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# Immune Response Produced by *Brucella ovis* and Mutants in *virB10* and *virB11* in Murine Model

[1]Marisela Leal-Hernandez, [2]Enrique Salas Tellez, [2]Alma Nunez Del Arco, [3]Rodolfo A. Ugalde,
[1]Victor R. Tenorio-Gutierrez, [1]Rigoberto Hernandez-Castro,
[4]Francisco Suarez Guemes and [1]Efren Diaz-Aparicio
[1]CENID Microbiologia, Instituto Nacional de Investigaciones Forestales,
Agricolas y Pecuarias, Carretera Federal Mexico, Toluca Km. 15.5, Cuajimalpa, DF, 05110, Mexico
[2]Facultad de Estudios Superiores Cuautitlan, Universidad Nacional Autonoma de Mexico,
Carretera Cuautitlan-Teoloyucan Km 2.5, Cuautitlan Izcalli, Estado de Mexico 54740, Mexico
[3]Instituto de Investigaciones Biotecnologicas-Instituto Tecnologico de Chascomus (IIBINTECH),
Consejo Nacional de Investigaciones Cientificas y Tecnicas
Universidad Nacional de General San Martin (CONICET-UNSAM), Buenos Aires, Argentina
[4]Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autonoma de Mexico,
Ciudad Universitaria, D.F., 00900, Mexico

**Abstract:** The aim of this research was to evaluate the humoral and cellular response, produced in mice inoculated with *Brucella ovis* and mutants in *virB10 and virB11,* measuring IgG1, IgG2a, IgG2b and IgM levels and cytokines production IL-2, IL-4 e IFN-γ, in lymphocyte culture. Mice of 6-weeks-old Balb/c were immunized intraperitoneally with each strain of *B. ovis* and the mutants with $3\times10^{13}$ CFU $mL^{-1}$ in PBS. For the cellular response, they were injected 2 times. In the humoral response the predominant antibodies were IgG2b and IgM in the different strains. The cytokine production with the wild type were indicative of Th2 and low response of IFN-γ and IL-2, that is indicative of an answer Th1 type characteristic of intracellular bacteria. The Reo 198 strain, showed Th1 type response. The mutant Δ*virB11* only presented response to IFN-γ 24 h that decreases after 48 h and with the mutant *virB10*:: Gm the shown levels of the different cytokine were low, this is indicative that the genes *virB10* and *virB11* of *B. ovis* need are complete to induce a suitable immune response. In the virulence in mice only recovered the Reo and wild type strains until 1 week postinoculation and the mutants can recover until 4 and 5 weeks postinoculation. One concludes the immune response by the different strains of *B. ovis* was moderate and can be attributed to that the murine model not was the more recommended to evaluate to cellular response would presumably have been specific to specie by the poorly or inability response showed when were mice inoculated with the different strains of *B. ovis*.

**Key words:** *Brucella ovis*, immune response, cytokines, antibodies, mouse, *virB* mutants

## INTRODUCTION

The genus *Brucella* is bacteria of gram-negative, facultative intracellular pathogens that cause a severe infectious disease in many animal species, including humans. *B. ovis* and *B. melitensis* causes ovine brucellosis, a disease that induces major economic losses in countries in which sheep husbandry is an important industry (Cassataro *et al*., 2005).

Animal resistance to intracellular pathogens such as *B. abortus* depend mainly on the induction of specific, cell-mediated immunity (Zhan and Cheers, 1995; Zhan *et al*., 1996). Murine studies show that the vaccine induces a significant cell response characterized by the production of both Interferon-gamma (IFN-γ) and Interleukin 2 (IL-2) (Pasquali *et al*., 2001; He *et al*., 2001). Cytokines that allow for the differentiation and activation of several cell populations including cytotoxic T lymphocytes, helper T lymphocytes, macrophages and Natural Killer (NK) cells. These cell populations research in a coordinated manner towards intracellular infection control or elimination (Golding *et al*., 2001).

**Corresponding Author:** Marisela Leal Hernandez, CENID Microbiologia, Depto de Bacteriologia,
Instituto Nacional de Investigaciones Forestales, Agricolas y Pecuarias,
Carretera Federal Mexico-Toluca Km. 15.5, Cuajimalpa, DF, 05110, Mexico

Murine $CD4^+$ T cells have been divided into at least 2 subtypes (Th1 and Th2) on the basis of the cytokine profile they secrete upon antigen stimulation Th1 cells characteristically secrete IL-2 and IFN-γ while Th2 lymphocytes typically produce IL-4, IL-5 and IL-10 (Zhan *et al*., 1995).

Cell-mediated immunity plays a critical role in protection against virulent *Brucella* infection, although Antibodies (Ab) specific to the O polysaccharide of the lipopolysaccharide and certain membrane proteins can confer protection in some host species (Cassataro *et al*., 2005).

IFN-γ is crucial during the early stages of infection, since its activity mainly focuses mononuclear phagocytes, by increasing their phagocytic/bactericidal activity. This promotes antigen processing/presentation resulting in increased production of cytokines that stimulate inflammation, Nitric Oxide (NO) production and the expression of Class II Major Hystocompatibility Complex (MHC) (Arestegui *et al*., 2001).

Recently, operons coding for export mechanisms specializing in transfer of a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells have been described. These complexes, named Type IV Secretion Systems (T4SS), are present in *Bordetella pertussis* (*ptl* genes), *Agrobacterium tumefaciens* (*virB* genes), *Escherichia coli* (*tra* genes), *Legionella pneumophila* (*doticm* genes and *lvh* genes) and *Helicobacter pylori* (*cag* genes) (Sieira *et al*., 2000). The *B. abortus* T4SS, encoded by the *virB* operon, is essential for intracellular survival, as well as establishing persistent infection by *Brucella* sp. in the murine reticuloendothelial system (Roux *et al*., 2007; Rolan and Tsolis, 2008). Mutational inactivation of the T4SS reduces the ability of *B. abortus* to survive and/or replicate in human epithelial cell lines (HeLa cells), murine bone marrow-derived macrophages and macrophage-like cell lines and similar phenotypes have been described for *Brucella melitensis* and *Brucella suis virB* mutants (Rolan and Tsolis, 2007).

The aim of this research, was evaluate the humoral and cellular immune response, produced in mice inoculated with *Brucella ovis* and mutants in *virB10* and *virB11*, measuring IgG1, IgG2a, IgG2b and IgM levels and cytokines production IL-2, IL-4 and IFN-γ, in lymphocyte culture.

## MATERIALS AND METHODS

**Bacterial strains, media and culture conditions:** Bacterial strains used were: *Brucella ovis* Reo 198 and *B. ovis virB10*:: $Gm^r$ and *B. ovis ΔvirB11*:: $Gm^r$ (*ΔvirB11)*. Strains were cultured on Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, MI) on a rotary shaker (200 rpm) for 20 h in tryptic soy broth at 37°C. Bacterial inoculums for infection of mice were cultured on TSA containing 10% of sheep blood. Both mediums were supplemented with 5% of bovine serum (Invitrogen). When, necessary, the following antibiotics were added: kanamycin (100 μg $mL^{-1}$), gentamicin (2.5 mg $mL^{-1}$) and ampicillin (200 mg $mL^{-1}$) (SIGMA Aldrich St Louis Missouri).

**Immunization of mice:** Four groups of 5 mice of 6 weeks old Balb/c were immunized intraperitoneally with each strain of *B. ovis* and the mutants with $3\times10^{13}$ CFU $mL^{-1}$ in PBS. For the cellular response, they were injected 2 times and the 2nd inoculation was 15 days after.

**Virulence in mice:** Groups of 15 mice were inoculated with each strain for determination of the virulence, quantifying the survival of the strains in the spleen after 1-5 weeks post-inoculation (p.i.). At this time 3 mice were sacrificed and their spleens were removed, weighed and homogenized in 1 mL of PBS. Tissue homogenates were serially diluted with PBS and plated on tryptic soy agar with 5% of bovine serum and 10% of blood. The numbers were expressed in colony forming units CFU.

**Humoral response:** For Indirect ELISA was used *B. ovis* bacterial extract as antigen and the response was evaluated using the commercially-kit Mouse monoclonal antibody isotyping reagents (Sigma Aldrich St Louis Missouri) it's contain the isotypes IgG1, IgG2a, IgG2b and IgM. The assay was performed following manufacturer's specifications. The antigen was used at 23.3 μg per well. Results were expressed in Optical Densities (OD).

**Lymphocyte culture and cytokine induction:** Mice spleens were mixed from each group, washed 3 times with Hanks' solution and placed in a Petri dish containing 5 mL of RPMI 1640 medium (Gibco Laboratories), containing 100 U $mL^{-1}$ penicillin and 100 μg $mL^{-1}$ streptomycin, over gauze to retain tissue debris. They were then macerated and the cell suspension was transferred to a conical tube with 5 mL of the same medium and centrifuged at 400× g for 3 min. The pellet was resuspended in 0.17 M $NH_4Cl$ for 5 min at 4°C to lyses the erythrocytes; it was washed 3 times with RPMI and then resuspended in RPMI enriched with 10% bovine fetal serum, 200 mM L-glutamine and 0.1 mM of nonessential amino acids. We used a different culture plate per strain, approximately $6.5\times10^6$ lymphocytes from each group were inoculated with *B. ovis* Reo 198, wild type, *virB10*:: Gm and

Δ*virB11*, respectively, distributed in 5 wells of the culture plate (Nunc, Rochester, New York, USA), each well was inoculated with 10 μL of the corresponding strain and the plates were incubated at 37°C with 5% $CO_2$. The concanavalin A was used as a positive control for non-specific induction of cytokines. Supernatants containing the cytokines were collected at 24, 48, 72, 96 and 120 h after inoculation and frozen until use.

**Determination of cytokines *in vitro* response:** To evaluate the response induced for the mice inoculated with *B ovis* and the mutants were used commercial kits for mouse IFN-γ, IL-2 and IL-4 (Duoset ELISA; R and D Systems, Minneapolis, Minnesota, USA), as per the manufacturer instructions. The concentration used for capturing and detector antibodies was 2000 and 300 ng $mL^{-1}$ for IL-2; 4000 and 400 ng $mL^{-1}$ for IFN-γ and 720 and 36 μg $mL^{-1}$ for IL-4. The standard curve was prepared by serial dilutions of a standard stock solution: 1000 pg $mL^{-1}$ for IL-2 and IL-4 and 2000 pg $mL^{-1}$ for IFN-γ.

For the results in ELISA between groups to the different back days to the inoculation, realized the statistical analysis by means of the t-test with a value of significance of $p<0.05$.

## RESULTS AND DISCUSSION

For the evaluation of the humoral response at *B. ovis* and mutants was used Indirect ELISA with *B. ovis* sonicated bacterial extract antigen.

In the estimation of the immune response of *B. ovis* (Fig. 1), the levels of IgG1 were lowest in all of strains. For the IgG2a the behavior was similar. Only 4 weeks after inoculation increase the levels for IgG2b. In the case of IgM the mayor levels was in the first week and after decrease gradually. Between the different IgG's the behavior of levels was similar.

**Cytokine response by *B. ovis* and *virB* mutants:** The results obtained in the induction of cytokines (Fig. 2) by the Reo 198 strain the major levels of IFN-γ were 24 and 120 h. A low IL-2 response showed at 24 h until 72 h and remained low. IL-4 showed decrease of 24 h until 72 h, presenting a increased at 96-120 h. In the wild type strain the cytokine with major production was IL-4 and peaked at 72 h and decrease. The levels of IFN-γ showed increase of 24 h until 96 h returned to the 1st sample at 120 h. In the case the mutant *ΔvirB11* induced a rapid response from IFN-γ at 24 h, with a marked reduction at 48 h. While, in the mutant *virB10*:: Gm the levels of cytokines were lowest of different strains.

**Virulence in mice:** One week after infection fewer bacteria were recovered from mice infected with wild type strain ($9\times10^2$ CFU $spleen^{-1}$) (Fig. 3) and the major strain recovered was Reo 198 ($1.2\times10^4$ CFU $spleen^{-1}$). Reo 198 and wild type were undetectable in the spleen after second week. While, that both mutants Δ*virBll* and *virB10*:: Gm were recovered until 3 and 4 weeks $2\times10^2$ CFU and $1\times10^2$ CFU $spleen^{-1}$, respectively. The complemented mutants Δ*virBllc* and *virB10*:: Gmc were recovered until 5 and 4 week, respectively.

In literature do not exist antecedent of studies in the murine model of the immune response of strains of *B. ovis*, it have been only evaluated with the use of subcellular fractions like inducers of the immune response.

*Brucella* is considered a facultative intracellular pathogen that can survive and replicate within phagocytic and non phagocytic cells (Celli, 2006). Resistance to facultative intracellular bacterial pathogens such as *Brucella* depends on acquired cell-mediated resistance and activation of macrophages by IFN-γ producing T lymphocytes (Zhan *et al*., 1995).

We vaccinated groups of 5 Balb/c mice with the Reo198, wild type strains and the *virB11* and *virB10* mutants of *B. ovis* to evaluate the humoral response and the levels of predominant antibodies were IgG2b and IgM (Fig. 1). Jimenez *et al.* (1994) vaccinated groups of 5 mice with Hot Saline (HS) extracted from *B. ovis* Reo 198 strain with and without adjuvant. Five weeks after the vaccination, mice were challenged with *B. ovis* PA. The presence of adjuvant resulted in large and highly significant increases in antibodies of IgG1, IgG2a and IgG2b isotypes and with the infection with *B. ovis* were produced predominantly IgG2a, IgG3 antibodies with IgG1 at the lowest level.

In this research, we evaluated the cytokine production with IFN-γ, IL-4 and IL-2 to determinate the inductor capacity that showed the *virB* mutants and the reference strains. The results obtained in the response cytokine with the wild type strain were indicative of Th2 response because the IL-4 showed increased. The innate susceptibility of Balb/c mice to numerous intracellular pathogens has been linked to hypoproduction of IFN-γ and preferential Th2 type cytokine response involving increased activity of Th2 type cells (Ulett *et al*., 2000).

Nevertheless, the wild type provokes a moderate answer of IFN-γ and IL-2, which is indicative of an answer Th1 type characteristic of intracellular bacteria (Splitter *et al*., 1996).

The results obtained when the mice were inoculated with the Reo 198 strain, was a poorly response, the cytokine of major production was IFN-γ and then follow by IL-2 it was guide around Th1 type response. The fact

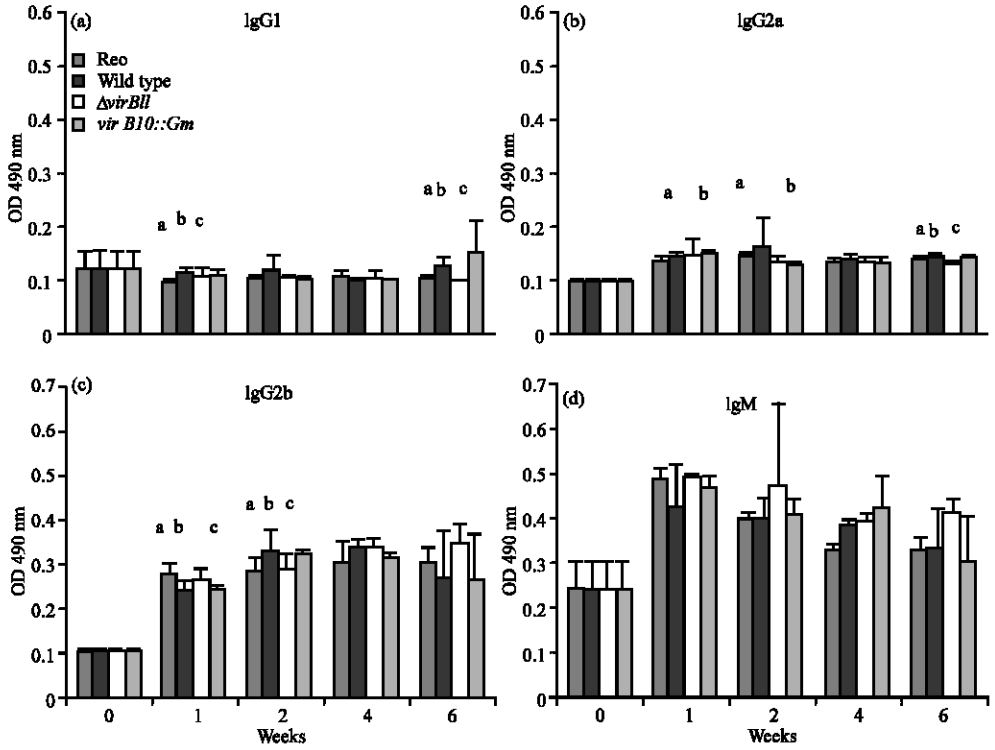


Fig. 1: Average optical densities of indirect ELISA to evaluation the immune response of *B. ovis:* Reo 198, wild type, *virB10*:: Gm and *ΔvirB11* mutants. In mice during 6 weeks after immunization, a-c, the different literals showed a significantly different

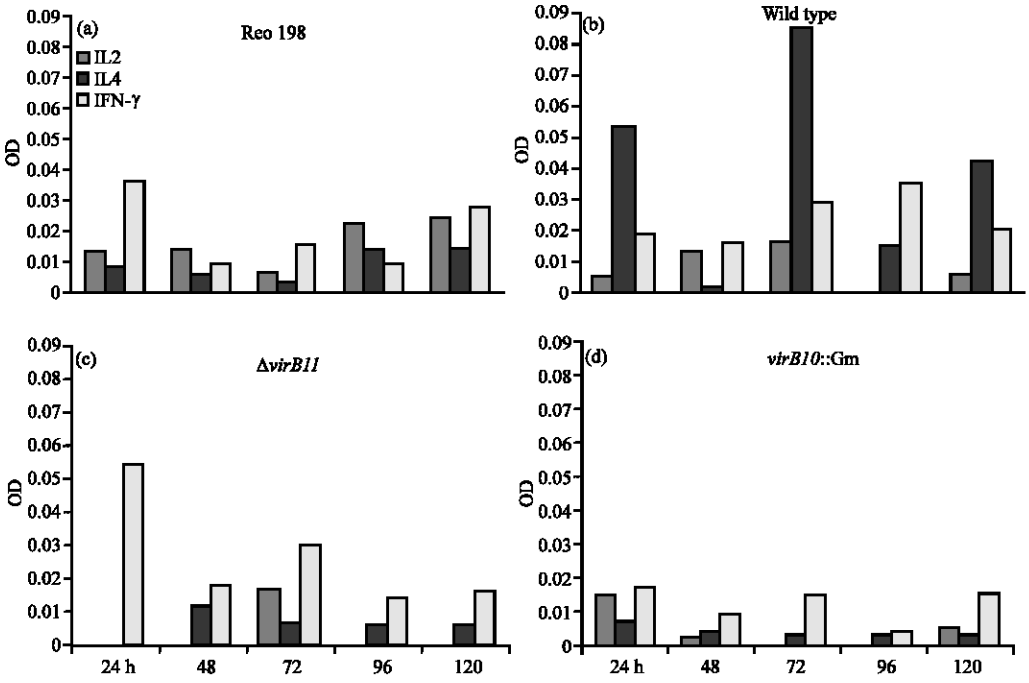


Fig. 2: Response of cytokines produced for the mice inoculated with *B ovis* and the mutants using a ELISA, with supernatans of primary culture of mice spleen cell culture, stimulated with *B. ovis:* Reo 198, wildtype, mutants *virB10*:: Gm and *ΔvirB11*:: Gm

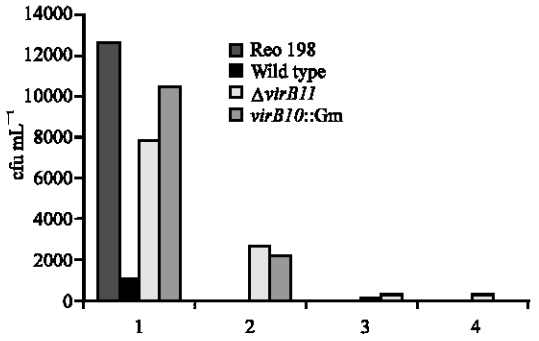


Fig. 3: Proliferation in mice. Mice were infected intraperitoneally with *Brucella ovis* Reo 198, wild type and *virB10*:: Gm and Δ*virB11* mutants. Recovery of viable bacteria of spleens in infected mice at different times post-inoculation

that the response to IL2 and IFN-γ was low can be due to that the Reo 198 strain has been adapted to the conditions of the laboratory causing that its virulence is smaller.

Sieira *et al.* (2000) using *virB* polar and no polar mutants of *B. abortus* found that *virB10* and downstream sequences (*virB11-ORF12-ORF13*) are essential for *Brucella* pathogenesis in mice and suggest that the integrity of the *virB* operon is required for wild-type virulence. In this research, with the mutant Δ*virB11* only presented response of IFN-γ to 24 h that decreases after 48 h and with the mutant *virB10*:: Gm the shown levels of the different cytokine were low, which is indicative that the genes *virB10* and *virB11* of *B. ovis* need are complete to induce a suitable immune response.

In a study, realized by Salas *et al.* (2005), employed different *B. ovis* subcellular fractions (OMP, IMP and CP) to evaluate a cytokines response in mice. The OMP fractions produced a high IL-2, IFN-γ and the highest DTH response. This is consistent with a classic Th1 lymphocyte response, associated with acquired cellular resistance and DTH. The IL-4 was only produced by the OMP fraction, demonstrating activation by the Th2 lymphocytes and a humoral response.

Maturation of the BCV into the replication niche is dependent upon the *virB* T4SS and therefore, this system constitutes an important virulence factor for intracellular survival of *Brucella* sp. (Rajashekara *et al.*, 2006; Spera *et al.*, 2006). Sieira *et al.* (2000) was carried one infection with Balb/c mice injected intraperitoneally with $10^4$ CFU of *B. abortus* 2308 wild-type or mutant *virB* strains. The mice were sacrificed 15 days p.i. and the number of *Brucella* bacteria recovered from spleens was one log up the inoculated with the wild-type *B. abortus* 2308, whereas no viable bacteria were obtained from spleens of mice inoculated with the *B. abortus* polar mutant. The number of bacteria recovered from spleens of mice inoculated with the non polar mutant was significantly lower than the number recovered from mice inoculated with the wild type but significantly higher than CFU recovered from mice inoculated with the *B. abortus* polar mutant. And their complements recovered virulence, reaching viable counts similar to those of the wild-type parental strain. At difference of the results showed by Kim *et al.* (2003) were *B. abortus virB* mutants was cleared from the infected mice faster than wild type strain, the mutant did not replicate in mouse spleen, but it was not cleared from the infected mice soon. We in this study only recovered the Reo 198 and wild type strain until 1 week p.i and the mutants can recovered until 4 and 5 weeks p.i.

In spite of the splenomegaly showed in the group of mice inoculated with the strain wild type, only was recovered during the first week post inoculation. The weight obtained of the spleens to the different times was not indicative of the quantity of CFU obtained, by what there was not relation in the weight of the bacterium's recovered.

Interestingly, bovine and murine T cells exhibited a similar cytokine profile following stimulation with *B. abortus,* suggesting an analogous immune response between these 2 animal species and supporting the use of mice as a relevant model on study immunity to brucellosis (Splitter *et al.*, 1996).

Diverse authors have showed that in *B. abortus* and *B. melitensis* the use of the murine model was adequate to study evaluations of the mutants strains, as well as vaccines (Montaraz and Winter, 1986; Pasquali *et al.*, 2002; Gonzalez *et al.*, 2008).

## CONCLUSION

One concludes that in this study the response of cellular and humoral type caused by the mutants *virB* was moderate and can be attributed to that the murine model not was the more recommended to evaluate to cellular response would presumably have been specific to specie by the poorly or inability response showed when mice were inoculated with the different strains of *B. ovis*.

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