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Mini-review

Exploring *in vivo* and *in vitro* infection models in brucellosis research: A mini-review

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Abstract

Brucellosis is a serious disease that affects both animals and humans. It is caused by consuming unpasteurized dairy products that are contaminated with the *Brucella* bacteria. To study the pathobiology of this disease and develop preventive strategies, researchers rely on *in vivo* and *in vitro* models. A systematic literature search was conducted in January 2024, which revealed 38 studies that used these models in the previous four years. Mice were the most commonly used model for studying the disease's virulence genes, immune responses, vaccination, and treatment testing. Out of the 38 articles discussing infection models in brucellae, 6 used only *in vivo* models, 9 used only *in vitro* models, and 24 used both models. In addition, there were 32 studies with *in vitro* experiments, most of which utilized macrophages to study intracellular survival mechanisms and host-pathogen interactions. The studies mainly focused on *B. abortus*, as it had a significant impact on public and livestock health. Both *in vivo* and *in vitro* models were used to understand comprehensive intracellular mechanisms, immune responses, and treatment evaluations. However, there were several challenges in using these models, such as ethical concerns and host pathogen-specific immune responses. While both models provided important insights, the final selection choice of the model mostly depended on the research objectives, pathogen type, and availability of resources. Nevertheless, validation and understanding of these models are important to predict responses in the natural hosts.

Keywords: *Brucella*, *in vivo*, *in vitro*, Host-pathogen interactions, Infection model

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Introduction

Brucellosis remains a significant global health concern, affecting both humans and animals (Lai et al., 2021; Moriyón et al., 2023). An estimated 2.1 million new cases occur in humans every year, mostly in Africa and Asia and, to a lesser extent, in the Americas and Europe (Laine et al., 2023). Transmission in humans mostly occurs directly via consumption of unpasteurized dairy milk or products or occupational contact with infected animals or biologicals (Dadar et al., 2023; Vives-Soto et al., 2024). Indirect transmission occurs via contaminated environments or fomites where hygiene practices are compromised (Qureshi et al., 2023). Human-to-human transmission is rare (Tuon et al., 2017). Clinical onset varies in humans from undulant fever, headache, musculoskeletal pains, fatigue, and sweating in acute cases to osteomyelitis, abscesses, granulomas, and neurological manifestations in chronic cases (Qureshi et al., 2023). In animals, late-term abortion storms accompanied by fetal membrane retention and fever are characteristic signs. In males, it causes orchitis and epididymitis, leading to infertility. Infected animals remain carriers for life, and since vaccination and treatment in animals pose public health risks, culling the reactor animals remains the safest choice but has very limited implementations (Gwida et al., 2010; Dieste-Pérez et al., 2016). Moreover, milk from such animals poses serious public health threats, especially in countries where it is marketed unpasteurized and storage and transport conditions are not up to the mark (Jamil et al., 2021; Abnaroodheleh et al., 2023).

The gold-standard diagnostic tool "isolation of brucellae" remains in limited practice due to its potential hazard and advanced bio-safety requirements (e.g., level 3), and diagnosis mainly depends on serology. Thus, brucellosis poses a huge economic burden in terms of culling, production losses, diagnosis, vaccination and treatment, and surveillance costs (Franc et al., 2018; Khurana et al., 2021). Brucellosis is caused by different types of bacteria from the *Brucella* genus. Each type of bacteria prefers a specific host for infection. For example, *B. abortus* mainly infects cattle and buffaloes, *B. melitensis* mainly infects sheep and goats, *B. suis* infects mainly pigs, *B. canis* dogs, and *B. ovis* rams. Among these, *B. abortus, B. melitensis*, and *B. suis* are highly important as they can potentially infect humans. It's also worth noting that these bacteria can infect non-preferred hosts (Wareth et al., 2017; Celik et al., 2023). Although brucellae exhibit high genetic similarity (>95%), the molecular basis of these preferences remains largely unclear (Suárez-Esquivel et al., 2020; Bialer et al., 2021).

Brucellae also establish themselves as chronic intracellular pathogens by evading and manipulating host immune systems (de Figueiredo et al., 2015; Barrionuevo and Giambartolomei, 2019; Pellegrini et al., 2022). To understand these host-pathogen interactions, e.g., host adaptation, tissue tropism, intracellular niche, immune response, and immunometabolism of brucellosis and brucellae, *in vivo* and *in vitro* infection models serve as crucial tools (Tan and Nemeth, 2023). These tools help in understanding the underlying mechanisms of host and tissue tropism and the development of effective prevention and therapeutic strategies. Both models have been frequently used in brucellosis research, and a gap was found in the literature providing understanding and guidance for their strategic applications. The purpose of this review was to address this gap by examining the strengths and limitations of these models as well as the type of *Brucella* spp. tested in the literature and to find out how they contribute to brucellosis research.

Literature search criteria

A systematic literature search was done on 22.01.2024 by using the keywords "brucellosis," "host-pathogen interactions,"

"*in vivo* models," "*in vitro* models," "immune response," and "*Brucella* pathogenesis" in online databases. Only studies published within the last four years (2020-2023) and published in the English language were considered. This resulted in 12 articles via PubMed Central (PMC), two articles via Web of Science (WoS), 40 articles via Scopus, and, finally, 220 articles via Google Scholar. Only full-length peer-reviewed journal research articles were considered. After considering the keywords, abstracts, and duplicates, only two articles in PMC, 1 in WoS, 8 in Scopus, and 27 in Google Scholar fulfilled the final inclusion criteria. Hence, a total of 38 research articles were included in the study.

In vivo models for brucellosis

Out of the total 38 studies, 30 used *in vivo* models, out of which 24 studies used both *in vivo* and *in vitro* models, and six studies used solely *in vivo* models. Mice were the most preferred model used in 29 studies. Guinea pigs (*Cavia porcellus*), ewes, and moth larvae (*Galleria mellonella*) were the least used, with one study for each model (Table 1). These models were useful for studying the relevance of the virulent genes associated with specific *Brucella* strains (Sidhu-Muñoz et al., 2020), evaluation of cellular and humoral response in testing immunization potency of vaccine candidates and vaccine delivery systems (Sadeghi et al., 2020), determination of bacterial load and tissue damage via histopathology in specific organs (Gomes et al., 2021; Vu et al., 2021; Tsai et al., 2022), verification of *in vitro* RNA expression predictions (Oliveira et al., 2021), function evaluation of pseudogenes (Zhang et al., 2022) and even evaluation of cost-effective and efficient new *in vivo* infection models e.g. *Galleria mellonella* larvae (Wang et al., 2023).

Mice models have been widely used since mice are easy to handle, and most immunological and genetic tools have been standardized using these models, especially for studying chronic brucellosis (Silva et al., 2011; Bryda, 2013). This makes them favorable for studying pathogenesis and testing vaccines (Sadeghi et al., 2020; Tupik et al., 2020; Wang et al., 2020) e.g., in understanding how *Brucella* infects, proliferates, and interacts within a specific host (Khan et al., 2020; Machelart et al., 2020; Wang et al., 2020; Altamirano-Silva et al., 2021). Although mice are not the natural hosts for *Brucella*, the bacterial splenic proliferation profiles are highly reproducible in these models (Silva et al., 2011), enabling us to understand the molecular and cellular mechanisms of infection, including how *Brucella* evade immune detection and establish chronic infection (Khan et al., 2020). Moreover, mice and other lab animals acted as a source of primary cells for *in vitro* experiments (Saadat et al., 2021). Mice and specific hosts have been used to understand the structural and functional impact of vaccine derivatives in the laboratory and natural hosts (Mena-Bueno et al., 2022).

In vivo models usually mimic the results obtained in the natural hosts, but this may not always be the case, e.g., *Brucella* mutants showed full virulence in a mouse model but attenuated in the natural host (Bellaire et al., 2003; Sidhu-Muñoz et al., 2020). Moreover, the immune status of the infected host determined the genetic requirements of *Brucella* for optimal growth (Potemberg et al., 2022). *In vivo* models are also a subject of ethical considerations and regulatory constraints (Li et al., 2021; Wang et al., 2023). Results obtained from animal models may not always translate directly to the actual hosts due to physiological and genetic differences (Solanki et al., 2021). This poses a challenge in developing and standardizing *in vivo* models for effective and acceptable treatment regimens or vaccine response predictions in the final hosts. Moreover, differences in the pathogen strains, *in vivo* model strains, and even the routes of infection, e.g., intra-nasal, intraperitoneal, etc., could play a role in the outcomes of the experiments (Budnick et al., 2020).

In vitro models for brucellosis

Out of the total 38 studies, 32 used *in vitro* models, and nine studies used solely *in vitro* models. Murine macrophages were the most frequently used *in vitro* models, i.e., 15 studies used RAW264.7, eight used murine bone marrow-derived macrophages (BMDMs), four used J774A.1, two used peritoneal, and one study used alveolar macrophages. This was followed by HeLa, used in three studies, MC3T3-E1, used in two studies, while a single study for each of the human choriocarcinoma cell lines (JEG-3 and BeWo). Other models included bone marrow-derived dendritic cells (BMDCs), mononuclear cells, primary lymphocytes, goat fibroblasts, L2, and lung epithelial cells, for which a single study was found in every case (Table 1).

In vitro infection models, such as cell cultures, were essential for dissecting specific interactions between host cells and *Brucella,* e.g., examination of osteoclast roles in osteoarticular brucellosis using BMDMs (Khalaf et al., 2020) and investigation of the interaction between *Brucella* Omp25 and SLAMF1 receptors in dendritic cells (Degos et al., 2020), helped to understand *Brucella*'s intracellular survival mechanisms, immune evasion (Sadeghi et al., 2020; Vu et al., 2021), and interaction with host cell pathways (Budnick et al., 2020; Sheehan et al., 2020). Specifically, our results showed that these models provided a suitable model for studying regulatory pathways, e.g., the role of RNases in virulence (Sheehan et al., 2020), intracellular specific transport and utilization mechanisms of biochemical messengers, e.g., the gamma-aminobutyric acid (GABA) (Budnick et al., 2020), expression of various genes in an intracellular environment, e.g., intracellular behavior in specific phagocytes (Sidhu-Muñoz et al., 2020), roles of various metalloproteinases, e.g., zinc-dependent metalloproteinase (ZnMP) in the intracellular adaptation, i.e. inside the endosome/lysosome (Gómez et al., 2020).

In vitro models also provided suitable alternatives to study intracellular pathogenic mechanisms of highly zoonotic pathogens, e.g., *B. melitensis* and *B. abortus* (Salmon-Divon and Kornspan, 2020; Altamirano-Silva et al., 2021), as predictors for vaccine safety (Khalaf et al., 2020) and evaluation of macrophage metabolic reprogramming due to stimulator of interferon genes (STING) in *B. abortus* infection (Gomes et al., 2021). Furthermore, the use of *in vitro* models was very useful in studying cytokines gene expression and proliferation assays of splenic lymphocytes (Saadat et al., 2021), the role of Omp 16 in *Brucella* infection using transcriptomic analysis in the macrophages (Zhou et al., 2021), RNA expression analysis in infected cells (Li et al., 2021; Oliveira et al., 2021), evaluating antimicrobial treatments in cellulo (Mode et al., 2022), and finally, transposition mutants encoding antimicrobial resistance (Rivas-Solano et al., 2023). Murine bone marrow-derived macrophages (BMDMs) and RAW264.7 were widely and classically used in studying *Brucella* pathobiology because survival and replication within macrophages are important aspects of *Brucella* pathogenesis and mice cell lines have been frequently established (Gómez et al., 2020; Hu et al., 2020; Altamirano-Silva et al., 2021; Potemberg et al., 2022). Although used less frequently, fibroblasts and neutrophils are also targets of *Brucella*, particularly through the intradermal route (Li et al., 2021). Lung epithelial cells were used to evaluate immune responses in cases of inhaled brucellosis (Alonso Paiva et al., 2023).

Although *in vitro* infection models have provided satisfactory results for many years, a need for a closer *in vivo* conditions' replicating system of the natural hosts remains there, e.g., most of the terminally differentiated cells *in vitro* are in a quiescent metabolic state (Eisenreich et al., 2019) and may not accurately reflect *in vivo* host immune responses for immunomodulatory therapy (Boraschi et al., 2021; Jansen et al., 2023). Moreover, the media conditions applied *in vitro* may also influence the metabolism of the infected cells (Eisenreich et al., 2019), as well as the type of the cells, e.g., primary cells or immortal cell lines (Segeritz and Vallier, 2017). For this, cell diversity and the complexity of the host-pathogen interactions *in vivo* in the natural host must be considered (Haddad et al., 2023).

Brucella spp.

Out of the total 38 studies, 28 used *B. abortus* or its derivative strains. Eight studies used *B. melitensis*, followed by four studies using *B. suis* and two studies each for *B. ovis* and *B. neotomae*. Single studies used each of the following strains: *B. canis*, *B. microti*, and *B. inopinata*. Seven studies used vaccinal strains, three each for S19 and Rev.1, and one for RB51. *B. abortus* was the most common focus of the studies due to its significant impact on livestock and public health. Thus, there is a great need to understand this particular species' pathogenesis and develop effective control measures (Budnick et al., 2020; Gómez et al., 2020; Vu et al., 2021).

Table 1: *Brucella* spp. *in vivo* and *in vitro* models included in the study.

No.	*Brucella* spp.	*In vivo* model	*In vitro* model *	Study reference
1	*B. melitensis, B. abortus, B. suis, B. neotomae*	Mice	V-raf/v-myc immortalized and primary BMDMs	Khan et al. (2020)
2	*B. abortus* 2308, S19, S19vjbR, *B. abortus* ΔvirB2		Murine bone marrow-derived macrophages (BMDMs), MC3T3-E1	Khalaf et al. (2020)
3	*B. abortus* 544, RB51, *B. melitensis* 16M, Rev.1	Mice	-	Sadeghi et al. (2020)
4	*B. abortus* 2308**	Mice	BMDCs	Degos et al. (2020)
5	*B. abortus* S2308**	Mice	Murine BMDMs, RAW264.7	Hu et al. (2020)
6	*B. abortus* 2308, RB51, znBAZ	Mice	Mononuclear cells	Wang et al. (2020)
7	*B. abortus* 2308	Mice	BMDMs	Tupik et al. (2020)
8	*B. melitensis* Rev.1		JEG-3	Salmon-Divon and Kornspan (2020)
9	*B. abortus* 2308**		RAW264.7	Gómez et al. (2020)
10	*B. ovis* PA**	Mice	J774.A1, HeLa	Sidhu-Muñoz et al. (2020)
11	*B. abortus* 2308**	Mice	Peritoneal macrophages	Budnick et al. (2020)
12	*B. abortus***	Mice	Peritoneal macrophages	Sheehan et al. (2020)
13	*B. melitensis* 16M, *B. abortus* 2308, *B. suis* bv. 1 str. 1330, *B. suis* bv. 5 str. 513, *B. microti* CCM4915, *B. neotomae* 5K33, *B. inopinata* B01	Mice	-	Machelart et al. (2020)
14	*B. melitensis* M5-90		Goat fibroblasts	Li et al. (2021)
15	*B. abortus* 544	Mice	RAW264.7	Reyes et al. (2021a)
16	*B. abortus* 544	Mice	RAW264.7	Reyes et al. (2021b)
17	*B. abortus* 2308	-	RAW 264.7, HeLa	Altamirano-Silva et al. (2021)
18	*B. abortus* 544	Mice	RAW264.7	Vu et al. (2021)
19	*B. abortus* 2308	Mice	BMDMs	Oliveira et al. (2021)
20	*B. abortus* S544, S19**	Mice	-	Solanki et al. (2021)
21	*B. suis* S2**		RAW264.7	Zhou et al. (2021)
22	*B. canis***	Mice	RAW264.7	Sun et al. (2021)
23	*B. abortus* 544	Mice	RAW 264.7	Huy et al. (2021)
24	*B. melitensis*	Guinea pigs	Primary lymphocytes	Saadat et al. (2021)
25	*B. abortus* S2308	Mice	BMDMs	Gomes et al. (2021)
26	*B. abortus* S2308	Mice	RAW264.7, BMDMs	Hu et al. (2022)
27	*B. melitensis* Rev.1**	Mice, Ewes	BeWo	Mena-Bueno et al. (2022)
28	*B. abortus***		RAW264.7	Mode et al. (2022)
29	*B. melitensis* 16M** , *B. abortus* 2308, S19	Mice	RAW264.7, BMDMs, J774A.1, MC3T3-E1, L2	Wells et al. (2022)
30	*B. abortus* 2308**	Mice		Tsai et al. (2022)
31	*B. melitensis* 16M**	Mice	RAW 264.7	Potemberg et al. (2022)
32	*B. melitensis* strain 63/9**	Mice		Zhang et al. (2022)
33	*B. ovis***	Mice	J774A.1	Tartilán-Choya et al. (2021)
34	*B. abortus* 544	Mice	RAW264.7	Reyes et al. (2023)
35	*B. abortus* A19, A19ΔVirB12, *B. suis* S2, *B. abortus* 104M	*Galleria mellonella* larvae, Mice	-	Wang et al. (2023)
36	*B. abortus* 2308	Mice	Alveolar macrophages (AM), Lung epithelial cells (LEC)	Alonso Paiva et al. (2023)
37	*B. abortus* 544**	Mice	J774A.1	Hop et al. (2023)
38	*B. abortus* 2308W**	-	RAW 264.7, HeLa	Rivas-Solano et al. (2023)

*Abbreviations: BMDMs; bone marrow-derived macrophages, BMDCs; bone marrow-derived dendritic cells; and derivative strains. BeWo is a cell line exhibiting epithelial morphology that was isolated from the placenta of a patient with choriocarcinoma. RAW cells are a macrophage-like, Abelson leukemia virus-transformed cell line derived from BALB/c mice. JEG-3 is a hypertriploid, clonally-derived, human cell line with epithelial morphology that was isolated from the Woods strain of the Erwin-Turner tumor.
**Derivative strains

Brucella belongs to a very diverse group, Rhizobiales, and thus, represents a suitable pathogen model for studying intracellular host-adaptation traits (Machelart et al., 2020), e.g., the role of RNases in bacterial pathogenesis and the functionality of the enzyme glutamate decarboxylase (GAD) system in the classical *Brucella* species (Budnick et al., 2020; Sheehan et al., 2020). Since understanding the specific immune response is crucial to understanding the role in acute and chronic brucellosis, different types of *Brucella* species would evaluate a specific protein, and every species had behavioral differences in similar types of hosts (Khan et al., 2020). Rough strains, e.g., *B. ovis*, were selected to evaluate the relevance of flagellar genes and transcriptional regulator MucR in their virulence (Sidhu-Muñoz et al., 2020; Tartilán-Choya et al., 2021) and *B. canis* was selected in one study due to its ignorance as a public health risk and less existing knowledge about the pathogenic mechanisms and virulence factors (Sun et al., 2021). One study chose *B. suis* since its attenuated strain S2 was essential and critical in controlling brucellosis in that particular region (Zhou et al., 2021). Overall, multiple studies explored various *Brucella* species depending on the needs and objectives of the experiments.

Perspectives from 2020-2023

Cell lines and mouse models are chosen for their ability to mimic disease processes in natural hosts as closely as possible, e.g., to study the specific mechanisms of *Brucella* infection and immune response (Khalaf et al., 2020; Khan et al., 2020). *In vivo* models helped understand the pathogen metabolic pathways in response to the host environments (Machelart et al., 2020), and a combination of these models demonstrated underlying intracellular mechanisms of virulence via metabolic and transcriptomics-based studies, differential behavior of pathogen species (Budnick et al., 2020; Sheehan et al., 2020), and the correlation between thioredoxin-interacting protein (TXNIP) and nitric oxide (NO) (Hu et al., 2020). Combining these models also enabled studying comprehensive immune responses, histopathology, bacterial load, and cell death in *B. abortus* infection, e.g., inflammasomes activation (Tupik et al., 2020), the role of $CD8^+$ tissue-resident memory T cells (Wang et al., 2020), the interaction of *Brucella* outer membrane protein (Omp) 25 with signaling lymphocytic activation molecule family 1 (SLAMF1) in dendritic cells (DC) (Degos et al., 2020) and role of STING in controlling acute and chronic brucellosis (Khan et al., 2020). These models also helped evaluate metabolic intermediates, e.g., succinic acid (SCA) (Huy et al., 2021) and multifunctional proteins, e.g., heme oxygenase 1 (HO-1) in *Brucella* infections (Hu et al., 2022). Moreover, gene expression and intracellular multiplication dynamics in the spleen (Sun et al., 2021), candidate vaccine evaluation (Oliveira et al., 2021), surrogate and endogenous G-protein coupled receptor (GPR) 84 agonists (Reyes et al., 2021a), and selection pressure identification using transposon sequencing (Tn-seq) (Zhang et al., 2022) in *Brucella* infections needed combination of both models.

Evaluation of novel immune defense factor, e.g., biogenesis of lysosome-related organelles complex-1 subunit 1 (BLOS1) (Wells et al., 2022), function evaluation of novel bacterial defense systems, e.g., DNA-binding proteins from starved cells (Dps) (Hop et al., 2023) as well as host immune system, e.g., cGAS/STING cytosolic DNA sensing pathway in inhalation brucellosis (Alonso Paiva et al., 2023) or evaluating modulator effects of Sirtuin1 activators (Reyes et al., 2023) also used a combination of both systems. It wasn't surprising that on several occasions, *in vitro* and *in vivo* results didn't match; hence a combination of both models was necessary to address the gaps between the variation in the results, e.g., the role of *Brucella* Omp 25 results *in vivo* and *in vitro* (Degos et al., 2020). These involve issues like species-specific model limitations, difficulty in replicating chronic aspects of the disease, and varying immune responses. Also, finding a practical treatment strategy against brucellosis would need to investigate potential candidates in both *in vivo* and *in vitro* (Reyes et al., 2021b). In summary, the findings from these infection models have enhanced understanding of *Brucella*'s behavior, e.g., molecular pathogenesis, host-pathogen interactions, and immune evasion mechanisms, contributing to more targeted diagnostic methods and treatment approaches. However, each model and even the pathogen will have a certain degree of variability (Silva et al., 2011; Hensel and Arenas-Gamboa, 2018; Carvalho et al., 2023). Standards of reproducibility and repeatability would reduce the degree of errors (Hirsch and Schildknecht, 2019). However, the choice of the model ultimately will depend on the specific research question and objectives of the experiment (Allweiss and Dandri, 2016).

Conclusions

In vivo and *in vitro* models have played a significant role in increasing the understanding of pathobiology, immune responses, and preventive measures in brucellosis research. *In vivo* models have helped discover the *Brucella* infection mechanisms, host-bacterial interactions, and the dynamics of the host immune responses. *In vitro* models have provided detailed insights into the intracellular processes involved. While both models have their advantages, challenges are associated with each, e.g., *in vivo* studies can produce species-specific and route-dependent responses and raise ethical questions. *In vitro* models can help to address these challenges by reducing the need for animal experiments, addressing ethical questions, and minimizing the risk of bio-risk transmission but on the other side, they don't represent *in vivo* conditions. Therefore, the choice of model will depend on various factors, such as the objectives of the experiment, the type of pathogen, the route of infection, and the available resources and trained personnel. Different types of *in vitro* and *in vivo* environments will represent the situation differently and will not fully predict the situation in the natural environment. Therefore, it is important to validate and understand these infection models to minimize the chances of errors.

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