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BRUCELLA SPP. ANTIGENS IMPACT IN THE PATHOGENESIS AND DIAGNOSIS OF HUMAN BRUCELLOSIS

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Abstract

Introduction. Brucellosis remains one of the most widespread and underdiagnosed zoonotic infections, with over 500,000 new human cases annually and continued endemic circulation in livestock. *Brucella melitensis, B. abortus, B. suis*, and *B. canis* are the key pathogenic species causing chronic disease with nonspecific clinical symptoms. Brucella antigens are central to the pathogenesis and immune response, yet current diagnostic tests lack sufficient specificity, and no licensed human vaccine is available. This highlights the urgent need for more accurate antigen-based tools for both diagnosis and prevention.

Aim. To review and evaluate the main *Brucella* antigens with proven or potential diagnostic and vaccine value, focusing on their structural features, immunogenicity, and applicability in translational research.

Search strategy: A systematic review was conducted following PRISMA guidelines. Literature searches were performed in PubMed, Scopus, and Web of Science (2018–2025) using predefined keywords related to *Brucella* antigens, diagnostics, and vaccine development. After multistage screening of titles, abstracts, and full texts, 106 studies were included in the final analysis, as illustrated in the PRISMA flow diagram.

Results. Outer membrane proteins (Omp16, Omp19, Omp25, Omp31), periplasmic proteins (BP26, Cu/Zn-SOD, L7/L12), heat shock proteins (Hsp60, Hsp70), and Type IV secretion system proteins (VirB) demonstrate distinct immunogenic profiles. Among them, L7/L12, Omp19, and SOD show the highest promise in terms of serological sensitivity and protective immune stimulation. A subset of these antigens is currently being explored as part of recombinant subunit vaccine formulations.

Conclusion. Molecularly defined *Brucella* antigens offer tangible opportunities to improve human brucellosis diagnostics and support the rational development of safer, targeted vaccines. Their documented immunological relevance justifies further preclinical evaluation and standardization in applied immunodiagnostics.

Keywords: *Brucellosis, Brucella spp., outer membrane proteins, antigens, diagnostics, vaccine development, human brucellosis, heat shock proteins, lipopolysaccharides, proteins, type IV secretion system (T4SS) proteins.*

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Резюме

ВЛИЯНИЕ АНТИГЕНОВ *BRUCELLA* SPP. НА ПАТОГЕНЕЗ И ДИАГНОСТИКУ БРУЦЕЛЛЕЗА У ЧЕЛОВЕКА

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Введение. Бруцеллез остаётся одной из наиболее распространенных и недодиагностируемых зоонозных инфекций, с более чем 500 000 новых случаев среди людей ежегодно и устойчивой энзоотией среди сельскохозяйственных животных. *Brucella melitensis, B. abortus, B. suis* и *B. canis* являются основными патогенными видами, вызывающими хронические заболевания с неспецифическими клиническими проявлениями. Антигены *Brucella* играют ключевую роль в патогенезе и

формировании иммунного ответа, однако существующие методы диагностики недостаточно специфичны, а лицензированной вакцины для человека до сих пор не существует. Это подчёркивает необходимость создания более точных антиген-ориентированных средств диагностики и профилактики.

Цель. Провести обзор и оценку основных антигенов *Brucella* spp., обладающих подтвержденной или потенциальной диагностической и вакцинной значимостью, с акцентом на их структурные особенности, иммуногенность и прикладную ценность.

Стратегия поиска: Систематический обзор проведён в соответствии с рекомендациями PRISMA. Поиск литературы выполнен в базах PubMed, Scopus и Web of Science за период 2018–2025 гг. с использованием заранее определённых ключевых слов, связанных с антигенами *Brucella*, диагностикой и разработкой вакцин. После многоэтапного отбора по заголовкам, аннотациям и полным текстам в итоговый анализ было включено 106 исследований, что отражено на схеме PRISMA.

Результаты. Наружные мембранные белки (Omp16, Omp19, Omp25, Omp31), периплазматические белки (BP26, Cu/Zn-SOD, L7/L12), белки теплового шока (Hsp60, Hsp70) и компоненты системы секреции типа IV (VirB) демонстрируют разную степень иммуногенности. Среди них белки L7/L12, Omp19 и SOD обладают наибольшим потенциалом как серологические маркеры и компоненты вакцинных конструкций. Часть этих антигенов рассматривается в составе рекомбинантных субъединичных вакцин.

Заключение. Молекулярно охарактеризованные антигены Brucella открывают реальные перспективы для совершенствования диагностики и разработки целенаправленных и безопасных вакцин против бруцеллеза человека. Их иммунологическая значимость обосновывает необходимость дальнейшей доклинической оценки и внедрения в прикладную иммунодиагностику.

Ключевые слова: *бруцеллёз, Brucella spp., антигены, наружные мембранные белки, диагностика, вакцинопрофилактика, тепловые шоковые белки, липополисахариды, белки, белки системы секреции типа IV.*

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Түйіндеме

BRUCELLA SPP. АНТИГЕНДЕРІНІҢ АДАМ БРУЦЕЛЛЁЗІНІҢ ПАТОГЕНЕЗІ МЕН ДИАГНОСТИКАСЫНА ӘСЕРІ

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Кіріспе. Бруцеллез – жыл сайын 500 000-нан астам адамда тіркелетін, үй жануарлары арасында кеңінен таралған және жиі анықталмайтын зооноздық инфекция. *Brucella melitensis, B. abortus, B. suis* және *B. canis* – аурудың негізгі қоздырғыштары, олар созылмалы және спецификалық емес белгілермен сипатталатын инфекцияларды тудырады. *Brucella* антигендері ауру патогенезінде және иммундық жауапта маңызды рөл атқарады. Алайда қазіргі диагностикалық тесттердің спецификалығы төмен және адамға арналған лицензияланған вакцина әлі жасалмаған. Бұл диагностика мен алдын алуға арналған антигендік негіздегі құралдарды жетілдіру қажеттігін көрсетеді.

Мақсат. *Brucella* spp. антигендерінің құрылымдық сипаттамалары, иммуногендік қасиеттері және қолданбалы әлеуетін бағалай отырып, олардың диагностикалық және вакциналық маңыздылығын талдау.

Іздеу стратегиясы. Жүйелі шолу PRISMA нұсқауларына сәйкес жүргізілді. Әдебиеттерді іздеу PubMed, Scopus және Web of Science деректер базаларында 2018–2025 жылдар аралығында Brucella антигендері, диагностикасы және вакцина әзірлеуіне қатысты алдын ала анықталған кілт сөздер арқылы орындалды. Тақырыптар, аңдатпалар және толық мәтіндер бойынша көпсатылы іріктеуден кейін қорытынды талдауға 106 зерттеу енгізілді, бұл PRISMA диаграммасында көрсетілген.

Нәтижелер. Omp16, Omp19, Omp25, Omp31 секілді сыртқы мембраналық белоктар, BP26, Cu/Zn-SOD, L7/L12 периплазмалық белоктары, Hsp60, Hsp70 жылулық шок белоктары және VirB секреция жүйесінің белоктары әртүрлі иммуногендік белсенділік көрсетті. L7/L12, Omp19 және SOD антигендері серологиялық сезімталдық пен қорғаныштық жауапты индукциялау бойынша ерекше әлеуетке ие. Бұл антигендер рекомбинантты субъединикалық вакциналар құрамында зерттелуде.

Қорытынды. Молекулалық деңгейде сипатталған Brucella антигендері адам бруцеллезін диагностикалау мен мақсатты, қауіпсіз вакциналарды құрастыру бағытында маңызды мүмкіндіктер береді. Олардың иммунологиялық маңыздылығы доклиникалық зерттеулер мен қолданбалы иммунодиагностикада одан әрі зерттеуді қажет етеді.

Түйінді сөздер: *бруцеллез, Brucella spp., антигендер, сыртқы мембраналық белоктар, диагностика, вакцина, жылулық шок белоктары, липополисахаридтер, белоктар, IV типті секреция жүйесінің белоктары*

Дәйексөз үшін:

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Introduction

Brucellosis is a globally prevalent zoonotic disease, with the highest endemicity reported in the Mediterranean region, Central Asia, sub-Saharan Africa, and Latin America. The causative agents, Gram-negative facultative intracellular coccobacilli of the genus *Brucella*, affect both public health and livestock productivity by causing reproductive failure in animals and systemic illness in humans [4,39]. Transmission to humans typically occurs via ingestion of raw milk or dairy items, exposure to airborne infectious particles, or direct contact with livestock carrying the pathogen or their secretions. Occupations involving close animal contact-such as farming, slaughtering, and veterinary work-remain the highest risk [13,93]. The global distribution of brucellosis prevalence, based on epidemiological data published between 2015 and 2024, is illustrated in Figure 1.

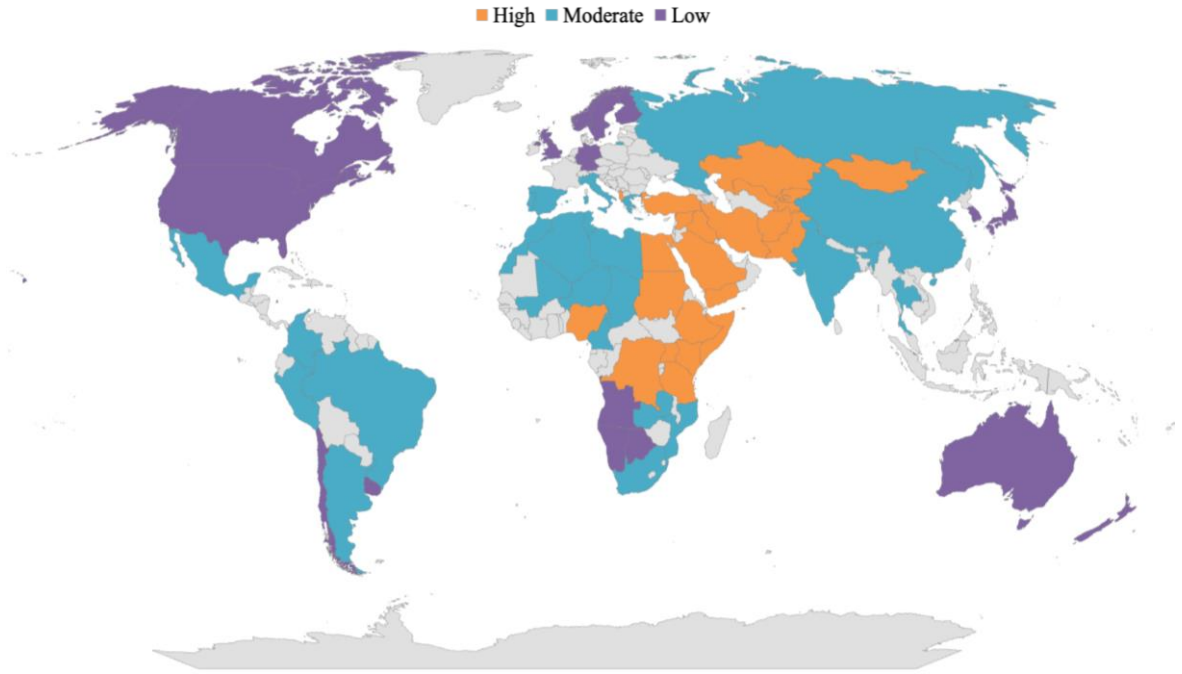

Figure 1. Global distribution of human brucellosis prevalence based on reviewed epidemiological literature (2015–2024). The color scale indicates prevalence levels: high (orange), moderate (blue), and low (purple). Only countries with available data are shown.

Twelve *Brucella* species have been formally identified, with *B. melitensis*, *B. abortus*, *B. suis*, and, to a lesser extent, *B. canis* recognized as the primary human pathogens. *B. melitensis*, commonly found in sheep and goats, is the most virulent and often causes chronic systemic disease [16,76]. The clinical manifestations of brucellosis are nonspecific-fever, fatigue, arthralgia-and may resemble various febrile conditions, complicating clinical recognition, especially in chronic or subclinical infections [12].

Bacteriological diagnosis through blood culture remains the gold standard, yet is often limited by low sensitivity in chronic cases and long incubation times. Serological methods such as the Rose Bengal test (RBT), standard agglutination test (SAT), and enzyme-linked immunosorbent assay (ELISA) are widely used but exhibit variable performance depending on disease stage, endemicity, and host immune status [39,92]. The development of antigen-based diagnostic platforms-particularly those incorporating recombinant outer membrane proteins (OMPs) like Omp28 and Omp31-has improved specificity and reproducibility in ELISA assays [3,38]. Additional methods such as fluorescence polarization assays and paper-based ELISAs offer promise for point-of-care testing in field conditions [41].

Despite the availability of effective veterinary vaccines such as *B. abortus* S19 and *B. melitensis* Rev.1, no vaccine is currently licensed for human use. These animal vaccines, although valuable for controlling livestock infections, are unsuitable for human application due to residual virulence, safety concerns in vulnerable populations, and interference with serological diagnostics [59,17]. These challenges underscore the need for safe, well-defined *Brucella* antigens with diagnostic and immunogenic potential.

Among the most relevant immunological targets are outer membrane-associated proteins (OMPs), structural endotoxins like lipopolysaccharides (LPS), stress-inducible proteins such as Hsp60 and Hsp70, antioxidant defense enzymes including Cu/Zn-superoxide dismutase (SOD), and molecular determinants of virulence encoded by the type IV secretion system (T4SS). Each of these antigen classes contributes to different facets of *Brucella* pathogenicity-including immune evasion, intracellular persistence, and inflammation modulation. For example, recombinant Omp19 and Omp31 have demonstrated protective efficacy and strong immunoreactivity in experimental models [29]. LPS and HSPs suppress innate immune signaling and facilitate chronic infection [50], while T4SS proteins are indispensable for intracellular replication and resistance to host clearance mechanisms. Their systematic characterization forms the basis for the development of next-generation serological assays and subunit vaccines [19].

Search strategy

A systematic search of the literature was conducted in the PubMed, Scopus, and Web of Science databases for publications between 2018 and 2025. The following combinations of keywords and Medical Subject Headings (MeSH) terms were used: *Brucella* spp., *Brucella melitensis*, *Brucella abortus, Brucella suis*, diagnosis, epidemiology, antigen, outer membrane proteins (Omp16, Omp19, Omp25, Omp31), periplasmic proteins (BP26, L7/L12, SodC), heat shock proteins (Hsp60, Hsp70, DnaK, GroEL), lipopolysaccharides (S-LPS, R-LPS), type IV secretion system (VirB), vaccine candidate, and serodiagnosis.

Selection process

– Initial search retrieved 4,460 records

– Title screening excluded 2,806 studies as duplicates or irrelevant

– Abstract screening excluded 554 additional records due to thematic or methodological mismatch

– Full-text review excluded 138 articles because of methodological overlap or insufficient data

– **Final inclusion:** 106 publications that fulfilled all eligibility criteriaInclusion criteria

– Peer-reviewed articles indexed in PubMed, Scopus, or Web of Science

– Published in English

– Publication period: 2018–2025

– Studies providing data on antigen structure, immunogenicity, pathogenesis, or diagnostic/vaccine potential of *Brucella* spp.

Exclusion criteria

– Duplicate records

– Studies unrelated to *Brucella* antigens or diagnostics

– Reviews without original data (unless of high scientific value)

– Articles with insufficient methodological transparency

Distribution of included studies

– Introduction: 16

– Sections 1.1–1.3 (epidemiology, diagnostics, preventive challenges): 24

– Section 2.1 (Outer membrane proteins): 17

– Section 2.2 (Periplasmic proteins): 16

– Section 2.3 (Heat shock proteins): 12

– Section 2.4 (Lipopolysaccharides): 14

– Section 2.5 (Type IV secretion system proteins): 12

– Section 2.6 (Other antigens): 8

Epidemiological relevance of brucellosis as a persistent zoonotic threat

Brucellosis ranks among the most widespread and economically significant zoonoses, particularly in regions with inadequate veterinary infrastructure. The disease reduces livestock productivity through abortions and infertility, and it continues to pose a substantial risk to human health due to zoonotic spillover [4,93]. Although official data report around 500,000 new human cases annually, modelling studies estimate the true global incidence at over 2 million, reflecting widespread underdiagnosis and underreporting, particularly in Asia and Africa [41]. The global distribution of human brucellosis prevalence is shown in Figure 2, based on data from reviewed epidemiological studies published between 2015 and 2024.

High-income countries have largely controlled brucellosis through systematic test-and-slaughter programs and livestock vaccination.

However, in endemic zones such as Central Asia, East Africa, and parts of Latin America, the disease remains entrenched. Field investigations in Kenya and Uganda have shown a clear epidemiological connection between infected livestock and human brucellosis within the same communities [17,29].

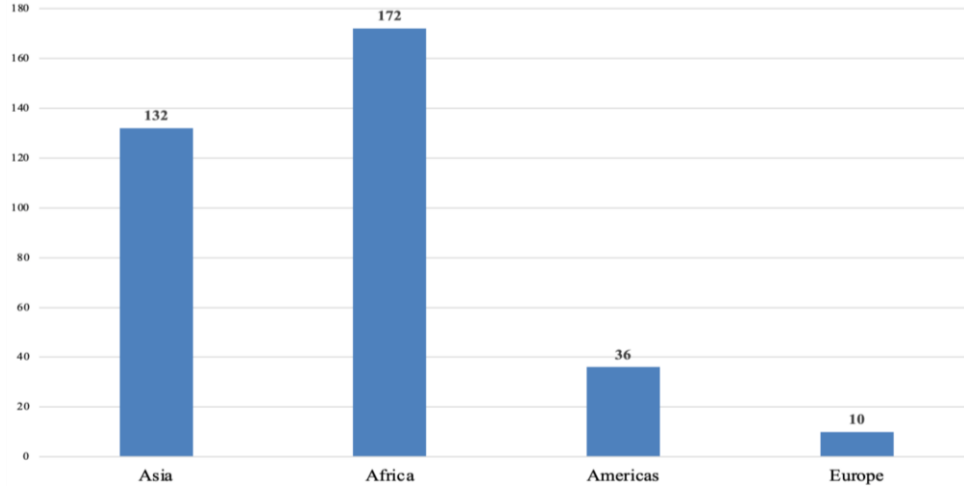

Figure 2. Geographical distribution of brucellosis-related studies included in the review. The majority of studies were conducted in Africa (n=172) and Asia (n=132), followed by the Americas (n=36) and Europe (n=10), reflecting regional research focus and burden.

Medically important Brucella species

Among the twelve identified Brucella species, human brucellosis is primarily attributed to *B. melitensis, B. abortus, B. suis*, and, less frequently, *B. canis*. *B. melitensis*, predominantly found in sheep and goats, is the most pathogenic and commonly identified in endemic regions. Its high bacterial concentration in placental tissues and unpasteurised milk increases the risk of human infection via ingestion or contact [74,50].

B. abortus, associated with cattle, presents a significant occupational risk in areas with insufficient veterinary control. Although generally less aggressive, it accounts for a substantial proportion of human cases [19]. *B. suis*, primarily affecting pigs, causes a more chronic and suppurative form of brucellosis, often involving abscess formation and osteoarticular complications. Human infection occurs mostly in hunters, swine farmers, and abattoir workers [74,17]. *B. canis*, though rare, is occasionally reported in immunocompromised individuals and poses a risk in canine breeding or veterinary settings. Additionally, emerging species such as *B. inopinata* and marine mammal–associated *Brucella* strains have demonstrated zoonotic potential and require surveillance [18].

Challenges in diagnosis and limitations of current vaccines. The diagnosis of brucellosis remains challenging due to the non-specificity of symptoms, low bacterial loads, and overlapping clinical features with other infections. Culture, while definitive, is often impractical in chronic cases due to delayed growth [13,12]. Serological tests (RBT, SAT, ELISA) are widely used but suffer from false-positive results due to cross-reactivity with other Gram-negative bacteria and false negatives following antimicrobial therapy [24,89].

Molecular assays, particularly real-time PCR, have significantly improved the sensitivity and specificity of *Brucella* detection, enabling early diagnosis and species differentiation [60,90]. However, their routine use is limited by high costs, the need for specialised infrastructure, and the lack of standardised protocols across laboratories, which hinders result comparability and widespread implementation [35].

On the prophylactic front, live attenuated veterinary vaccines - such as *B. melitensis* Rev.1 and *B. abortus* S19- have played a central role in controlling brucellosis in livestock. Nevertheless, their use in humans is contraindicated due to the risk of residual virulence, abortion in pregnant individuals, and post-vaccination seroconversion, which can interfere with serological diagnostics [27,40]. These limitations have prompted the search for alternative human vaccine strategies that combine safety with robust protective immunity.

Modern approaches to *Brucella* vaccine development increasingly emphasize recombinant subunit platforms incorporating well-characterized antigens such as Omp16, Omp19, Omp25, Omp31 (outer membrane proteins), stress-response molecules like Hsp60 and Hsp70, and antioxidant enzymes including Cu/Zn-superoxide dismutase (SOD). These components have demonstrated the capacity to activate both humoral and cellular branches of the immune system in experimental settings. To enhance immunogenicity, they are tested in combination with adjuvants or delivered via attenuated vectors such as *Salmonella enterica* or viral platforms, aiming to stimulate mucosal and systemic immunity [37].

Immunological significance of *Brucella* antigens

Brucella antigens are central to the pathogen's intracellular lifestyle, immune modulation, and chronic persistence. OMPs-such as Omp16, Omp19, Omp25, and Omp31—facilitate adhesion, immune evasion, and survival within host phagocytes, making them attractive targets for diagnostics and recombinant vaccines [73,91]. The smooth form of LPS (S-LPS), present in *B. melitensis* and *B. abortus*, reduces TLR4-mediated activation and impairs pro-inflammatory signaling, whereas rough LPS variants, typical of *B. canis*, are less immunosuppressive [44].

Intracellular survival of *Brucella* is facilitated by the type IV secretion apparatus (T4SS), encoded within the virB gene cluster, which disrupts phagolysosomal maturation and enables the establishment of replicative compartments [5]. Antioxidant enzymes like Cu/Zn-SOD allow *Brucella* to neutralize reactive oxygen species within phagocytes, supporting its persistence [25]. Moreover, TIR-domain-containing effector proteins (BtpA, BtpB) mimic host signaling molecules and inhibit IL-12 and IFN-γ responses, suppressing protective Th1 immunity [84].

Transcriptomic analyses of infected human immune cells confirm downregulation of genes involved in inflammation and antigen presentation [55]. In parallel, recent findings show that *Brucella* antigens disrupt dendritic cell function-especially in plasmacytoid DCs-by modulating SLAMF7/8 signaling, thereby impairing interferon responses and T cell activation during chronic infection [106,43].

The main groups of *Brucella* spp. antigens and their characterisation

Outer Membrane Proteins (OMPs)

Outer membrane proteins (OMPs) of *Brucella* spp. are integral components of the bacterial cell envelope and play a central role in pathogenesis, immune evasion, and intracellular survival. These membrane-associated proteins are involved in maintaining structural stability, regulating molecular exchange, and facilitating nutrient uptake. Due to their localization on the bacterial surface and ability to elicit immune responses, they represent valuable candidates for serodiagnostic assays and the design of recombinant vaccines [28,6].

Structurally, *Brucella* OMPs are grouped by molecular weight into three categories: high molecular weight proteins (88–94 kDa), porins (36–38 kDa), and low to medium-sized immunogenic proteins (25–34 kDa) [28]. Among these, the 25–34 kDa group includes Omp25 and Omp31, which are highly conserved across pathogenic species and have been most extensively studied due to their strong immunogenicity and translational relevance [8,63].

Omp25 plays a multifaceted role in virulence and immune modulation. It downregulates TNF-α secretion in macrophages, facilitating bacterial persistence by dampening host inflammatory responses [49]. Although masked by smooth lipopolysaccharide (S-LPS) during natural infection, recombinant Omp25 has demonstrated strong humoral and cellular immunogenicity in animal models, particularly when used with adjuvants [75].

Omp31 is absent in *B. abortus* but present in *B. melitensis* and *B. suis*. It is involved in iron acquisition and outer membrane permeability, and elicits strong antibody responses-especially in infections caused by rough strains such as *B. ovis* [70]. Its immunodominance and species-specific expression make it a valuable antigen for differential diagnosis and targeted immunoprophylaxis [57].

Porin proteins Omp2a and Omp2b form trimeric β-barrel channels that regulate outer membrane permeability. Despite over 85% sequence identity, species-specific

mutations result in functional divergence. While less immunogenic than Omp25 or Omp31, these porins contribute to resistance against environmental stressors and antimicrobial peptides, and may serve as auxiliary components in multivalent vaccine formulations to enhance protective breadth [87, 23].

Smaller outer membrane lipoproteins such as Omp16 and Omp19 also play significant roles in *Brucella* survival and host interaction. Omp16, homologous to peptidoglycan-associated lipoproteins, is essential for cell viability. Conditional knockdown of Omp16 impairs membrane integrity and triggers elevated pro-inflammatory cytokine responses in host cells [104]. Omp19 acts as a protease inhibitor, aiding survival in hostile environments such as phagosomes and mucosal surfaces. Notably, recombinant *Lactococcus lactis* expressing Omp19 has induced mucosal immunity and protection in oral vaccination models, supporting its utility for needle-free immunization [20].

The diagnostic application of OMPs is well substantiated. Recombinant forms of Omp25, Omp31, Omp16, and Omp19 have been successfully incorporated into ELISA platforms, demonstrating improved specificity and sensitivity over traditional LPS-based tests [7,58]. Their reduced cross-reactivity with other Gram-negative bacteria and suitability for DIVA (Differentiating Infected from Vaccinated Animals) strategies enhance their value in both human and veterinary diagnostics [68,11].

A major advantage of OMP-based antigens is their high sequence conservation across *Brucella* species, enabling the development of cross-protective vaccines. Bioinformatics-driven epitope mapping has facilitated the design of multi-epitope constructs incorporating conserved OMP regions, several of which have shown promising immunogenicity and protection in preclinical studies [97]. Moreover, their recombinant nature and lack of residual virulence make them safer alternatives to whole-cell vaccines, particularly for human use [75,97].

Periplasmic proteins of *Brucella spp.* play essential roles in maintaining cell envelope integrity, evading host immune responses, and surviving intracellular stress conditions. Unlike classical surface-exposed virulence factors, these proteins are often hidden from direct immune detection but exhibit significant immunogenic potential, making them relevant candidates for diagnostic and vaccine development. The most studied include BP26, Cu/Zn-SOD (SodC), DsbA, HtrA, PrpA, BepC, and Omp19.

Also referred to as Omp28, BP26 is a highly conserved protein (~26 kDa) expressed across smooth *Brucella* species [7]. Initially misclassified as an outer membrane protein, it was later localized to the periplasmic space. Structurally, BP26 shares homology with phage channel proteins and has demonstrated interactions with host extracellular matrix components such as collagen and vitronectin, suggesting a potential role in adhesion [58].

Immunologically, BP26 elicits strong antibody responses in both infected and vaccinated hosts. It has been widely applied in indirect ELISA and lateral flow tests with high sensitivity and specificity [7, 58]. Its utility as a DIVA (Differentiating Infected from Vaccinated Animals) marker is supported by data from BP26-deficient vaccine strains (e.g., *B. melitensis* Rev.1 Δbp26) [68]. Recombinant BP26 has also shown the capacity to induce Th1-biased responses and partial protection in murine models [11, 97].

SodC is a periplasmic metalloenzyme that catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen, providing crucial protection against the oxidative burst of phagocytes. It is required for survival of *Brucella* within macrophages, particularly under phagolysosomal stress [78].

Although SodC induces relatively modest humoral responses, it triggers robust T-cell immunity, including IFN-γ production. Immunization with SodC, especially in combination with antigens like L7/L12 and Omp25, enhances protection in murine models [22, 32]. Recombinant vaccines incorporating SodC have shown significant immunogenicity in BALB/c mice and are considered valuable components in multivalent vaccine formulations [83].

DsbA is a periplasmic thiol-disulfide oxidoreductase essential for the folding of exported proteins via disulfide bond formation. Disruption of dsbA expression in *Brucella* leads to compromised stress resistance and severely reduced intracellular survival [61].

Mutants lacking DsbA display defects in outer membrane protein maturation and envelope homeostasis. These properties make DsbA a potential target for rational attenuation of live vaccines, preserving immunogenicity while reducing virulence [66].

HtrA is a periplasmic serine protease upregulated in response to stress stimuli such as heat, oxidative agents, and acidic pH. It degrades misfolded proteins and stabilizes the bacterial envelope under host-imposed stress [105].

Deletion of htrA impairs *Brucella* survival in macrophages and increases sensitivity to antimicrobial peptides. Live attenuated vaccine strains lacking htrA have demonstrated safety and the ability to elicit Th1-skewed immune responses, making HtrA a valuable marker for stress response and vaccine design [33].

PrpA is an immunomodulatory periplasmic enzyme capable of catalyzing the conversion of L-proline to D-proline and inducing polyclonal B-cell activation in a T-cell-independent manner. It promotes hypergammaglobulinemia and upregulates TGF-β, contributing to immune suppression and *Brucella* persistence [48].

Although not protective alone, PrpA enhances cellular immunity when incorporated into multivalent vaccine formulations, helping to reduce bacterial loads [83]. Elevated anti-PrpA antibodies in chronic brucellosis cases suggest potential use in immune profiling.

BepC, a TolC-like outer membrane channel, functions as part of *Brucella's* tripartite efflux systems that expel bile salts, antimicrobial peptides, and antibiotics. Knockout of bepC results in heightened sensitivity to these agents and reduced virulence in murine models [81].

While not strongly immunogenic under natural infection, BepC is currently being explored as a DIVA marker and a possible target for efflux pump inhibitors to enhance antibiotic efficacy. Although classified as an outer membrane lipoprotein, Omp19 anchors to the inner leaflet and acts functionally as a periplasmic protease inhibitor. It protects *Brucella* against proteolytic degradation in the gastrointestinal tract and within lysosomes. Omp19-deficient mutants show reduced infectivity after oral administration and impaired intracellular survival [58,33].

Recombinant unlipidated Omp19 (U-Omp19) has demonstrated adjuvant properties, enhancing mucosal

immunity and antigen presentation. It also serves as a DIVA marker, as Rev.1 Δomp19 mutants do not induce detectable anti-Omp19 antibodies [81].

Heat shock proteins (HSPs) play essential roles in the adaptation, survival, and immune interactions of *Brucella* spp. under host-induced stress. Among the most functionally and immunologically relevant are the chaperone proteins Hsp60 (GroEL), Hsp70 (including DnaK), and the ATP-independent serine protease HtrA. These molecules are not only critical for intracellular proteostasis but also serve as immunodominant antigens with significant implications for serodiagnosis and vaccine development.

GroEL is a 60 kDa chaperonin involved in protein folding, expressed under stress conditions and essential for *Brucella* spp. survival in the intracellular environment. It promotes immune responses, especially in combination with outer membrane proteins (OMPs) such as Omp25 and Omp31. Co-immunization studies have demonstrated enhanced protection in murine models, indicating synergistic effects between GroEL and surface antigens [1]. Transcriptomic analyses of the *Brucella melitensis* Rev.1 vaccine strain revealed downregulation of GroEL under acidic stress, which may correlate with attenuated virulence [71]. Furthermore, GroEL expression is modulated in response to antibiotics such as polymyxin B, highlighting its role in stress adaptation. Post-translational modifications including lysine acylation affect GroEL and other virulence-associated proteins, linking structural regulation with immune evasion and intracellular persistence. Studies also indicate a role for GroEL in subverting autophagic host defenses, particularly via LC3-dependent mechanisms in macrophages [105].

Hsp70 proteins, including DnaK, contribute to protein homeostasis and immune activation. DnaK has been identified in the vaccine strain *Brucella* 104M through proteogenomic profiling, confirming its constitutive expression and immunogenicity [54]. Functionally, DnaK facilitates refolding of misfolded proteins and promotes macrophage activation via NF-κB pathways, leading to pro-inflammatory cytokine production [30,34,56]. Its dual role in stress resilience and host immune signaling underscores its potential as a diagnostic and vaccine antigen. Structural modeling of Hsp70 family members, including insights into inhibitor interactions, further highlights their biomedical relevance [98].

Beyond ATP-dependent chaperones, ATP-independent serine proteases such as HtrA also contribute critically to protein homeostasis and virulence. HtrA is a periplasmic protease essential for *Brucella* virulence and protein quality control. It degrades misfolded proteins and modifies outer membrane components, facilitating adaptation to oxidative and thermal stress [30,51]. Experimental deletion of the *htrA* gene results in reduced virulence and impaired intracellular survival of *Brucella* within macrophages. HtrA regulates multiple virulence-associated functions, including bacterial adhesion, invasion, and biofilm development [52]. It may also interfere with host immune responses by modulating cellular signaling pathways. Given its ability to function without ATP, its induction under cellular stress, and the high degree of structural preservation across *Brucella* strains, HtrA is regarded as a strong candidate for use in both diagnostic strategies and antigen-specific vaccine development [51].

Although less extensively studied, small heat shock proteins (sHSPs) such as Hsp20-like proteins are believed to contribute to immediate bacterial stress responses without requiring ATP. These proteins assist in preventing protein aggregation and stabilizing membrane integrity during infection. Their expression is often upregulated under heat or oxidative stress and may aid *Brucella* spp. in rapid adaptation to intracellular host environments [56]. While their immunogenicity remains less characterized compared to major HSPs, their functional relevance and evolutionary conservation warrant further investigation as potential diagnostic or vaccine adjuncts.

HSPs of *Brucella* spp. function at the intersection of virulence regulation, stress adaptation, and immune interaction. Their expression is modulated by transcriptional regulators such as MucR, further embedding them in broader regulatory networks [71]. The conserved structure and antigenicity of HSPs, particularly GroEL, DnaK, and HtrA, across multiple *Brucella* species including *B. melitensis*, *B. abortus*, and *B. suis*, enhance their relevance for cross-protective vaccine strategies. Diagnostic applications, including lateral flow assays targeting Hsp70, show promise for rapid field detection [56]. In vaccine design, co-formulation of HSPs with OMPs or adjuvants has demonstrated increased efficacy in animal models, offering pathways for subunit vaccine development [1].

In summary, HSPs are multifunctional proteins essential for *Brucella* spp. virulence, intracellular survival, and immune evasion. Their structural conservation and immunogenic potential make them compelling candidates for targeted diagnostics and subunit vaccine platforms against brucellosis.

Lipopolysaccharides (LPS)

Lipopolysaccharide (LPS) is a major surface component of *Brucella* spp. and represents the principal immunodominant antigen in smooth strains. Structurally, *Brucella* LPS consists of three classical domains: lipid A, a non-repeating oligosaccharide core, and an O-polysaccharide, also known as the O-antigen. Unlike enterobacterial LPS, the lipid A of *Brucella* is modified with very long-chain fatty acids and exhibits hypoacylation and hypophosphorylation, leading to low endotoxicity and reduced recognition by Toll-like receptor 4 (TLR4) [72].

The O-polysaccharide is composed exclusively of 4-formamido-4,6-dideoxy-α-D-mannopyranose (N-formylperosamine) residues, primarily linked by α-(1→2) glycosidic bonds. This conserved homopolymer, consisting of approximately 90–100 repeating units, provides serological specificity and supports immune evasion through molecular mimicry [10].

Brucella spp. display two LPS phenotypes: smooth (S-LPS), with a complete O-antigen chain, and rough (R-LPS), lacking or having a truncated surface polysaccharide. Smooth strains, including *B. melitensis*, *B. abortus*, and *B. suis*, exhibit species-specific antigenic profiles: *B. melitensis* primarily expresses M-epitopes, *B. abortus* A-epitopes, and *B. suis* shows a mixed pattern [21]. In contrast, rough strains such as *B. canis* and *B. ovis* produce R-LPS and are generally less virulent in humans [42].

Functionally, the O-antigen plays a central role in immune evasion. It shields outer membrane proteins, interferes with opsonization and complement activation, and reduces neutrophil recruitment. The low immunostimulatory potential of *Brucella* LPS, due to its weak interaction with TLR4, allows intracellular invasion with minimal inflammation [100]. Furthermore, the branched structure of the core

oligosaccharide masks pathogen-associated molecular patterns (PAMPs), enhancing immune concealment [101].

The O-antigen also contributes to intracellular survival. In smooth strains, it impairs phagosome-lysosome fusion and delays phagosomal maturation, facilitating replication within host macrophages [65]. Conversely, R-LPS mutants, which lack this protective barrier, are more susceptible to immune clearance due to increased membrane permeability and exposure of antigenic determinants [53].

Immunologically, the O-antigen elicits the dominant humoral response during natural infection. Specific antibodies, particularly of the IgM and IgG2 subclasses, arise early and form the basis of classical serological assays, for example, the Rose Bengal assay, the complement fixation reaction, and enzyme-linked immunosorbent assays (ELISAs) [15]. However, these tests are limited by cross-reactivity among smooth *Brucella* species and with unrelated Gram-negative bacteria. A notable example is *Yersinia enterocolitica* O:9, which expresses an identical terminal saccharide epitope [47].

Vaccination further complicates serodiagnosis. Live attenuated strains such as *B. abortus* S19 and *B. melitensis* Rev.1 generate anti-O-antigen antibodies indistinguishable from those induced by infection, hampering surveillance and eradication programs [26]. To overcome this, the rough strain *B. abortus* RB51 is used in veterinary settings. Its lack of O-antigen prevents interference with serological testing and aligns with DIVA (Differentiating Infected from Vaccinated Animals) principles [62].

Despite its immunodominance, the O-antigen is a thymus-independent antigen and fails to activate T-helper cells or induce lasting immunological memory. Thus, while anti-LPS antibodies can mediate opsonization in vitro, effective protection against brucellosis relies primarily on Th1-driven cellular immunity [64].

From a vaccine perspective, native LPS is suboptimal due to its residual endotoxin-like properties and limited capacity to induce cell-mediated responses. Nevertheless, detoxified LPS preparations and conjugate vaccines—linking the O-antigen to protein carriers such as tetanus toxoid or bovine serum albumin—have demonstrated improved immunogenicity and partial protection in murine models. However, these strategies remain experimental and face ongoing challenges, including cross-reactivity and potential diagnostic interference [36].

Type IV secretion system proteins (VirB)

The type IV secretion apparatus (T4SS), whose genetic components are organized within the virB operon (virB1 to virB12), represents one of the principal virulence mechanisms in *Brucella spp.* and is indispensable for intracellular survival, immune evasion, and chronic persistence in the human host. Structurally and functionally analogous to conjugative systems of Gram-negative bacteria, the T4SS mediates the translocation of effector proteins across the bacterial envelope into host cells, enabling *Brucella* to subvert key cellular processes such as vesicle trafficking, apoptosis, autophagy, and innate immune signaling. Recent studies have also identified novel T4SS effectors, such as RS15060, which contribute to intracellular replication and chronic infection, further underscoring the role of this system in bacterial virulence [94,95].

The *virB* operon was initially characterized in *B. suis* and subsequently found to be highly conserved across *B. melitensis* and *B. abortus*. Its expression is upregulated in response to phagosomal acidification within the *Brucella*-containing vacuole (BCV), typically peaking within the first 4–6 hours post-internalization. The assembled T4SS spans both bacterial membranes and comprises three structural modules: (i) the cytoplasmic ATPase complex (VirB4, VirB11), (ii) the inner membrane platform (VirB3, VirB6, VirB8, and the N-terminal of VirB10), and (iii) the outer membrane core complex (VirB7, VirB9, and the C-terminal of VirB10) [79].

VirB2, the major pilin subunit, is essential for pilus assembly and is involved in effector delivery. VirB4 and VirB11 act as ATPases energizing translocation, while VirB9 and VirB10 are critical for the stability of the secretion channel. Disruption of these genes abolishes secretion function and abrogates intracellular replication in both murine and ruminant models [88]. Other components such as VirB1 (a peptidoglycan hydrolase), VirB7 (lipoprotein), and VirB12 (surface-exposed protein) are not essential for secretion per se but contribute to apparatus stability and host-pathogen interactions [85].

The *Brucella* T4SS delivers several effector proteins that actively reprogram host cell biology. VceC induces endoplasmic reticulum (ER) stress by binding the chaperone BiP/Grp78, promoting membrane remodeling and facilitating BCV maturation and ER association [103]. RicA targets the small GTPase Rab2, disrupting ER-Golgi trafficking and interfering with phagosome maturation [45]. TIR-domain-containing effectors BtpA and BtpB inhibit Toll-like receptor (TLR) signaling by mimicking host adaptor proteins, suppressing NF-κB activation and proinflammatory cytokine production. These activities collectively enable Brucella to persist within macrophages and prevent effective antigen presentation, contributing to the stealthy and chronic nature of human brucellosis [46, 80].

Due to their conserved nature and immunological relevance, several VirB proteins have been investigated as serodiagnostic and vaccine targets. Antibodies against VirB9, VirB10, VirB11, and VirB12 have been consistently detected in sera from naturally infected humans and animals, with recombinant forms used in diagnostic ELISA formats demonstrating sensitivity and specificity above 90% [85,95]. Notably, VirB12, while non-essential for secretion, is surface-exposed and highly immunodominant, making it a promising DIVA (Differentiating Infected from Vaccinated Animals) marker. In parallel, PCR assays targeting VirB genes have proven useful for identifying virulent *Brucella* field isolates and for molecular epidemiology in endemic regions [99].

In the context of vaccine development, VirB proteins offer a rational strategy to disrupt *Brucella* intracellular persistence. Subunit vaccine candidates incorporating VirB9 and VirB11 have shown the ability to induce Th1-skewed cellular responses, which are essential for protection against intracellular pathogens [69]. Additionally, T4SS effectors such as VceC and BtpB have demonstrated immunomodulatory and protective potential in experimental murine models, contributing to reduced bacterial loads and altered cytokine profiles following challenge. Nevertheless, limitations remain concerning antigen delivery systems, adjuvant selection, and cross-protection across *Brucella* species, warranting further research into optimized multivalent vaccine constructs [67,88].

Other Potential Antigens

Beyond the well-characterized antigenic determinants of *Brucella* spp., such as lipopolysaccharides (LPS), outer membrane proteins (OMPs), and type IV secretion system

(T4SS) components, a range of less-studied but immunologically promising proteins have been identified through immunoproteomic approaches and computational epitope prediction. These include cytoplasmic enzymes with atypical surface exposure, ribosomal constituents, periplasmic transporters, and transcriptional regulators. Despite the limited availability of in vivo validation, their evolutionary conservation, stress-induced upregulation, and demonstrated immunogenicity in infected hosts suggest a potential role in both serodiagnostics and rational vaccine design [14].

Among the most notable are cytosolic metabolic enzymes: noteworthy examples include the cytosolic enzymes enolase, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and MDH (malate dehydrogenase). These multifunctional or "moonlighting" proteins, despite the absence of conventional secretion motifs, have been identified at the bacterial cell surface, where they engage in interactions with host factors. Enolase, in particular, has been shown to bind plasminogen, potentially promoting degradation of the extracellular matrix and facilitating bacterial dissemination. GAPDH has been implicated in adhesion processes through binding to host fibronectin and other extracellular components. Importantly, MDH has shown selective immunoreactivity with sera from naturally infected, but not vaccinated, animals, indicating its diagnostic value as a marker of early-stage infection [77].

Ribosomal proteins, particularly the L7/L12 ribosomal stalk complex, are evolutionarily conserved antigens that elicit strong Th1-biased immune responses. L7/L12 has been widely investigated as a subunit vaccine component and diagnostic antigen, especially when combined with outer membrane proteins to improve assay specificity [86]. Some ribosomal proteins beyond L7/L12, such as putative 30S subunit components, have been tentatively detected as reactive in proteomic analyses, but their diagnostic relevance is unclear due to limited characterization and high sequence conservation [96].

The *Brucella* cell surface protein 31 (BCSP31), a 31-kDa conserved protein originally described as a transport-related factor, is frequently targeted in PCR-based diagnostics and has demonstrated reliable immunogenicity in serological assays. Its application as a capture antigen in lateral flow devices and ELISA formats has been well documented, although its precise physiological function remains incompletely defined [76].

Additional periplasmic transporter proteins, including OppA (oligopeptide-binding protein), LAO-binding proteins (specific for L-arginine and ornithine), and elements of the Sec-dependent secretion system, have been identified as seroreactive antigens in ruminants naturally infected with *Brucella*. Although not surface-exposed in the classical sense, their elevated periplasmic abundance during infection and consistent recognition by host antibodies support their utility as internal markers in multiplex serodiagnostic platforms [2].

Regulatory proteins, notably the quorum-sensing transcriptional regulator VjbR and the cold-shock protein CspA, display altered expression profiles during intracellular adaptation. VjbR regulates the transcription of multiple virulence-associated genes and is involved in modulating host immune responses, while CspA contributes to bacterial survival under host-induced stress. Both proteins have demonstrated modest T-cell immunogenicity in preliminary experimental models, but their utility as diagnostic or vaccine antigens remains to be validated in comprehensive immunological studies [31].

The invasion-associated protein IalB is a membrane-associated factor essential for *Brucella* adhesion and intracellular survival. Deletion of the ialB gene in *B. suis* leads to reduced virulence, highlighting its role in pathogenesis. While its immunogenicity is moderate, IalB remains a potential target for subunit vaccines or DIVA diagnostics [9].

Discussion

Modern serological diagnostics of brucellosis have progressively transitioned from crude bacterial lysates toward the use of defined, molecular antigens. This refinement addresses the need for improved test specificity, particularly in endemic areas where cross-reactivity with *Yersinia enterocolitica* O:9 remains a major confounder. The adoption of recombinant antigens has not only enhanced specificity but also enabled species-independent detection and improved assay reproducibility across laboratories.

The enzyme-linked immunosorbent assay (ELISA) continues to be the primary platform for brucellosis screening in both humans and animals. Commercially available ELISA kits frequently incorporate recombinant or purified proteins such as Omp31, BP26, ribosomal protein L7/L12, and Cu/Zn superoxide dismutase (SOD), all of which demonstrate consistent immunoreactivity in infected sera. L7/L12 and SOD, in particular, show sustained serological responses throughout the course of infection, making them valuable markers for detecting both acute and chronic brucellosis.

The fluorescence polarization assay (FPA), recommended by the World Organisation for Animal Health, employs fluorescein-labeled O-polysaccharides derived from smooth lipopolysaccharide (S-LPS). While FPA offers rapid and highly sensitive detection, its reliance on high-quality LPS extraction and its inability to distinguish between *Brucella* species limit its broader diagnostic applicability. New approaches incorporating synthetic oligosaccharide analogues or recombinant proteins into FPA formats are currently under investigation to overcome these limitations.

Despite these advances, existing antigen-based assays still fall short in early-stage detection and multi-host application. To address these gaps, multiepitope recombinant constructs combining outer membrane proteins (e.g., Omp16, Omp19) with intracellular proteins such as enolase, malate dehydrogenase (MDH) are being developed. These composite antigens offer higher specificity by minimizing cross-reactivity with non-pathogenic microorganisms and may improve diagnostic accuracy during early infection.

Among the most promising diagnostic antigens, Omp16, Omp19, and Omp31 consistently exhibit strong immunogenicity across various *Brucella* species. Although Cu/Zn-SOD is periplasmic in localization, it elicits both humoral and cellular responses and has shown excellent diagnostic potential. Ribosomal proteins L7/L12, which induce early-phase antibody responses particularly in ruminants, are emerging as valuable components of non-LPS diagnostic panels. Additionally, several cytoplasmic metabolic enzymes—such as enolase, MDH, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—function as “moonlighting” proteins that become surface-exposed under intracellular stress conditions. Alongside regulatory proteins like VjbR, these antigens broaden the diagnostic landscape and may enhance sensitivity in latent infections.

In parallel, the pursuit of a safe and effective human brucellosis vaccine remains an urgent but unmet objective. Currently available live attenuated veterinary vaccines (e.g., *B. melitensis* Rev.1) are not suitable for human use due to residual virulence and interference with serological surveillance. This has shifted the focus to subunit and DNA-based vaccine strategies utilizing well-characterized, immunogenic proteins.

Promising antigen candidates validated in murine models include Omp16, Omp19, Omp25, Omp31, BP26, Cu/Zn-SOD, and L7/L12. DNA vaccines encoding L7/L12 and Omp16 have demonstrated strong Th1-type responses marked by IFN-γ and TNF-α secretion and a significant reduction in splenic bacterial burden following challenge. Cu/Zn-SOD, in addition to its antioxidant function, has proven highly immunogenic and effective in stimulating cell-mediated responses. Preliminary studies suggest that IalB may contribute to protection in DNA vaccine constructs, although its immunogenicity and protective efficacy remain lower than those of more established *Brucella* antigens.

The rational design of multivalent subunit vaccines rests on the inclusion of structurally and functionally diverse antigens that cover different stages of *Brucella* pathogenesis. Combinations such as Omp25, Omp31, and BP26 have already achieved diagnostic performance exceeding 94% sensitivity and 100% specificity in human ELISA formats. The inclusion of stress-responsive metabolic and regulatory antigens could improve the identification of persistent or subclinical *Brucella* infections, particularly in cases characterized by diminished levels of circulating antibodies.

Nevertheless, several translational challenges remain. Preclinical safety and immunogenicity data must be rigorously validated in relevant animal models. The scalable production of recombinant proteins under GMP conditions, optimization of adjuvants (e.g., CpG-ODNs, monophosphoryl lipid A), and selection of effective delivery platforms (such as viral vectors, mRNA, or nanoparticle systems) are critical next steps. Initial studies on intranasal and oral immunization strategies have already yielded encouraging results, demonstrating both favorable safety profiles and robust mucosal immunity.

In summary, the incorporation of defined *Brucella* antigens into diagnostic assays and vaccine platforms represents a promising path forward. The combined use of outer membrane, periplasmic, and cytoplasmic proteins offers an opportunity to develop highly specific, broadly applicable tools for the detection and prevention of human brucellosis. A comparative overview of immunogenicity levels for the most studied *Brucella* antigens is presented in Figure 3.

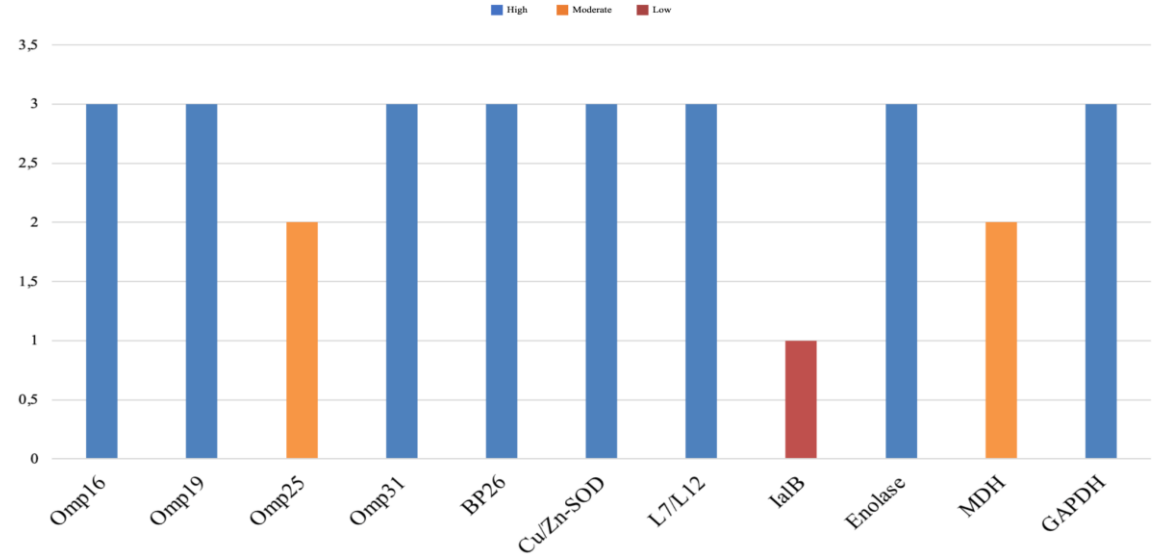

Figure 3. Comparative immunogenicity of *Brucella* antigens. The chart summarizes relative immunogenicity levels based on Th1/Th2 cytokine profiles, antibody responses, and T-cell reactivity data extracted from experimental studies. Only antigens with available and quantifiable immune data are included. Immunogenicity categories: high (blue), moderate (orange), and low (dark red).

Conclusion

Brucellosis remains a significant zoonotic threat that necessitates both the refinement of diagnostic tools and the development of effective preventive strategies. Traditional serological methods often exhibit insufficient sensitivity and specificity, particularly in chronic or latent infections, reinforcing the need for highly specific, antigen-based diagnostic assays.

Simultaneously, the formulation of a subunit vaccine represents a safe and scientifically grounded alternative to live-attenuated vaccines. Based on immunological relevance and accumulated experimental data, a panel of *Brucella* antigens has been identified as promising candidates: ribosomal protein L7/L12, Cu/Zn-SOD, and outer membrane proteins Omp16, Omp19, Omp25, and Omp31. Their combined inclusion ensures broad-spectrum immunogenicity while maintaining a favorable safety profile. This integrated approach may substantially contribute to the global effort to control human brucellosis.

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