Brief Report

Enhanced Isolation of Brucella abortus from Lymphoid Tissues of Mice Orally Infected with Low Doses in a Two-Step Procedure

Ana Beatriz Sánchez-Argáez , Estefania Herrera-Torres , Martha Cecilia Moreno-Lafont , Leopoldo Flores-Romo , Rubén López-Santiago *

Posted Date: 29 April 2025

doi: 10.20944/preprints202504.2475.v1

Keywords: Brucella abortus; oral infection; mouse models; isolation of Brucella

 doi:10.20944/preprints202504.2475.v1

Brief Report

Enhanced Isolation of *Brucella abortus* from Lymphoid Tissues of Mice Orally Infected with Low Doses in a Two-Step Procedure

Ana B. Sánchez-Argáez [1], Estefania Herrera-Torres [1], Martha C. Moreno-Lafont [1], † Leopoldo Flores-Romo [2] and Rubén López- Santiago [1] *

[1] Departamento de Inmunología. Escuela Nacional de Ciencias Biológicas. Instituto Politécnico Nacional. Prol. de Carpio y Plan de Ayala s/n. Del. Miguel Hidalgo. CP 11340. Ciudad de México. México; sanchezargaezana@gmail.com (A.B.S.-A.); estefa_h@hotmail.com (E.H.-T.); mlafont@ipn.mx (M.C.M.-L.).

[2] Departamento de Biología Celular. Centro de Investigación y de Estudios Avanzados (CINVESTAV). Instituto Politécnico Nacional. Av. IPN No. 2508. Del. Gustavo A. Madero. CP 07360. Ciudad de México. México; (L.F.-R.) leflores@cinvestav.mx

* Correspondence: rsantiag@ipn.mx

Abstract: The main aspects of brucellosis have been studied in animal models to better understand the pathogenesis of the disease and develop vaccines and drugs for its treatment. Mice are the most common animal model of brucellosis. To verify that the infection has been successfully induced, it is necessary to assess the presence of *Brucella* spp. in the experimentally infected mice. The most common way to detect the presence of *Brucella* is by microbiological isolation from spleen and liver. However, high doses of *Brucella* are needed by most models to successfully isolate the bacteria. In this study we propose a method consisting in culturing homogenates of the organs recovered from orally infected mice in selective broth to increase the bacterial load. This made it possible to recover *Brucella abortus* from Peyer's patches, mesenteric lymph nodes, spleen, and feces of mice orally inoculated with a low dose of 5 x 10^6 CFU, as early as one hour and up to five weeks post inoculation.

Keywords: *Brucella abortus*; oral infection; mouse models; isolation of *Brucella*

1. Introduction

Brucellosis is a disease that develops in mammals, including humans, and is caused by bacteria of the genus *Brucella*. It is considered an important anthropozoonosis distributed worldwide, with some authors suggesting that new human cases are reaching 1.6-2.1 million per year, an allarming number when compared to the 500,000 cases frequently reported [1, 2]. Humans acquire the infection by consuming products derived from infected animals or by their handling. Brucellosis continues to be a health problem in Latin American countries, China, Russia, and Arabian nations even though, in countries such as the United States, Canada, Japan and New Zealand, it has been successfully eradicated in cattle and, therefore, in humans. However, the lack of control in other countries and the increase in immigrants carrying the disease put the countries that have eradicated brucellosis at risk again [3]. The pathophysiology of brucellosis is poorly described, and there is no consensus on an adequate classification of its clinical course. Then, a more detailed and precise description of brucellosis is of great importance. In scientific research, the use of animal models is required to study the main human diseases, including brucellosis. In the *Brucella* infection model, isolation of the pathogen is vital to confirm successful infection, regardless of the administration route of the bacteria. Reviewing the literature, we found that, for many years, animal models of brucellosis were induced by other ways rather than the natural infection pathway. These models induce systemic infection, usually inoculating the bacteria intravenously or intraperitoneally, which not only makes it easier to recover the bacteria by classic methods as microbiological isolation but also evades important natural

 doi:10.20944/preprints202504.2475.v1

defense mechanisms of the host [4, 5]. To better comprehend the development of the disease it is important that the animal model simulates a natural infection to mimic the natural route of bacterial entry and bacterial load which cause the disease. Nonetheless, oral inoculation models mimicking the natural route of entry of this pathogen appear to require higher doses of *Brucella* since the bacteria are subjected to several barriers in the gastrointestinal tract. Apparently, that is one of the reasons why other research groups employ high doses of inoculation, ranging from 1×10^9 to 2×10^{10} CFU [6, 7]. Therefore, in this work we propose a method that allows the enrichment of the small bacterial load of *Brucella abortus* in selective broth medium that can be recovered from lymphoid and non-lymphoid tissues for subsequent isolation and characterization, using a murine model of infection with low doses of bacteria through the natural entryway, the oral route.

2. Materials and Methods

2.1. Mice and Infection

To induce infection, BALB/c mice aged 4–6 weeks were orally inoculated with a stainless-steel feeding tube (Sigma-Aldrich, St. Louis, MO). The experiments in this work followed the ARRIVE guidelines and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

The animals received 100 µL sodium bicarbonate (0.35 M, Técnica Química, Mexico) to buffer the stomach pH 15 minutes prior infection with *Brucella*. Mice were orally administered with 100 mL of 1×10^6, 5×10^6, or 10×10^6 colony forming units (CFU) of *B. abortus* to induce infection. The bacterial suspensions were adjusted to a 0.4 OD at 540 nm (Spectra 20, Bausch and Lomb, Ontario, CAN) in injectable water.

2.2. Tissue samples

Ten groups of three mice were sacrificed at different timepoints ranging from one hour up to five weeks post infection. Peyer's patches (PP), mesenteric lymph nodes (MLN), and spleen were removed, and each tissue was mechanically homogenized in one mL cold sterile PBS (Sigma-Aldrich, St Louis, MO). When needed, 100 µL of each cell suspension were used for serial dilutions and the remaining 900 mL were reserved for the selective broth culture method.

2.3. Bacteria isolation by direct plating

Using 100 mL of cell suspension obtained from each tissue, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were made with sterile distilled water to lyse the cells and release the intracellular bacteria. Then, 100 µl of each dilution was plated by duplicate on plates with trypticase soy agar (TSA) (BD Bioxon, Mexico) supplemented with Modified *Brucella* Selective Supplement (reconstituted as indicated by manufacturer) (Oxoid™, Thermo Fisher, Waltham, MA) and incubated at 37 °C in 5% CO_2 for 48 h. The growth of CFU was monitored for up to 2 weeks. The number of CFU was calculated by applying the following formula: CFU/mL = (No. CFU x inverse of dilution)/volume of plated suspension.

2.4. Bacteria isolation by selective broth medium enrichment

The remaining 900 µl of cell suspension from each sample were placed in independent conical tubes (15 mL, Falcon™, Corning, Somerville, MA) with 3 mL trypticase soy broth (TSB) supplemented with Modified *Brucella* Selective Supplement (Oxoid™, Thermo Fisher, Waltham, MA). The tubes were incubated at 37 °C for 72 h in an orbital shaker at 150–180 rpm (Barnstead Lab-line Max^{Q} 4000).

2.5. Obtention of fecal samples and bacteria isolation

Six additional mice were used to obtain fecal samples. The mice were divided in two groups (control and infected) and were administered with injectable water or 5×10^6 CFU of *B. abortus*. The

 doi:10.20944/preprints202504.2475.v1

feces from these mice were recollected from 1 h up to 5 weeks post administration. The feces of each animal were weighted to obtain approximately 0.1 g and subsequently homogenized with 2 mL TSB supplemented with Oxoid™ Modified *Brucella* Selective Supplement and strained through a cell strainer (40 mm, Corning, Somerville, MA) to eliminate any remaining large particles. Once strained, 70 µL of the suspension were placed in conical tubes containing 3.93 mL TSB supplemented with Modified *Brucella* Selective Supplement (30 µL/mL) (Oxoid, Thermo Fisher, Waltham, MA). The tubes were incubated at 37°C for 72 h in an orbital shaker at 150–180 rpm. Finally, after 72 h, 20 mL of each tube was plated on TSA (BD Bioxon, Mexico) plates and incubated at 37°C for 48 h.

3. Results

First, we sought to demonstrate that *Brucella* could be recovered even when using lower doses to infect. To do this, different groups of mice were inoculated with the selected doses of bacteria. Using the proposed method, the bacteria could not be recovered from the mice infected with the lowest dose of *B. abortus* (1 x 10^6 CFU). However, we were able to recover the bacteria from the mice that were infected with 5 x 10^6 and 10 x 10^6 CFU in all the organs tested with the selective culture broth (Table 1). Based on these results, the dose of 5 x 10^6 CFU was chosen for subsequent experiments.

Table 1. Growth of *B. abortus* 2308 in selective *Brucella* medium, from Peyer's patches (PP), mesenteric lymph node (MLN), and spleen of three mice infected with different doses of bacteria.

Dose	Tissue								
	PP			MLN			Spleen		
1 x 10^6	**ND**	**ND**	**ND**	**ND**	**ND**	ND	ND	ND	ND
5 x 10^6	+	+	+	+	+	+	+	+	+
10 x 10^6	+	+	+	+	+	+	+	+	+

(+) Bacterial growth, (ND) Not detected.

Once demonstrated that the bacteria could be recovered even with low doses, we attempted to recover it with the usual TSA plaque growth technique. Bacterial growth (reported as CFU) was inconsistent between the different tissues and mice from the same group and only observed at certain time points through the kinetics. These results were not consistent and repeatable in three additional experiments performed (Table 2).

Table 2. Detection of *B. abortus* 2308 by direct plate count from Peyer's patches (PP), mesenteric lymph node (MLN), or spleen of three mice orally infected with 5 x 10^6 CFU.

Tissue	Time post infection											
	1 h			2 h			48 h			72 h		
PP	**3.33 x 10^2**	**ND**	**ND**	**ND**	**1.2x10^3**	**ND**	**ND**	ND	ND	ND	ND	ND
MLN	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Spleen	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

(ND) Not detected.

Tissue	Time post infection								
	7 d			4 week			5 week		
PP	**ND**	**ND**	**ND**	**ND**	**2.6 x 10^3**	ND	ND	ND	ND
MLN	ND	UC	ND	5.1 x 10^6	ND	ND	ND	ND	ND
Spleen	ND	ND	ND	ND	ND	UC	ND	UC	ND

(ND) Not detected, (UC) Uncountable.

 doi:10.20944/preprints202504.2475.v1

Since, it was not possible to recover *Brucella* by the classic direct plating technique, we decided to use the selective culture broth enrichment technique and analyze the progression of the infection at different time points, ranging from early stages to acute and chronic phases. Using this technique, the bacteria was recovered as early as 1 h post infection from PP and MLN and from feces, 2 h post infection. In the spleen, it was detected 72 h after infection, indicating that *Brucella* disseminated systemically. The results obtained from these experiments demonstrate that *Brucella* is not only capable of breaching the intestinal barrier and remain both in PP and MLN but it is also being released in feces during shedding. These findings show a consistent behavior throughout the time lapse and the correlation with the results of the spleen shows that the bacteria is systemically disseminated while remaining in the entryway. Interestingly, the bacteria was not detectable after a week of infection in PP, feces, and spleen, but remainedp constant in MLN through the whole shedding kinetics. The presence of *Brucella* was monitored up to 5 weeks, corresponding to a chronic infection. The results shown in Table 3 correspond to three repetitions of each time post infection.

Table 3. Growth of *B. abortus* 2308 in selective broth for *Brucella*, from Peyer's patches (PP), mesenteric lymph node (MLN), spleen or feces of mice orally infected with 5 x 10^6 CFU.

Tissue	Time post infection																				
	1h			2h			48h			72h			7d			4week			5week		
PP	+	+	+	+	+	+	+	+	+	+	+	+	**ND**	ND	ND	+	+	+	ND	ND	ND
MLN	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND
Spleen	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	ND	ND	ND	+	+	+	+	+	+
Feces	ND	ND	ND	+	+	+	+	+	+	+	+	ND	ND	ND	ND	+	ND	+	+	+	+

(+) Bacterial growth, (ND) Not detected.

4. Discussion

Due to its zoonotic potential, *Brucella abortus* is one of the most common causes of human brucellosis, being oral and inhalation intake the most common ways of infection. With an infective dose ranging from 10 to a 100 bacteria and the lack of prophylactic treatments, the infection may last for years [8]. A successful and more realistic *Brucella* animal model would be one induced by the administration of *Brucella* orally or intragastrically. Different research groups have tried to establish a model of oral infection, but among the difficulties they have been found is the need to administrate high doses of *Brucella* (≥ 10^{10} CFU) in order to detect viable bacteria in the organs of the mouse [9] The need for these high doses to infect mice by this route might be related to the hostile environments the bacteria confronts, such as the acid pH of the stomach, the epithelial barrier of the intestine, the competition with the mouse microbiota, and other factors including molecules and cells of the intestinal immune system [10-13]. Both in an oral model of infection or when natural infections occur, the bacteria are subjected to all the above mentioned barriers. Consequently, the number of bacteria that can overcome these barriers must be minimal and the bacterial load established in the organs is lower than the required to recover *Brucella*, making it difficult to recover by the traditional methods such as agar culture media. In this work, the murine infection model was implemented with a dose of 5 x 10^6 CFU, which is considered a low dose, between 200 and 4000 times lower than those usually used. Still, the technique implemented in this work aimed at detecting the few bacteria that were able to survive the innate immunity mechanisms. The technique used showed that with low doses, in orally inoculated BABL/c mice, *Brucella* can pass through gut-associated lymphoid tissue as PP and MLN and remains there up to five weeks. The behavior of both MLN and PP was very similar. We reported the presence of *Brucella* within 1 h after inoculation, indicating that *Brucella* was able to cross the physical and chemical barriers of the gastrointestinal tract and successfully arrive to the intestine. It was interesting to continue detecting *Brucella* up to 5 weeks after inoculation.

The detection of viable *Brucella* in these tissues throughout the different time points led us to question whether *Brucella* only initially transits through the intestine and proceeds to spread

 doi:10.20944/preprints202504.2475.v1

systemically or if it stablishes a niche in the intestine. We also found the bacteria at times that have been reported as part of the chronic stage (4–5 weeks) of *Brucella*'s infection cycle. Interestingly, we agree with another group that not finding the bacteria at one week of infection is most likely because the bacterial load was very low, or it is in tissues different from the ones studied in our research [9].

Using the enrichment technique, we found that in our model the bacterium is found in the spleen at 72 h, indicating that the infection is already systemic, and it remains in the spleen, where it can be recovered from for at least up to 5 weeks. With the use of this technique, it is possible to study all the organs in which *Brucella* spreads from the early times of infection and where it is still detected several weeks later, without resorting to higher doses than those of the natural infection.

The use of a selective medium is necessary when attempting to culture tissues that are in constant contact with other antigens, as in the case of mucosa-associated lymphoid tissue. Selective media for *Brucella* increase the chances of successfully culturing the bacterium. It has been proposed and used due to their high sensitivity, and our results confirm this. Even though molecular biology techniques, such as PCR, are more sensitive, they also increase the complexity of the essays because of the need for special equipment, specially trained people, and expensive reagents [14].

The constant elimination of *Brucella* through feces in mice also suggests another possible source of infection, as it has been shown previously that *Brucella* can stay viable in manure up to 2 months if the environmental conditions are favorable [15]. With the technique we used, it was possible to detect the presence of *Brucella* in feces at different times of infection, throughout the acute, systemic, and chronic stages of the infection. These results are important so that in the future, research groups employing the oral model of infection can take this into consideration if they deem necessary to isolate their experimental groups. Additionally, it seems important to study feces both as a source of contagion or a good sample for detection of *Brucella* in cattle. The use of manure in agriculture is a common practice, making it a source of pathogens derived from animals and facilitating the spreading of zoonotic diseases. Manure contaminated with *Brucella* can originate outbreaks both in farm animals and farm workers, yet scarce information is available about the risk it presents on a daily basis, and the consumption of contaminated food remains the main source of infection in humans [16, 17]

5. Conclusions

The results of this work show that the selective agar medium is not adequate for direct isolation of *Brucella* from orally inoculated BALB/c mice when using low doses of *Brucella*. We demonstrated that the selective culture broth allows to recover and isolate *Brucella* from BALB/c mice inoculated orally if low doses of bacteria are employed. These results introduce the possibility of mimicking the natural infection with lower doses thus making the mouse model more adequate and relatable, getting us closer to better understand brucellosis.

Author Contributions: A.B.S.-A. conceived the study, designed and performed experiments, analyzed data, produced figures, wrote and edited the manuscript; E.H.-T. performed the fecal isolation experiments, analyzed data, produced figures, wrote and edited the manuscript; M. C. M.-L. Edited the manuscript and secured funding; L. F.-R. Conceived and supervised the study R. L.-S. secured funding and supervised the study. .

Funding: Financial support was provided by SIP 20232507, Instituto Politécnico Nacional.

Institutional Review Board Statement: The animal study protocol was approved by the Research Ethics Committee from ENCB-IPN (CEI-ENCB), with register number CEI-020-2017.

Informed Consent Statement: Not Applicable

Data Availability Statement: The data presented in this study is available on request from the corresponding author. The data is not publicly available due to some manuscripts in preparation are closely related to the results included in this paper.

Conflicts of Interest: The authors declare no conflict of interest.

 doi:10.20944/preprints202504.2475.v1

Abbreviations

The following abbreviations are used in this manuscript:

CFU	Colony Forming Units
MLN	Mesenteric Lymph Nodes
PP	Peyer's Patches
TSA	Trypticase Soy Agar

References

1. Laine, C.G., et al., *Global Estimate of Human Brucellosis Incidence.* Emerg Infect Dis, 2023. **29**(9): p. 1789-1797.
2. Pappas, G., et al., *The new global map of human brucellosis.* Lancet Infect Dis, 2006. **6**(2): p. 91-9.
3. Moreno, E., J.M. Blasco, and I. Moriyón, *Facing the Human and Animal Brucellosis Conundrums: The Forgotten Lessons.* Microorganisms, 2022. **10**(5).
4. Baldwin, C.L. and M. Parent, *Fundamentals of host immune response against Brucella abortus: what the mouse model has revealed about control of infection.* Vet Microbiol, 2002. **90**(1-4): p. 367-82.
5. Grilló, M.J., et al., *What have we learned from brucellosis in the mouse model?* Vet Res, 2012. **43**(1): p. 29.
6. Pasquali, P., et al., *Brucella abortus RB51 induces protection in mice orally infected with the virulent strain B. abortus 2308.* Infect Immun, 2003. **71**(5): p. 2326-30.
7. von Bargen, K., et al., *Cervical Lymph Nodes as a Selective Niche for Brucella during Oral Infections.* PLoS One, 2014. **10**(4): p. e0121790.
8. Kaden, R., et al., *Brucella abortus: determination of survival times and evaluation of methods for detection in several matrices.* BMC Infect Dis, 2018. **18**(1): p. 259.
9. Rungue, M., et al., *NLRP6-associated host microbiota composition impacts in the intestinal barrier to systemic dissemination of Brucella abortus.* PLoS Negl Trop Dis, 2021. **15**(2): p. e0009171.
10. Golding, B., et al., *Immunity and protection against Brucella abortus.* Microbes Infect, 2001. **3**(1): p. 43-8.
11. High, K.P., et al., *Outcome and immune responses after Brucella abortus infection in young adult and aged mice.* Biogerontology, 2007. **8**(5): p. 583-93.
12. López-Santiago, R., et al., *Immune Response to Mucosal.* Front Immunol, 2019. **10**: p. 1759.
13. Paixão, T.A., et al., *Establishment of systemic Brucella melitensis infection through the digestive tract requires urease, the type IV secretion system, and lipopolysaccharide O antigen.* Infect Immun, 2009. **77**(10): p. 4197-208.
14. Yagupsky, P., P. Morata, and J.D. Colmenero, *Laboratory Diagnosis of Human Brucellosis.* Clin Microbiol Rev, 2019. **33**(1).
15. El-Sayed, A. and W. Awad, *Brucellosis: Evolution and expected comeback.* Int J Vet Sci Med, 2018. **6**(Suppl): p. S31-S35.
16. de Figueiredo, P., et al., *Pathogenesis and immunobiology of brucellosis: review of Brucella-host interactions.* Am J Pathol, 2015. **185**(6): p. 1505-17.
17. Morales-Estrada, A.I., et al., *Isolation, identification, and antimicrobial susceptibility of Brucella spp. cultured from cows and goats manure in Mexico.* Archivos de medicina veterinaria, 2016. **48**: p. 231-235.