since the interleukin-4 (IL-4) gene has a C-T substitution at position 590 which is associated with increased production of IL-4, Rezazadeh *et al.* investigated the potential association of this polymorphism with susceptibility to human brucellosis. The prevalence of the T allele of IL-4 polymorphism was significantly higher in the group with brucellosis than in controls, and furthermore patients with brucellosis had a higher frequency of intermediate producer genotype (CT) while low producer genotype (CC) was higher in the control group. Multiple logistic regression analysis demonstrated that patients who were heterozygous (CT) for interleukin-4 promoter polymorphism had a significantly higher risk for brucellosis with an odds ratio of 4.2, demonstrating an association between IL-4 590 promoter polymorphism and contracting human brucellosis [51].

Macrophage Function

Host resistance to *B. abortus* was found to be significantly related to macrophage function and immune mechanisms in cattle naturally resistant or susceptible to brucellosis [57, 58]. Studies to determine the genetic basis for resistance to *B. abortus* in cattle found that natural resistance to brucellosis in cattle is a complex phenotypic trait determined by two or more interacting genes resulting in complex genetic types [59-61]. Twenty year classical breeding studies involving over 300 progeny of unvaccinated and unexposed sexually mature bulls and unvaccinated and unexposed 180 day pregnant outbred heifers phenotyped resistant or susceptible by standardized *in vivo* intraconjunctival challenge with 1 x 10^7 cfu of *B. abortus* Strain 2308 established that the population frequency of natural resistance to *Brucella* could be significantly increased with one round of selective breeding [59, 61, 62]. In compliance with and approval by local, national and international health and animal protection and welfare requirements, the standard challenge is administered by bilaterally placing 50 µl containing 5 x 10^6 cfu of *B. abortus* Strain 2308 in the conjunctiva sac such that the inoculum drains from each eye *via* the nasolacrimal duct into the nasal cavity, thus simulating a natural *per os* and/or aerosol exposure as would be expected to occur when ruminants are exposed by a *Brucella* induced abortion in a natural herd setting. Fortunately, classical genetic studies can now be greatly accelerated by embryo transfer, cloning technologies and now by deep sequencing methodologies. In these experiments, the frequency of natural resistance to

standardized experimental *B. abortus* challenge was increased from 20% to almost 59% in one generation when mating a resistant bull to resistant heifers.

Solute Carrier 11A1 (*SLC11A1*) (formerly Natural Disease Resistance-Macrophage Protein 1 (*NRAMP1*) Candidate Gene

The solute carrier gene superfamily (SLC) encodes a group of integral membrane proteins that includes passive transporters, ion coupled transporters, and exchangers that traffic crucial compounds into and out of cells and organelles [63]. Currently the human SLC superfamily consists of 43 gene families encoding 298 individual transporter proteins [64]. A number of human disease syndromes are linked to SLC transporter gene defects, including hypertension [65], deafness [66], and hemochromatosis [67], and these integral membrane transporters proteins are prime candidates to be exploited as drug delivery systems or drug targets in the treatment of disease.

The SLC11 family consists of two member genes that encode proton coupled metal ion transporters, *SLC11A1* and *SLC11A2* (formerly known as *DMT1*). Originally characterized in mice, *SLC11A1* mapped to the mouse *Bcg/Lsh/Ity* locus, a region conferring resistance to infection by the live attenuated Bacillus Calmette-Guerin (BCG) strain of *Mycobacterium bovis*, *Leishmania donovani*, and *Salmonella enterica* serovar Typhimurium in the murine host [68]. *SLC11A1* is a highly conserved gene, with orthologs found in both prokaryotes and eukaryotes, while homologs have been identified in mycobacteria [69], yeast [70], *Drosophila* [71], chickens [72], swine [73], dogs [74], horse [75], deer [76], cattle [77], and humans [78]. *SLC11A1* expression occurs mainly in macrophages and is interferon-gamma-inducible. *SLC11A1* expression in macrophages is also modulated by exposure to bacterial lipopolysaccharide (LPS) [79].

The SLC11A1 gene product is a polytopic integral membrane protein made up of 10-12 transmembrane domains. It is a 548 amino acid protein containing three putative phosphorylation sites, two SH3 binding motifs, and a single exofacial glycosylation site [80]. Bovine *SLC11A1* also contains a highly conserved binding-protein-dependent transport system inner membrane component signature that alludes to the putative divalent cation transporter function of SLC11A1. Localization studies demonstrate that the protein product is recruited to the late endocytic compartment in a Lamp1 (lysosomal-associated membrane protein-1)-positive compartment and remains associated with this compartment as it matures to a phagolysosome [81], supporting the theory that SLC11A1 restricts replication of infecting pathogens by altering the intracellular environment. Research into the function of SLC11A1 suggests that the protein may modulate phagosomal pH [82], as well as alter divalent cation concentrations, including iron, within the phagosome [83, 84].

The disease resistance association of *SLC11A1* inferred by the mapping the gene to the *Bcg/Lsh/Ity* locus was confirmed in experiments in which innate resistance to intracellular pathogens was abrogated in *SLC11A1* knockout mice [85]. In addition, the phosphoglycoprotein gene product of *SLC11A1* was determined to be absent in macrophages from *Bcg* susceptible mouse strains, with anti-SLC11A1 antiserum failing to detect mature SLC11A1 protein in macrophages isolated from C57BL6/J and BALB/c Bcg^s mice [86]. A single, non-conserved glycine-to-aspartic acid amino acid substitution at position 169 results in a nonfunctional gene product and susceptibility of murine macrophages to *BCG*. In contrast to macrophages from $SLC11A1^{G169}$ mice, in which a 100-kDa mature SLC11A1 protein is detected, SLC11A1 specific antiserum fails to detect an immunoreactive protein in macrophage lysates from susceptible mouse strains bearing the $SLC11A1^{D169}$ allele [86]. This disrupting amino acid substitution is not found in SLC11A1 proteins sequenced from other species, including deer, bison, cattle, dogs, and humans. However, DNA sequence analysis and disease association studies in humans identified a functional microsatellite polymorphism in the human *SLC11A1*. In the human *SLC11A1* gene, polymorphisms in a GT dinucleotide microsatellite in the 5' promoter of the gene correlate with both autoimmunity and infectious disease susceptibility [87]. A similar microsatellite length polymorphism was found in the 3' untranslated region of the bovine *SLC11A1*gene [80]. Single Stranded Conformation Analysis (SSCA) indicated that length polymorphisms within this region of the gene were associated with disease resistance to *B. abortus* in cattle. Standardized *in vivo* wild type *B. abortus* challenge studies in unvaccinated, unexposed 180 day pregnant first calf heifers revealed that microsatellite lengths of 13 GT dinucleotide repeats were found in cattle resistant to challenge by *B. abortus*, while microsatellite lengths of 14, 15, and 16 GT dinucleotide repeats were found in cattle susceptible to *B. abortus* challenge [80]. As expected for a multigenic trait, a minority cattle with $\leq$ 13 GT were susceptible, and a minority $\geq$ 14 GT cattle were resistant, i.e. the association of natural resistance was not perfect for the GT polymorphisms of *SLC11A1* in these studies [80]. Analysis of immune correlates related to natural resistance to *Brucella* revealed a differential response in macrophage activation between resistant and susceptible cattle, along with the associated segregation of specific alleles of bovine *SLC11A1*, a gene involved in macrophage activation [60]. *In vitro* assays demonstrated that macrophages from cattle with the resistant phenotype had a greater ability to kill *B. abortus* than did those from animals with the susceptible phenotype [88]. When an expression vector carrying the bovine *SLC11A1* resistant allele (GT13) was transfected into a susceptible mouse macrophage cell line, the phenotype switched from susceptible to resistant in an *in vitro* macrophage-killing assay with *B. abortus* challenge [89]. Alternately, when the expression vector carrying a susceptible allele from bovine *SLC11A1* (GT16) was transfected, there was no change in the susceptible phenotype. The same study found that a significantly higher level of *SLC11A1* mRNA present in transfected cells carrying the resistant allele than in the transfected cells carrying the susceptible allele.

Horin [90] confirmed that the nucleotide sequence polymorphism due to a variation in the number of GT dinucleotide repeats is found in the 3' untranslated region (nucleotide positions 1781–1804) of the *SLC11A1* gene, and identified different sequences with variable numbers of GT repeats, particularly GT_{10}. The variation in the number of GT

repeats was related to a variation in the number of 5' adjacent Gs. Deeper sequencing revealing nearly the complete structure of the bovine NRAMP 1 gene identified a novel polymorphism within intron X consisting of insertion of three guanine nucleotides at positions 37, 40 and 98 relative to the intron X start point, and scans of several cattle breeds suggested that the two intron X alleles identified are stable and widespread in the *Bos taurus* population [91]. Paixao *et al.* compared the frequency of *SLC11A1* 3'UTR polymorphisms between Holsteins (*Bos taurus taurus*) and Zebu (*Bos taurus indicus*), including the Nelore, Guzera, and Gir breeds. A marked difference in the frequency of alleles was detected between the Zebu and Holstein cattle that had only the GT13 genotype. The Nelore breed had the most heterogeneous genotype with four allelic combinations, namely, homozygous GT13, homozygous GT14, heterozygous GT13/GT14, and heterozygous GT13/GT15. The authors proposed that the allelic frequencies in different breeds of cattle may be useful in the future for planning breeding strategies for selection of naturally resistant cattle [92]. In an attempt to identify additional polymorphisms, Martinez *et al.* further characterized the *SLC11A1* gene in different breeds of Colombian Creole *Bos taurus* and *Bos indicus*, identifying six new variants among a total of 11 single nucleotide mutations, of which five occurred in the coding sequence (three are missense mutations), one in the promoter region and five in introns [93, 94].

PROPOSED ROLE OF *SLC11A1* DINUCLEOTIDE REPEAT INSTABILITY ASSOCIATED WITH BRUCELLOSIS RESISTANCE

In humans, instability in DNA dinucleotide and trinucleotide repeats is associated with several neurodegenerative and tumorigenic diseases. Genome wide instability of dinucleotide repeats is observed in tumors isolated from patients suffering from Hodgkin's disease and hereditary nonpolyposis colon cancer [95, 96]. The archetypal triplet repeat disease, Huntington's disease, is categorized as a translated polyglutamine (polyQ) repeat disease, with its functionally associated unstable trinucleotide repeat (CAG), located in the first exon of the huntington (Htt) gene, laying down an expanded tract of glutamines within the expressed sequence of the gene [95]. Repeat lengths of eight to 39 are found in normal individuals, while repeat lengths of 37 or more are found in affected individuals [97]. It is thought that the expressed polyQ tract results in a toxic gain of function of the mutant protein, such as formation of neuronal intranuclear inclusions, interference with cytoskeletal and vesicular transport, and impaired gene expression [98].

Microsatellite repeat diseases are not restricted solely to translated expansions within mutant protein. Repeat diseases with untranslated repeat expansions found in the 5' and 3' untranslated regions of the gene, as well as within the introns of the gene, have also been identified. Friedreich's ataxia (FRDA) is one such disease, containing an expanded GAA trinucleotide repeat in the first intron of the frataxin gene and inhibiting expression of the gene [99]. Although the mechanism of this altered gene regulation is unknown, one possibility is that changes in polymorphic microsatellite repeat can hinder binding of transcriptional factors through the localized formation of tightly packed heterochromatin or through disruption of synergistic protein-protein interactions and cooperative binding of the proteins to the DNA.

PROTEIN-PROTEIN INTERACTIONS AND COOPERATIVE DNA BINDING OF REGULATORY FACTORS

Nucleotide variations found in bovine *SLC11A1* cDNA do not appear to be associated with natural disease resistance to *B. abortus* in cattle. Functionally associated nucleotide variants may be overlooked in under-represented alleles due to reduced expression levels of these alleles, and this is particularly important since it has been reported that individuals carrying the susceptible allele for *SLC11A1* have reduced mRNA expression in both humans and cattle [80, 87]. Screening of the genomic complement of DNA for the sequence variation should provide a more accurate accounting of allelic variation in each individual since the DNA template would not be affected by allelic differences in expression. To date, the only bovine *SLC11A1* polymorphisms proposed to be associated with *B. abortus* natural disease resistance in cattle is the dinucleotide microsatellite length polymorphism found in the 3' untranslated region of the gene. While it is suggested from previous studies that variable microsatellite lengths affect macrophage bactericidal activity, the mechanism of this effect is not known. It is possible that changes in the 3'UTR microsatellite length may alter the angular orientation of two or more flanking transcription regulation sites specific for cooperatively binding transcription factors and reduce the efficiency of the synergistic binding of these proteins to the DNA molecule. In this case, polymorphisms in microsatellite length would alter transcription in a helical-phase dependent manner. Cooperative binding is a phenomenon whereby protein-protein interactions between two or more DNA binding proteins reduce the free energy of binding to DNA so that together proteins bind more tightly to DNA than each factor alone. This is a key mechanism for generating specificity within multicomponent nucleoprotein complexes [100]. DNA bending facilitates the protein-protein interactions between factors whose binding elements are separated by long distances across the DNA molecule [101]. Studies on cooperatively binding lambda repressor proteins have shown that changes in the angular orientation of the protein-binding site along the DNA alpha helix affects the strength of this protein-protein interaction [102]. Normally the two binding sites for lambda repressor have a center-to-center distance of 25 bp or 2.4 helical turns. Insertion of 10 or 11 bp (1 helical increment) has no effect on cooperativity up to a distance of 80 bases or 8 turns of the a-helix. However, the insertion of non-helical increments abolishes co-operativity. This is due to the energetic penalty of twisting the DNA in order to place the proteins into a favorable angular orientation to interact cooperatively.

DISCREPANCIES IN THE ASSOCIATION OF THE POLYMORPHISMS OF THE CANDIDATE GENE, *SCLC11A1*, WITH PHENOTYPIC NATURAL DISEASE RESISTANCE TO BRUCELLOSIS

Evidence Supporting the Association of *SLC11A1* with Natural Resistance against Brucellosis

Ganguly *et al.* evaluated the Murrah breed of buffalo (*Bubalus bubalis*) to identify four (GT13, GT14, GT15 and

GT16) polymorphisms of the 3'UTR of *SLC11A1* gene and evaluated the association of these polymorphisms with *in vitro* macrophage function. Non-vaccinated and serologically negative buffalo were divided into three genotypic groups: homozygous (GT)13 genotype; heterozygous [(GT)13/(GT)n, where n ≠ 13]; and non-(GT)13 [(GT)n/(GT)n, where n ≠ 13], and macrophages were challenged with *Brucella* LPS to measure H_2O_2 and NO production. The homozygous [(GT)13/(GT)13] or heterozygous [(GT)13/(GT)n], where n=14, 15 or 16] (GT)13 allele, was significantly ($p<0.01$) associated with increased production of H_2O_2 and NO, identifying the (GT)13 allelic variant to be significantly association with the improved macrophage function in buffalo [103]. Martinez *et al.* further investigated the association between resistance to brucellosis infection and *SLC11A1* SSCP genotype using a macrophage *in vitro* killing assay employing a virulent *B. abortus* strain. A significant association was found between the *B. abortus* macrophage *in vitro* killing assay phenotypes and the bovine *SLC11A1* 3' UTR genotypes, which suggested that the GT [12] A allele may be associated with resistance [94].

Borriello *et al.* tested water buffalo cows for the presence of anti-*B. abortus* antibodies and their corresponding *SLC11A1* genotype and compared seropositive and seronegative groups with their respective *SLC11A1* genotypes. The seropositive buffaloes had the *SLC11A1*A+ (*SLC11A1*AA or *SLC11A1*AB) genotype while the seronegatives had the *SLC11A1*A- (*SLC11A1*BB) genotype with an odds ratio of 4.37. Additionally, monocytes from *SLC11A1*BB buffalo had significantly higher levels of *SLC11A1* mRNA than *SLC11A1*AA buffalo as well as a significantly enhanced ability to control the *in vitro* intracellular replication of several *Brucella* species, leading Borriello *et al.* to conclude that selection for the *SLC11A1*BB genotype may be a valuable tool for the control of brucellosis in water buffalo in endemic areas [104]. Capparelli *et al.* further evaluated the 3' untranslated region of the water buffalo *SLC11A1* gene to identify two alleles (*SLC11A1*A and *SLC11A1*B) in two independent populations of *Brucella*-seropositive and -seronegative control buffalo, and the *SLC11A1*AA genotype was associated with susceptibility. Moreover, macrophages from *SLC11A1*AA buffalo had a lower *SLC11A1* mRNA level and a higher level of viable intracellular *Brucella* when compared with macrophages from *SLC11A1*BB buffalo. Also, monocytes and macrophages from *SLC11A1*AA subjects displayed a higher number of viable intracellular bacteria compared to *SLC11A1*BB animals, adding biological relevance to the association of genotypes with resistance or susceptibility [105]. Furthermore, Capparelli *et al.* tested water buffalo cows for the presence of anti-*B. abortus* antibodies and the *SLC11A1* genotype. They detected four alleles (*SLC11A1*A, -B, -C, and -D) in the 3' untranslated region of the *SLC11A1* gene, and found that the *SLC11A1*BB genotype was represented among only the seronegatives, providing evidence that this genotype apparently confers resistance to *B. abortus*. The monocytes from the *SLC11A1*BB (resistant) buffaloes had a higher basal level of *SLC11A1* mRNA and a lower number of viable intracellular bacteria than did the monocytes from *SLC11A1*AA (susceptible) genotyped buffaloes. Capparelli *et al.* proposed that the higher basal level of the antibacterial protein SLC11A1 probably provides the *SLC11A1*BB buffalos with the capability of controlling bacteria immediately after their entry inside the cell [106].

Evidence Refuting the Association of *SLC11A1* with Natural Resistance against Brucellosis

Continuing their investigation of the bovine *SLC11A1* gene, Paixao *et al.* evaluated the association between *SLC11A1* 3'UTR polymorphisms and resistance against bovine brucellosis in experimental and natural infections. In experimentally infected mixture of vaccinated and unvaccinated pregnant cows, abortion occurred in 42.1% of cows with a resistant genotype (SSCA(r) and in 43.1% of those with a susceptible genotype (SSCA(s). Additionally, the percentages of *B. abortus* positive cultures of the SSCA(r) genotype were 86 and 84% in serologically positive and negative cows from a farm with a very high prevalence of naturally-occurring bovine brucellosis. Hence, they found no association between the *SLC11A1*-resistant allele and the resistant phenotype in either experimental or naturally occurring brucellosis. Moreover, no differences were observed in the rates of intracellular survival of *B. abortus* within macrophages from cattle with susceptible or resistant genotypes. Thus, the investigators concluded that polymorphisms at the *SLC11A1* 3'UTR are not associated with resistance against *B. abortus* in cattle and that *SLC11A1* 3'UTR polymorphisms are not suitable markers of natural resistance against bovine brucellosis [107]. Bravo *et al.* examined polymorphisms of the *SLC11A1* gene in human patients with brucellosis and healthy controls and found no significant differences in the alleles studied, concluding that variants of the *SLC11A1* gene do not appear to affect susceptibility or protection in human brucellosis [108]. Kumar *et al.* evaluated samples from Indian zebu (*Bos indicus*) and crossbred (*Bos indicus x Bos taurus*) cattle that were homozygous (GT)(13)/(GT)(13). Cattle that were positive on three serological brucellosis tests with a history of abortion were grouped as "affected"; whereas the animals that were negative in all serological tests and completed third lactation without any history of abortion were grouped as "non-affected." The presence of (GT)(13) allele even in homozygous condition failed to provide resistance to brucellosis in a naturally infected herd [109]. In studies of bovine tuberculosis, even with high-level expression of *SLC11A1* proteins in peripheral blood cells and granulomatous lesions heavily labeled epithelioid macrophages and Langhans cells, active tuberculosis was progressive [110]. In contrast to infections with *Salmonella*, *Leishmania*, and *Mycobacterium*, the expression of the *SLC11A1* gene was found to be of limited importance for the natural resistance of mice to *B. melitensis* [111].

The Challenge of the Association of the *SLC11A1* with Resistance to Brucellosis

It is not surprising that the prevailing hypothesis that polymorphisms at the bovine *SLC11A1* 3'UTR are associated with genetic resistance against *B. abortus* in cattle is being challenged, simply fulfilling the fundamental premise of the scientific process by which a hypothesis is only valid until it is proven wrong. As observed from the preceding series of publications, the role of the *SLC11A1*

gene in resistance or susceptibility to ruminant brucellosis continues to be a hypothesis under serious investigation since the Adams and Templeton laboratories in the late 1980's and early 1990's demonstrated that resistance to *B. abortus* in cattle was a heritable trait and that polymorphisms of *SLC11A1* 3'UTR were associated with resistance, while fully recognizing that resistance to brucellosis was a complex phenotypic trait determined by two or more interacting genes. Clearly, data from other laboratories support (or refute) this hypothesis that polymorphisms at the bovine *SLC11A1* 3'UTR are (are not) associated with genetic resistance against *B. abortus* in cattle. The authors of other publications suggest that *SLC11A1* 3'UTR polymorphisms are not suitable markers of natural resistance against bovine brucellosis, while the results from other laboratories support the hypothesis.

There are a number of differences in designs, phenotyping protocols, performance standards and execution of the experiments from various laboratories that make it difficult to directly confront the discrepancies in the results and the subsequent interpretation of the data. The genetic background of the experimental animals is particularly relevant for several reasons: i) natural resistance/susceptibility to *B. abortus* is multigenic and all the genes encoding this phenotype have not been clearly defined; ii) even within a genetically-defined population of cattle, the correlation between the *in vivo* phenotype -as determined by experimental infection and the *in vitro* phenotype -as determined by *in vitro* killing assays- is not absolute; iii) there are likely breed-specific differences in frequencies of natural resistance/susceptibility to infectious diseases in cattle, e.g. 100% frequency of bovine *NRAMP1* $SSCA^r$ alleles in the Brazilian Holstein cattle reported by Paixao *et al*. [107]. Additionally, adoption of internationally standardized phenotyping of bovine brucellosis resistance or susceptibility by *in vivo* challenge with 1 x 10^7 cfu of wild type *B. abortus* Strain 2308 in unvaccinated, unexposed first calf heifers at 180 ± 30 days gestation without recovery of the pathogen from any tissues or secretions from the dam, placenta and fetus should constitute the resistance phenotype which would be highly desirable to evaluate the phenotypes in virtually any bovine population anywhere. Accordingly, the criteria applied by Paixao *et al.* [107] to determine the *in vivo* phenotype of the experimental cattle ("for the purpose of this study, susceptibility to brucellosis was characterized by the occurrence of abortion and by the intensity of inflammatory lesions in several organs") were certainly different if not less stringent than those used in original phenotyping experiments of Adams and Templeton [58, 61-62, 112]. Certainly, according to Hutt's original description of natural disease resistance, cattle should not be vaccinated for brucellosis or have had natural exposure to the *Brucella* prior to phenotyping by virulent challenge as occurred in the Paixao *et al.* [107] experiments in which 29 of the 42 heifers were previously vaccinated with either S19 or RB51 ("The inclusion of vaccinated cattle in this study was due to the fact that a significant percentage of the female bovine population are vaccinated, and therefore a marker for natural resistance should be suitable for both vaccinated and non vaccinated cattle"), or regarding the unconfirmed vaccination status of the cattle herd used in the natural *Brucella* infection study, in which there were "no records of vaccination against brucellosis were available for this herd." The obvious concern with cattle, whether vaccinated or not, naturally exposed to *B. abortus* is that it is not possible to confirm the time of exposure relative to gestation, the dosage of exposure and the virulence of the wild type organism any or all of which seriously compromise the phenotype classification. From the data cited above of the phenotyping experiments reviewed here and the subsequent discussion of the data, it is obvious that the procedures for *in vivo* phenotyping resistance against *Brucella* in ruminants are complex and should be conducted using internationally accepted standardized guidelines for challenge conditions and phenotyping assessment protocols to improve the efficiency of identifying the subtleties of the multigenic traits of natural resistance mechanisms against brucellosis.

SUMMARY

Natural resistance against brucellosis was reviewed from historical and biological perspectives with regard to animals, humans and *Brucella* spp. Unfortunately, brucellosis continues to be a serious worldwide bacterial zoonosis of major significance to animal and human populations. Host genetic, innate and adaptive immune factors significantly influence the outcome of brucellosis as does the enabling strategies of intracellular *Brucella* to evade host factors resulting in a delicate co-evolutionary balance for long term survival for both host and pathogen. Given this series of complex host-pathogen interactions, it seems clear that control of brucellosis and resulting disease would rarely be controlled by a single gene, although expression of an allele at one locus can significantly affect disease pathogenesis in individuals while at the herd and population levels, many genes are likely to be operational in controlling the spectrum of disease expression. Natural (innate) resistance mechanisms includes the complex of host cell surface receptors for *Brucella* pathogen-associated molecular patterns, Toll-IL-1 receptor mediated pathways, factors mediating effective macrophage and dendritic cell maturation and activation, carbohydrate binding proteins, antimicrobial peptides, and inflammatory cytokines orchestrated and regulated by the host genome. Heritability of natural resistance has long been recognized as a complex multigenic trait, however new tools for understanding the genetic basis for innate resistance are now providing a deeper knowledge to identify genes and polymorphisms associated with resistance or susceptibility. For example, polymorphisms in TGFbeta 1, E-selectin, TLR4, CD14 genes and the IL-4 promoter were found to be associated with a higher risk for contracting human brucellosis, while the Fc gamma RIIa was not decisive for susceptibility to brucellosis. Polymorphisms of the 3'UTR of the candidate gene, *SLC11A1*, have been investigated extensively in numerous host species yielding variable degrees of association with natural (innate) resistance to brucellosis. Publications supporting or refuting the hypothesis of associating *SLC11A1* 3'UTR polymorphisms with resistance to brucellosis in cattle and water buffalo were reviewed clearly indicating that further investigation will be required using standardized *in vivo* phenotyping guidelines to resolve the controversy and potential use of the *SLC11A1* 3'UTR polymorphisms as a basis for selecting ruminants naturally

resistant to *B. abortus*. Given the rate of development of new genetic tools coupled with rigorously controlled phenotyping protocols, it is anticipated that applying genetic selection as additional approach to controlling infectious diseases, such as brucellosis, in domestic animals will become increasingly feasible in the future.

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ABBREVIATIONS

BCV = *Brucella*-Containing Vacuole

CR3 = Complement Receptor 3 (CD11b/CD18)

DC = Dendritic Cell

Fc = Fragment, crystallizable of Immunoglobulins

LAMP1 = Lysosomal-Associated Membrane Protein 1

LBP = Lipopolysaccharide Binding Protein

LFA-1 = Leukocyte Function-Associated Antigen 1 (CD18/CD11a)

MAL = MyD88-Adapter-Like

MBP = Mannose Binding Protein

MPR = Mannose 6-Phosphate Receptors

NK = Natural Killer Cell

NOD = Nucleotide-Binding Oligomerization Domain

NRAMP1 = Natural Resistance-Associated Macrophage Protein 1 gene

PAMP = Pathogen-Associated Molecular Patterns

PRR = Pattern-Recognition Receptors

SLC11A1 = Solute Carrier 11A1 gene

TLR = Toll-like Receptor

TRIF = Toll-IL-1 Receptor (TIR)-Domain-Containing Adapter-Inducing Interferon-Beta

UTR = Untranslated Region

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