Veterinary Microbiology 239 (2019) 108447

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Rough *Brucella neotomae* provides protection against *Brucella suis* challenge in mice

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ARTICLE INFO

Keywords:
Brucella neotomae
Live-attenuated vaccine
Rough phenotype
Immunity
wboA glycosyltransferase
Lipopolysaccharide

ABSTRACT

Brucellosis is one of the most common zoonotic diseases worldwide. Almost 500,000 new human cases occur each year; yet there is no vaccine for human use. Moreover, there is no universal *Brucella* vaccine that would provide protection against all pathogenic species of *Brucella*. We generated a rough, live-attenuated *B. neotomae* strain by deleting the *wboA* gene encoding a glycosyltransferase. This strain lacks the O-side chain in its lipopolysaccharide (LPS) and thus the vaccinated animals can be differentiated serologically from the field-infected animals. We tested the efficacy of rough *B. neotomae* strain to stimulate dendritic cells compared to the smooth wild type strain. Based on TNF-α production, our data suggests that a significantly higher stimulation was obtained when dendritic cells were stimulated with the rough vaccine strain compared to the smooth wild type *B. neotomae*. Furthermore, the rough mutant was cleared from mice within 6 weeks even at a dose as high as 2 x 10^8 CFU. Vaccinated mice showed significantly higher level of protection against a virulent *B. suis* 1330 challenge compared to the control mice. Antibody titers in the mice and cytokine production by the splenocytes from the vaccinated mice showed a Th1 mediated immune response that correlated with the protection.

1. Introduction

More than a century after its discovery, brucellosis is still one of the most common zoonotic diseases worldwide (Corbel, 1997). The major pathogenic species of *Brucella* to humans and their preferred hosts are; *B. melitensis* (sheep), *B. abortus* (cattle) and *B. suis* (pigs). Although brucellosis has been eradicated from developed countries it is still endemic in the Mediterranean regions, Middle East, Latin America, and parts of Asia with more than 500,000 new cases of human infection appear every year. The symptoms of brucellosis in humans can vary from a mild fever, fatigue and myalgia (Saltoglu et al., 2004) to splenomegaly, hepatomegaly and lymphadenopathy (Pappas et al., 2006) to complicated situations such as arthritis, spondylitis, meningitis, and endocarditis (Shakir et al., 1987; Solera et al., 1999); yet there is no commercially available vaccine for human use (Wang and Wu, 2013). Every year in the USA millions of dollars are spent on the surveillance and control of brucellosis. One of the major concerns is the feral swine population that carries *B. suis* and imposes a threat to live-stock as well as humans (Sandfoss et al., 2012; Stoffregen et al., 2007). There is no successful vaccine that can prevent *B. suis* infections in swine. *B. abortus* RB51 and S19 vaccine strains that prevent *B. abortus* infection in cattle are limited because of rifampicin resistance, and a smooth phenotype respectively (Dorneles et al., 2015). Strain Rev1 is the commercial vaccine against *B. melitensis* for small ruminants but it is limited because of the streptomycin resistance and induction of antibodies against O-polysaccharide (O-side chain) that interferes with the sero-diagnosis (Adone et al., 2005). None of these vaccines provide protection against all pathogenic species of *Brucella.* There is a need for a broad *Brucella* vaccine that can be used to protect animals as well as humans.

B. neotomae was first isolated in 1952 from desert wood rats in Utah, USA (Stoenner and Lackman, 1957). Unlike the virulent species of *Brucella*, *B. neotomae* does not establish chronic infections in immunocompetent mouse models of brucellosis (Moustafa et al., 2011). Previously, irradiated wild type *B. neotomae* has been shown to provide protection against all pathogenic species of *Brucella* in mice (Dabral et al., 2014; Moustafa et al., 2011). Until recently *B. neotomae* was considered non-pathogenic to humans. However, the recent reports of human infection caused by *B. neotomae* raises concerns and the wild

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https://doi.org/10.1016/j.vetmic.2019.108447
Received 21 May 2019; Received in revised form 26 July 2019; Accepted 3 October 2019

type strain needs to be handled and manipulated more carefully than previously thought (Suarez-Esquivel et al., 2017; Villalobos-Vindas et al., 2017). A live-attenuated strain that can generate long-lasting protective immunity without causing the disease can be developed and tested as a vaccine.

The major reason for the success of the test and slaughter program to eradicate brucellosis in USA was the ability to differentiate vaccinated from infected animals. Vaccine strain, *B. abortus* RB51, has a natural and stable IS711 transposon insertion in the *wboA* gene encoding for glycosyl transferase that polymerizes the O-side chain of the LPS (Vemulapalli et al., 2000). Thus, unlike infected animals, vaccinated animals do not produce antibodies against LPS and can be easily identified through serology. Rough strains of *B. melitensis* and *B. suis* have been shown to be attenuated compared to their smooth parental strains and cleared from the host after vaccination (Smith et al., 2019; Winter et al., 1996). To be an ideal vaccine candidate, a strain should be capable of inducing a protective immune response in the host, while only replicating for a short period of time. It has also been shown that compared to the smooth parental strain, rough strain RB51 is more immunostimulatory and offers a better option as a vaccine candidate (Surendran et al., 2011). We aimed to test the potential of a rough *B. neotomae* as a vaccine against *B. suis* challenge. We generated a rough strain of *B. neotomae* and tested its clearance, ability to induce specific immune responses and to provide protection against a *B. suis* challenge in mice.

2. Material and methods

2.1. Bacterial strains

Wild type smooth strain *B. suis* 1330 and rough strain *B. suis* 1330 Δ*wboA* (VTRS1) (Winter et al., 1996) were from our culture collection at Virginia Tech, Blacksburg, VA. Strain *B. neotomae* 5K33 (ATCC 23,459) was purchased from the American Type Culture Collection (ATCC). All *Brucella* strains were regularly grown on tryptic soy agar (TSA) or in tryptic soy broth (TSB) at 37 °C in a 5% CO_2 environment. *B. neotomae* was passaged twice in mice to recover a stable strain. Briefly, a mouse was injected intraperitoneally (I/P) with 2×10^8 CFUs of *B. neotomae*. After seven days the mouse was euthanized. Serial dilutions of spleen were prepared and plated on TSA. Individual colonies were tested using biochemical assays and *Brucella* specific PCR to confirm them as *B. neotomae*. One colony was picked to culture and inject another mouse and it was euthanized as before. Again, single colonies were screened. Stocks were prepared from one colony and used to create the deletion mutant.

2.2. Generation and characterization of a rough mutant

To create unmarked *wboA* gene deletion, we used a *sacB* counter-selection system as previously published (Kim et al., 2014). Briefly, 500 bp regions flanking the *wboA* gene were PCR-amplified from *B. neotomae* and were ligated using the *Kpn*1 restriction sites, which were then ligated into the suicide plasmid pNPTS138 between the *Pst*1 and *Sal*1 restriction sites. This plasmid encodes a kanamycin resistance gene to select primary integrants and a *sacB* gene for counter-selection on 10% sucrose to identify clones in which the plasmid has been lost in a second recombination event. Plasmids were transformed into *B. neotomae* by electroporation. Clones were screened by PCR to identify those carrying the deletion of 1209 bp in the *wboA* gene. The resulting deletion strain was named as BNW.

The rough phenotype of the resulting strain was confirmed via three methods: clumping in the presence of acriflavine (Braun and Bonestell, 1947), crystal violet staining of the colonies (White and Wilson, 1951), and the serum agglutination test (Schurig et al., 1984) as previously described. To perform the acriflavine test, neutral acriflavine was freshly diluted 1:1000 in distilled water, 30 µl were placed on a glass slide, and single colonies of the candidate strain were mixed in the solution with a sterile loop. The *wboA* mutant *B. neotomae* strain was tested and compared with wild *B. neotomae* and *B. suis* 1330 and *B. abortus* RB51 as smooth and rough controls respectively. For the crystal violet test, crystal violet was prepared from a stock solution to a dilution of 1:2000 in distilled water. The crystal violet solution was used to flood the surface of a TSA plate containing either the candidate mutant strain or a control strain and assessed for uptake of the dye by rough colonies. The third test, serum agglutination, was performed using mouse monoclonal antibody (Bru38) serum against the O-side chain of *Brucella*. Smooth colonies strongly agglutinate when mixed with the serum versus rough strains lacking the O-side chain.

2.3. Ethics statement

All mouse experiments were done in our AAALAC approved facility and the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) (protocol # CVM-15-072) at Virginia Tech. For retroorbital bleeding, mice were anaesthetized under isoflurane using a Vet Equip Mobile Laboratory Animal Anesthesia System. Mice were euthanized using an overdose of carbon dioxide followed by cervical dislocation. Mice infected with virulent *B. suis* 1330 do not develop clinical disease or exhibit any signs of suffering for the duration of the experiments conducted in this study. Therefore, no humane endpoints were utilized for the mice in this study.

2.4. Dendritic cell preparation and infection

Bone marrow-derived DCs (BMDCs or DCs) were generated, as previously described (Surendran et al., 2010). Briefly, tibias and fibulas of 6–8 weeks old female BALB/c mice were incised and bone marrow (BM) cells removed. Following red blood cell lysis using ammonium-chloride-potassium (ACK) buffer and filtration, the cells were re-suspended and plated in RPMI 1640 complete medium with 10% non-heat-inactivated fetal bovine serum and 20 ng/ml rGM-CSF (Invitrogen, Carlsbad, CA). The cells were incubated at 37 °C in 5% CO_2. Fresh media containing rGM-CSF was added at days 2, 4 and 5 and harvested on day 6. On day 6, DCs were harvested and plated at 5×10^5 cells/well in 24 well plates and infected with *B. abortus* 2308, RB51, *B. neotomae* and *B. neotomae* Δ*wboA*, at a multiplicity of infection (MOI) of 1:100. Infection was enhanced by a short spin at 1300 rpm (400xg) for 5 min at room temperature. The infected cells were incubated for 4 h at 37 °C in 5% CO_2. Control samples were maintained by incubating cells with medium (negative control) or *Escherichia coli* LPS 0111:B4 (Sigma) (positive control) (100 ng/ml) following the exact same procedure for infection. For cytokine measurement, culture supernatants from *Brucella* infected DCs were collected after 4, 24 and 48 h of incubation, centrifuged at 10,000 *g* for 10 min and stored at? ?-80 °C. TNF-α levels were subsequently measured using sandwich ELISAs (BD OptEIA). The infection was ended by washing the cells with gentamicin (Sigma, St. Louis, MO) at 30 µg/ml to kill extracellular bacteria. The cells were then washed twice with PBS to remove traces of gentamicin. Intracellular bacterial counts were determined by lysing the dendritic cells using 0.1% Triton-X100 and plating the serial dilutions on TSA.

2.5. Mice vaccinations and challenge

Clearance in mice: Six-eight weeks old female BALB/c mice were injected intraperitoneally (I/P) with one of four different doses (2.0×10^2, 2.0×10^4, 2.0×10^6, and 2.0×10^8 CFU) of BNW (n = 12). Three mice/group were euthanized at week 2, 4, 6 and 8 post infection. The spleens were homogenized and a series of dilution were plated on TSA. After 48–72 h of incubation the colonies were counted and the Log_{10} CFU/spleen were calculated.

a) Protection in mice: To determine the ability of strain BNW to confer

protection against a *B. suis* 1330 challenge, female BALB/c mice (n = 5) were vaccinated with a series of doses as previously described. Six weeks post vaccination, mice were challenged with 5.0×10^4 CFU wild type *B. suis* 1330 I/P. Two weeks post challenge, mice were euthanized and splenic bacterial CFU were determined.

b) Efficacy of the highest dose of vaccine was also compared to the rough strain of *B. suis* (VTRS1). This strain was created by deleting the *wboA* gene (previously designated as *rfbU*) and has been reported to provide protection against *B. suis* challenge (Winter et al., 1996). Briefly, mice (n = 5) were vaccinated with PBS (control), strain BNW (2×10^8 CFU, I/P), or strain VTRS1 (5×10^4 CFU, I/V). As mentioned above, after six weeks of vaccination, mice were challenged by I/P injection with 5.0×10^4 CFU wild type *B. suis* 1330. Two weeks post challenge, mice were euthanized and splenic bacterial CFU were determined.

2.6. Splenocyte culture and quantification of IL-4 and IFN-γ

Mice (n = 3) were vaccinated with either PBS, strain VTRS1 or strain BNW. Six weeks post vaccination, splenocytes were obtained as previously described (Vemulapalli et al., 1998). Approximately, 5×10^5 splenocytes/well were seeded in flat bottom 96 well cell culture plates and stimulated with heat inactivated (heating *B. suis* at 68 °C for 1 h) *B. suis* 1330 at an MOI of 1:100. Splenocytes were stimulated with medium alone as a negative control and with Concavalin A (1 μg mL^{-1}) as a positive control. After incubating cells for 5 days at 37 °C, the supernatants were collected and concentrations of IL-4 and IFN-γ were determined using commercial ELISA kits (BD OptEIA).

2.7. Antibody titers in plasma

A sandwich ELISA was performed to measure the levels of *B. suis* specific antibodies in the plasma (Jain-Gupta et al., 2012). Briefly, heat killed *B. suis* 1330 was prepared by heating the stock cultures at 80 °C for 1 h. The suspensions were centrifuged at 10,000 *g* for 10 min and supernatants were collected. Killing was confirmed by demonstrating no growth in TSB after 48 h of incubation. Supernatant containing the soluble antigens was adsorbed to wells of polystyrene plates (Nunc Maxisorp) at the protein concentration of 1.0 μg mL^{-1} in 50 μl of bicarbonate buffer (pH 9.6). After incubating overnight at 4 °C, plates were washed 4 times with phosphate buffer saline (PBS) containing 0.05% Tween-20. The wells were then blocked with 2% bovine serum albumin in PBS and incubated for 2 h at room temperature. The plates were washed 4 times as before. Mice plasma diluted at 1:1000 was added in duplicates to the wells and incubated for 3 h at room temperature. Again, the plates were washed 4 times and isotype specific goat anti-mouse horseradish peroxidase conjugates was added for 30 min at room temperature. After washing the plates 4 times, 100 μL of TMB substrate solution (KPL, Gaithersburg, MD) was added and incubated in dark for 20 min. The reaction was stopped by adding 100 μL/well of 0.18 M sulfuric acid, and the absorbance of the developed color was measured at 450 nm.

2.8. Statistical analysis

All statistical analysis was done using a two-tailed Student's *t*-test (Microsoft EXCEL). Significance was set at a p value of ≤ 0.05.

3. Results

3.1. Characterization of a rough mutant

The *wboA* gene encodes a glycosyl transferase that polymerizes mannose into the O-side chain of *Brucella* LPS (McQuiston et al., 1999). Deletion of *wboA* results in a rough phenotype in *Brucella*. *B. abortus* RB51 is a natural rough strain and contains a naturally occurring and stable transposon insertion in *wboA*. We did whole genome sequencing of the deletion mutant of *B. neotomae* generated in this study to confirm that only *wboA* gene was mutated and the deletion was in frame and did not have any polar effect. Phenotype of the rough mutant was confirmed using acriflavine agglutination, crystal violet staining and lack of agglutination with Bru 38 monoclonal antibodies. Rough strains have a charged outer membrane and therefore clump in neutral acriflavine and cause the solution to clear whereas smooth strains stay in suspension. In a crystal violet assay, rough colonies tend to absorb crystal violet and appear dark while the smooth strain remained colorless. Monoclonal antibody Bru38 is specific for the perosamine O-side chain for the *Brucella* species and only smooth *B. neotomae* showed agglutination when mixed with Bru38 (data not shown).

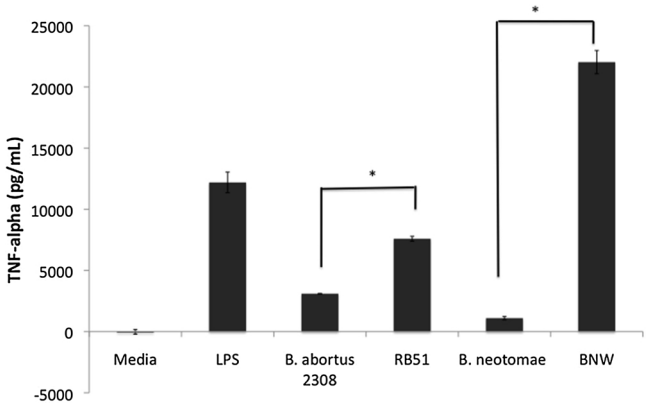

Fig. 1. *B. neotomae* rough vaccine strain BNW induces higher TNF-α secretion To assess the innate immune stimulation, BMDCs were collected from mice and infected with *B. abortus* 2308, *B. abortus* RB51, *B. neotomae* or strain BNW strains at a MOI 1:100. Levels of TNF-α were measured in supernatants, 4 h post infection using sandwich ELISA. *E. coli* LPS and media treated cells serve as positive and negative controls respectively. Both rough strains (RB51 and BNW) induced significantly higher (represented by *, $p \leq 0.01$) secretion of TNF-α compared to their respective smooth parental strains.

3.2. Innate immune response

At a MOI 1:100, rough strains (*B. abortus* RB51 and BNW) stimulated significantly higher production of TNF-α (Fig. 1) by the DCs compared to the respective smooth strains (*B. abortus* and *B. neotomae)*. The reported cytokine levels were detected at 4 h post infection. We also determined the intracellular *Brucella* CFU at 4, 24 and 48 h post infection to evaluate the differences in TNF-α production were not due to differences in the intracellular viability of the *Brucella* species. There was no significant difference in the intracellular CFUs of different strains at 4 h post infection (data not shown). However, intracellular bacterial counts of rough strain were significantly lower compared to the smooth strain at 24 and 48 h post infection and thus the cytokine level are not reported for those time points.

3.3. Clearance in mice

It is important that the live-attenuated vaccines are cleared from the host in at timely manner. Most wild type pathogenic strains of *Brucella* do not clear from the host and often lead to chronic infections. (Jain-Gupta et al., 2014). Vaccine strain, *B. abortus* RB51 is a naturally occurring rough strain and is shown to be cleared within 6–8 weeks from the vaccinated mice. Its attenuation cannot be attributed solely to the lack of O-polysaccharide as genome sequencing has revealed additional mutations (Moriyon et al., 2004). Genetically modified strains *B. suis* VTRS1 and *B. melitensis* VTRM1 has been shown to be attenuated in mice compared to their parental strains and their attenuation can be attributed to the lack of O-polysaccharide in LPS (Smith et al., 2019;

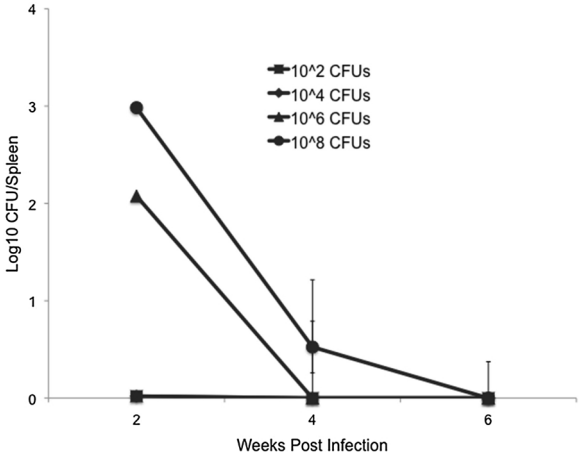

Fig. 2. *B. neotomae* Δ*wboA* clears within 6 weeks in mice Mice were vaccinated with a series of doses of strain BNW. At each time point three mice were euthanized to determine the clearance of bacteria from spleens. All the tested doses were completely cleared with in six weeks of vaccination.

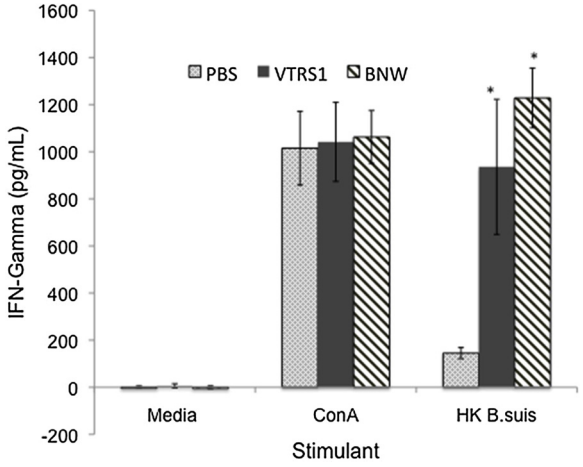

Fig. 4. IFN-γ but not IL-4 is produced by stimulated splenocytes obtained from vaccinated mice Mice (n = 3) were vaccinated with PBS (control), strain BNW or strain VTRS1. Almost six weeks post vaccination splenocytes were collected and stimulated with media (negative control), Concavalin A (ConA, positive control) and heat inactivated *B. suis* 1330. Levels of cytokines were determined in the cell culture supernatants. Compared to controls, a significantly higher (represented by *, $p \leq 0.01$) amount of IFN-γ was detected in splenocyte supernatants from strain BNW and strain VTRS1 vaccinated mice upon stimulation with heat inactivated *B. suis* 1330.

Winter et al., 1996). In this study, mice were vaccinated with four different doses of BNW and clearance was determined. All the four doses were well tolerated by the mice and didn't cause any ill effects or mortality. Mice vaccinated with 2×10^2 and 2×10^4 CFU cleared BNW within two weeks of vaccination (Fig. 2). Clearance before two weeks was not checked thus there is a possibility that it was already cleared. A dose of 2×10^6 CFU was cleared by the fourth week of vaccination. The highest dose (2×10^8 CFU) was cleared by the sixth week and was the dose chosen for the further experimentation in mice.

3.4. Protection against challenge

Strain BNW showed a dose dependent protection against *B. suis* 1330 challenge in mice (Fig. 3A). Within the time frame used in this study, doses of 2×10^2 CFU and 2×10^4 CFU failed to provide any protection against the challenge compared to the controls. A dose of 2×10^8 (Log_{10} 3.6 ± 0.5) showed significantly lesser splenic *Brucella* (higher protection) compared to the unvaccinated controls (Log_{10} 5.2 ± 0.2) as well as to the mice vaccinated with 2×10^6 CFU (Log_{10} 4.5 ± 0.2). Vaccinated mice didn't show any signs of sickness and thus a dose as high as 2×10^8 CFU can be used without causing any lethal effects. In the second mouse study, a rough mutant *B. suis* 1330 Δ*wboA* (VTRS1) was tested as a control. Compared to unvaccinated controls (Log_{10} 5.3 ± 0.1), both strains VTRS1 (Log_{10} 3.1 ± 0.4) and BNW (Log_{10} 3.3 ± 0.2) provided significant reduction in the *B. suis* counts in the spleens of vaccinated mice (Fig. 3B). However, there was no significant difference in the protection provided by the two vaccine strains.

3.5. Splenocyte activation and cytokine production

Compared to controls, splenocytes obtained from mice vaccinated either with strain VTRS1 or BNW produced significantly higher amounts of IFN-γ when stimulated with heat inactivated *B. suis* (Fig. 4) There was no significant difference in the IFN-γ production by the splenocytes from the strain VTRS1 vs. BNW vaccinated mice. No IL-4 was detected in the same samples indicating that the vaccine response was stimulating Th1 type immune responses.

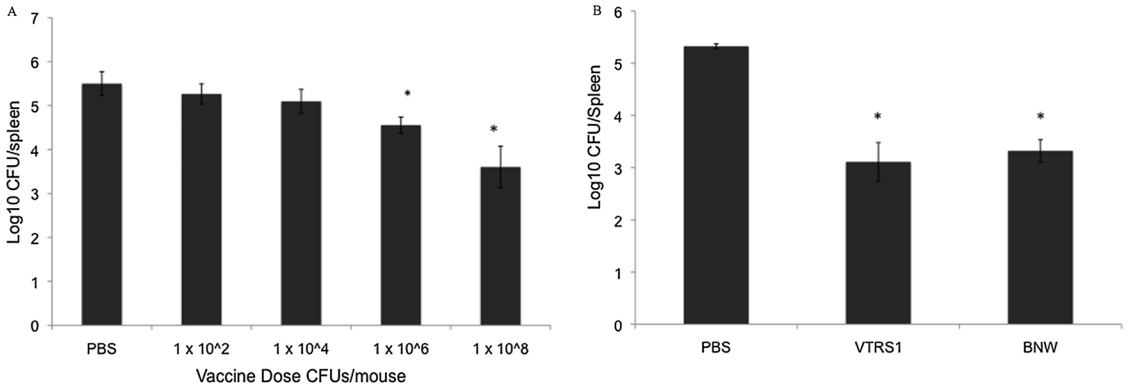

Fig. 3. a) A dose dependent protection was provided by BNW against *B. suis* challenge Mice were vaccinated with different doses of strain BNW. Six weeks post vaccination, mice were challenged with *B. suis* 1330 and euthanized two weeks later to determine the efficacy of vaccine. At a dose of 2×10^6 and 2×10^8 CFU, vaccine provided statistically significant protection compared to the PBS controls (represented by *, $p \leq 0.01$). **b) BNW provides the same level of protection as B. *suis* Δ*wboA* against a *B. suis* 1330 challenge:** Efficacy of homologous (VTRS1) and heterologous (BNW) vaccine strains was tested against *B. suis* 1330 challenge. Mice were vaccinated and challenged six weeks post vaccination. Mice were euthanized two weeks post challenge and bacterial CFU were determined in spleens as a parameter to compare the efficacies of the vaccines. Both vaccine strains provided significant protection (represented by *, $p \leq 0.01$) compared to the controls, however there was no difference between the vaccine strains.

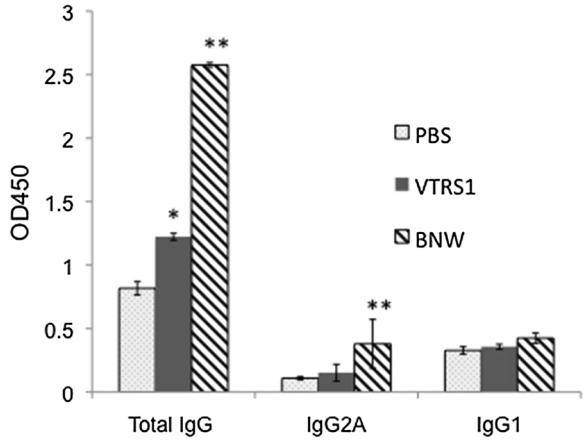

Fig. 5. *B. neotomae* rough vaccine strain BNW induces IgG and IgG2a production in mice Plasma was obtained from the vaccinated mice (n = 5) before challenging them and antibody titers were determined against *B. suis*. Compared to the controls, strain VTRS1 vaccinated mice produced significantly higher (represented by *, $p \leq 0.01$) IgG antibodies. BNW vaccinated mice produced significantly higher amounts of IgG and IgG2a compared to both controls as well as VTRS1 vaccinated mice (represented by **, $p \leq 0.01$).

3.6. Antibody titers

Before challenging the mice with *B. suis*, mice were bled and antibody production was determined in plasma. Compared to control mice, mice vaccinated with either strain VTRS1 or BNW produced significantly higher amount of IgG antibodies (Fig. 5). The subtyping shows the significant role of IgG2a subisotype and not that of IgG1, which is consistent with the role of a Th1 immune responses in the protection provided by the vaccines. Further, mice vaccinated with BNW produced significantly higher levels of both total IgG and IgG2a compared to mice vaccinated with VTRS1 but this could be due to the difference in the doses of two vaccine strains.

4. Discussion

Brucellosis occurs in humans as well as wild life and domestic animals. There is a need for a universal vaccine that should be safe and potentially could be used in humans too (Perkins et al., 2010). Also, there is an immediate need for a vaccine that can prevent *B. suis* infection in feral swine and its spread to other animals as well as to humans (Smith et al., 2019). It has been shown before that live-attenuated vaccines provide better protection compared to killed, subunit or DNA vaccines in the case of *Brucella* control but they also present a risk of reversion and causing infections. *Brucella* LPS is an amphipathic molecule with three covalently linked regions: O-polysaccharide (O-side chain), core oligosaccharide and lipid A (Kianmehr et al., 2015). Natural or synthetic rough LPS of *Brucella* lacks O-side chain and causes attenuation in the wild type strain. There are three major advantages for creating a rough strain: 1) rough vaccine strains do not interfere with the sero-diagnosis to differentiate between infected vs. vaccinated animals, which is of highest value in the “test and slaughter” program to control brucellosis in livestock; 2) rough strains created by deleting the *wboA* gene have been shown to be attenuated compared to their parental strains in mice and clears from host after vaccination; 3) it has been shown before that the rough strain *B. abortus*, RB51 stimulates higher innate immune responses compared to its smooth parental strain. We created a rough mutant of *B. neotomae* by generating an in-frame deletion of 1209 bp in *wboA* gene that encodes for a mannose glycosyl transferase. The resultant strain was confirmed both genotypically and phenotypically.

A strong innate immune response is required for facilitating a strong adaptive immune response and ultimately providing protection against a wild type *Brucella* challenge. Dendritic cells (DCs) have been previously shown to be an important target of *Brucella* infection (Billard et al., 2005). Differences between the activation and/or inhibition of DCs upon infection with smooth vs rough strains of *Brucella* have been reported before (Billard et al., 2007b; Surendran et al., 2011). We measured the levels of TNF-α and compared the stimulation provided by rough vs smooth *B. neotomae* strain. Upon activation DCs produce TNF-α that has been shown to play an important role in *Brucella* immunity (Billard et al., 2007a). TNF-α plays a critical role in enhancing the production of IL-12 which in turn up-regulates the production of IFN-γ by T-cells and natural killer (NK) cells (Trinchieri, 1997). Our results are consistent with the results published before which compare the DC stimulation and TNF-α production by rough vs smooth *B. abortus*. Compared to smooth wild type strain, DCs infected with rough mutant BNW produced significantly higher amount of TNF-α. These differences in TNF-α production were not due to the differences in intracellular viability of different *Brucella* strains. No differences in intracellular CFUs were determined at 4 h post infection. It has been previously established that these differences are not directly related to the difference in LPS of smooth vs rough strains (Billard et al., 2007a). However, the outer membrane proteins are more exposed on rough strains of *Brucella* due to the lack of O-polysaccharides (Gonzalez et al., 2008) and could have contributed to the differences in activation and cytokine production by DCs. This difference could also due to the difference in mechanism of entry and interaction between the pathogen and the host. It has been shown previously that smooth strains enter via lipid rafts and rough strains enter the host cells by phagocytosis and unlike vacuoles containing smooth strains, vacuoles with rough strains rapidly fuse with lysosomes (Porte et al., 2003). Our preliminary result shows that compared to smooth *B. neotomae*, the rough mutant stimulates higher dendritic cells activation *in vitro*. Further in-depth studies to test different MOIs, evaluation at different time points, and quantification of other cytokines like IL-12 are required to fully understand the mechanism of DC activation by rough *B. neotomae*.

To be an ideal vaccine, a vaccine strain should clear from the host but also persist long enough for the host immunity to generate a protective immune response. Strain BNW was tested at different doses in mice and it was found that at a dose as high as 2×10^8 CFU, the strain was cleared from the mice within 6 weeks. Vaccine strain RB51 is cleared within 6–8 weeks of vaccination in mice and is considered ideal to induce immune responses without imposing a risk of persisting in a mouse model. The clearance profile would be different in different animal species and should be tested appropriately. A dose dependent protection was seen after a challenge with pathogenic *B. suis* in the vaccinated mice. We did not test a dose higher than 2×10^8 CFU and there is a chance that a higher dose or a booster vaccination might enhance the protection in mice. From our results it can be suggested that a minimum of 3–4 weeks is required for the immune system to recognize the vaccine and generate protective immunity against a challenge. Mice vaccinated with either 2×10^2 CFU or 2×10^4 CFU did not show any protection against the challenge as the vaccine was cleared within two weeks of vaccination and failed to generate protective immune response. Strain VTRS1 is the rough strain of *B. suis* and was tested as a homologous vaccine against *B. suis* challenge while *B. neotomae* Δ*wboA* presents an option as a heterologous vaccine. When compared for their efficacies based on the endpoints of this study, no significant difference was found using either of the vaccine candidates. However, both the vaccines provided significantly higher protection against a *B. suis* challenge in mice compared to non-vaccinated controls. Thus, *B. neotomae* Δ*wboA* can be tested as a vaccine to provide protection against heterologous *Brucella* species. It has been shown that irradiated *B. neotomae* provided protection against *B. suis*, *B. abortus* and *B. melitensis* (Moustafa et al., 2011) This could be an avenue to develop a universal vaccine to prevent *Brucella* infection caused by any *Brucella* species. We further plan to test BNW vaccine strain against *B. melitensis* and *B. abortus* challenges.

To understand the underlying mechanism of protection provided by BNW, we looked at the cytokine production by the stimulated splenocytes and serum antibody titers in the vaccinated mice. In general, a Th1 type immune response is desirable to provide protection against *Brucella* challenge (Murphy et al., 2001a). It has been shown before that IFN-γ contributes to control *Brucella* infection in mice through its ability to activate macrophages that leads to increased microbial killing (Murphy et al., 2001b). Depletion of IFN-γ using neutralizing antibodies results in decreased resistance to *Brucella* infection in BALB/c mice (Baldwin and Parent, 2002). Thus, IFN-γ plays a critical role in controlling *Brucella* infection and thus a vaccine candidate should prime the host to produce higher amounts of IFN-γ to combat the challenge with a pathogenic strain. Compared to the saline control, splenocytes from mice vaccinated with either vaccine strain, VTRS1 and BNW, produced significantly higher amounts of IFN-γ upon stimulation with heat inactivated *B. suis* 1330. The amount of IL-4 was below the detection limit in all the samples indicating a clear role of Th1 type immune responses that protected mice against *B. suis* challenge. However, further work is required to determine the specific role of $CD4^+$ and $CD8^+$ T cells as both produce IFN-γ and have been shown to play critical roles in *Brucella* immunity. Results from the antibody titers in the vaccinated mice further substantiate the role of Th1 type immune responses in the protection provided by BNW. Mice vaccinated with either strain VTRS1 or BNW produced significantly higher IgG antibodies compared to the control mice. However, total IgG and IgG2a levels were significantly higher in BNW vaccinated mice compared to the mice vaccinated with VTRS1. These differences could be due to other genetic differences between the parental strains (*B. neotomae* and *B. suis*) itself or because of the difference in the vaccination doses of both strains. We used the previously reported dose for VTRS1 (5×10^4 CFU) that was much less than that the dose used for strain BNW (5×10^8 CFU). The difference in doses might have led to the differences in the antibody titers generated in the vaccinated mice.

In summary, we have shown that a rough attenuated strain of *B. neotomae* can provide protection against the heterologous challenge by *B. suis* in mice. This protection appears to be mediated by Th1 immune responses. Further experimentation should be done to test this vaccine strain against all pathogenic species of *Brucella* and to determine if this can be used as a universal vaccine to prevent brucellosis.

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