Antibody response of mice to *Porphyromonas levii* inoculation.

Blum, S., Leitner, G., Krifuchs, O., Ferber-Weismann, H. and Elad, D.*

Dept. of Clinical Bacteriology and Mycology, Kimron Veterinary Institute, Bet Dagan, Israel

* **Corresponding author:** Daniel Elad, DVM, PhD, Dept. of Clinical Bacteriology and Mycology, Kimron Veterinary Institute, P.O.Box 39, Bet Dagan, 50250 Israel. Phone: +972-3-9681688, Fax: +972-3-9688965; Email: danielad@moag.gov.il

Abstract

Three groups of 20 mice were inoculated subcutaneously once, twice or three times, respectively, with 10^9 colony forming units of *Porphyromonas levii* at 3, 5 and 7 weeks of age. Four mice were inoculated with saline and used as negative controls. An ELISA test was developed and used to assess the humoral reaction of mice to *P. levii*. Antibodies were present after the first inoculation and increased steeply after the second. The third inoculation had no significant effect on antibodies titers. These results indicate that *P. levii* induces a humoral immune response in mice in our model. Serum from mice challenged with a isolate of *P. levii* isolated from a case of Bovine Necrotic Vulvovaginitis was used to compare between different *P. levii* isolates from cows. Antibodies induced by this isolate cross-reacted with all the isolates studied and revealed a high degree of homology between these isolates.

Keywords: *Porphyromonas levii*, antibodies, mice, ELISA, Bovine Necrotic Vulvovaginitis

INTRODUCTION

Porphyromonas levii is a gram-negative, non-spore forming anaerobe bacterium (1) involved in a variety of infections in cattle, among which are foot rot (2) and a syndrome described in Israel, Bovine Necrotic Vulvovaginitis (BNVV) (3, 4). The latter syndrome affects mostly heifers and develops during the first week after calving (3). BNVV is of economic impact in BNVV affected herds (5, 6) and the development of ways to prevent the infection, such as vaccination, would be advantageous.

In order to assess anti-*P. levii* antibody production, we carried out a controlled experiment, using a murine model previously developed to test the virulence of *P. levii* isolates isolated from cases of BNVV (7), to study the humoral response of mice to *P. levii* vaccination and the cross reaction of antibodies elicited by a specific isolate against isolates from different herds. Moreover, to assess the extent to which isolate variation may influence the immune response we compared the reaction of the homologous isolate to that of five heterologous isolates.

MATERIAL AND METHODS

Bacteria isolation and identification

P. levii isolates were obtained from the genital tract of healthy and BNVV affected cows from different herds (Table 1). *P. levii* was isolated and identified as previously described (3). Bacteria were cultured on 5% sheep blood agar under anaerobic conditions (Pack Anaero, Mitsubishi, USA) at 37°C for 5 days. CFU counts were determined by turbidity (Hanna Instruments, UK), after calibrating turbidity and colony counts on an automatic colony counter (Symbiosis, UK).

Table 1: Source of *P. levii* isolates used in the present work.

Isolate	Herd	BNVV
AH1	Farm 1	Positive, (sporadic)
AH2		
BY	Farm 2	Negative, (isolated from cow mastitis)
Vlk	Farm 3	Negative
Shvl	Farm 4	Positive, (sporadic)
G7440	Farm 5	Positive, (endemic)

Animals and study design

Three groups of 20 Swiss mice each were inoculated subcutaneously in the left inguinal region with live 10^9 colony forming units (CFU) of *P. levii* (*G*7440) suspended in 0.5ml of saline. This isolate was taken from a case of BNVV in an endemic herd. One group of mice was injected at three, five and seven weeks of age, the second group was injected at five and seven weeks of age, and the third group was injected at seven weeks of age. Four mice were inoculated three times with saline and were used as negative controls. Mice were anaesthetized with chloroform, exsanguinated at nine weeks of age and subsequently euthanized. The experiment was approved by the Animal Care and Use committee at the Kimron Veterinary Institute and was performed by accepted national and international guidelines.

Serological assay

The level of anti-*P. levii* antibodies was evaluated using an in-house ELISA. The *G*7440 isolate was cultured as described above. Antigen was prepared by mechanical disruption of bacteria using glass beads in a cell homogenizer (Braun Melsungen, Germany). Glass beads and intact bacteria were removed by centrifugation and filtering of the homogenate through 0.2 µm pore-size membranes and the total protein concentration was assessed by the Bio-Rad Protein Assay (Biorad, USA). The test was performed in 96 well microplates (Nunc, Amstrup, Denmark) coated with 30µg/plate of *P. levii* antigen in carbonate-bicarbonate buffer (pH 9.6). Coating was done with 200 µl of antigen per well and incubation in 4°C overnight. Plates were washed 3 times with phosphate buffer saline (pH 7.6) containing 0.5% Tween 20, (PBST) and blocked with 3% casein (200 µl/well) for 45 minutes at 37°C. Following 3 additional washing steps, 100 µl of each serum diluted 1:500 in PBS, the dilution found to give the best results, was added to wells in duplicates and the plates were incubated at 37°C for 60 minutes. To each plate, negative and positive controls were added in duplicates. Negative control constituted a pool of naive mice serum whereas positive control consisted of a pool of hyperimmune sera from mice inoculated with *P. levii* with high titer of anti-*P. levii* antibodies. Subsequently, plates were washed again 3 times as described above, 100 µl of 1:10,000 dilution of peroxidase conjugated, goat anti-mice IgG heavy and light-chain (Jackson, CA, USA), linked to peroxidase diluted 1:5000 was added to each well, plates were incubated for 60 minutes at 37°C and washed again 3 times. Finally, bound antibodies were detected by adding 100 µl of 3,3',5,5'tetramethylbenzidine (Chemicon, CA, USA). Optical density (OD) was read in a microplate autoreader (Dynatech MR5000, Guernsey, UK) at 650 nm. Anti-*P. levii* antibodies titers were calculated using the following formula: (OD sample/OD negative control) – 1. Plates were valid when the ratio of positive and negative control was approximately 5.

Electrophoresis and immunoblot

One-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1-D-SDS-PAGE) was performed as follows. Bacterial cells disruption, determination of protein concentration and electrophoresis were performed as previously described (8). Electrophoresis was performed on 10% gel (Bis-Tris Gel with w/MOPS) (NOVEX, San Diego, CA, USA). For the immunoblot assay, a nitrocellulose membrane (0.2 µm) was blocked with 3% casein and incubated with 1:100 dilutions of serum from mice challenged with *G7740*. The blot was developed with goat anti-mouse IgG (H+L) alkaline peroxidase conjugate (1:1000) with a substrate of 3,3-diaminobenzidine tetrahydrochloride (ICN Pharmaceuticals, Inc). Molecular-weight markers for these gels were: NOVEX Marker 12 Standard Bands (200-2.5 kDa).

Statistical analysis

The differences between the groups was assessed by a two way two-sample t-test with Statistix v7 (Analytical Software, USA). P values of 5% or less were considered significant.

RESULTS

Descriptive statistics of the OD readings are presented in Table 2. The difference in antibody titers between mice inoculated once and those inoculated twice was statistically significantly different (p=0.0155) The third inoculation did not increase the antibody titers significantly (p=0.9328) relative those inoculated twice, although it did increased the significance relative to the mice injected only once (p=0.0046). Uninoculated mice showed no antibody titer.

Table 2: Optical density readings and descriptive statistics of mice inoculated with *P. levii*

	Inoculated once	Inoculated twice	Inoculated three times
Mean	1.46	3.95	3.89
SD	1.17	2.47	1.75
SEM	0.26	0.55	0.39

SD = Standard Deviation; SEM = Standard Error of the Mean

Inspection of total bacteria protein electrophoresis (Fig. 1) revealed little differences between the isolates studied. When immunoblot with antibodies elicited by isolate *G*7440 was performed, distinct reaction bands were present in all isolates (Fig. 2) and cross reaction and high similarity were observed between the isolates studied.

DISCUSSION

Local and systemic antibacterial treatments have been found to be unable to prevent or treat BNVV afflicted animals (3). While management improvements were beneficiary in some cases, they had no significant impact on the disease's incidence in others (unpublished data). BNVV is of economic importance in affected herds due to increased days between pregnancies (6), decreased milk yield and culling (5). The development of means to prevent the disease, such as a vaccine, may be therefore advantageous.

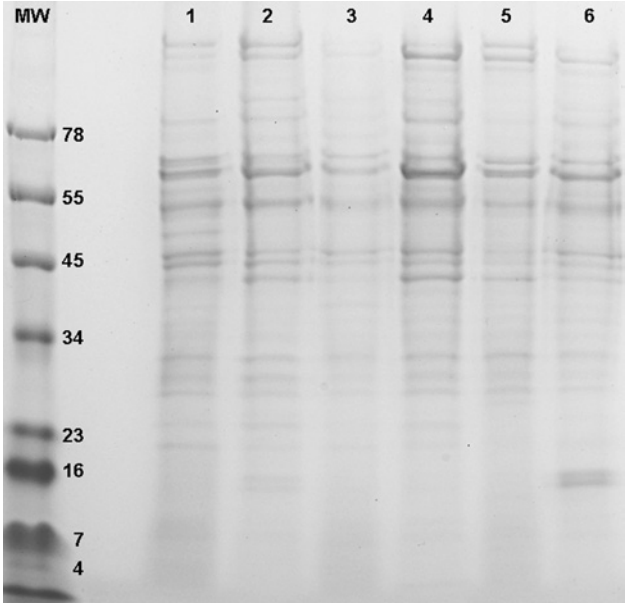

Fig. 1: Whole cell protein gel electrophoresis.
1) *AH*1; 2) *BY*; 3) *Vlk*; 4) *Shvl*; 5) *AH2*; 6) *G*7440.

For this purpose, we examined the humeral response of mice to *P. levii* and the ability of the antibodies to cross react with isolates from different herds. We have shown that inoculation of mice with *P. levii* induces the production of antibodies and that a booster injection significantly increases the antibodies titer. Moreover, no isolate variability between the isolates in cell protein structure and antibody affinity was observed. Consequently, it seems that in this mouse model a vaccine prepared from one isolate of *P. levii* was able to elicit a humoral reaction against isolates from different farms. The protective nature of antibodies elicited against *P. levii* is, however, unknown. *In vitro* work has shown that *P. levii* secretes an IgG2 protease impairing phagocytosis by neutrophils (9). In addition, *P. levii* also has low rates of phagocytosis by macrophages and induces macrophage chemotaxis and oxidative burst only when in circumstance of a high bacteria to cell ratios (2), meaning that this bacterium may evade being recognized when in low numbers during the first stages of infection. However, high-titer serum enhances macrophage phagocytosis of *P. levii* by opsonization (2). Although the humoral response and protective ability against *P. levii* in

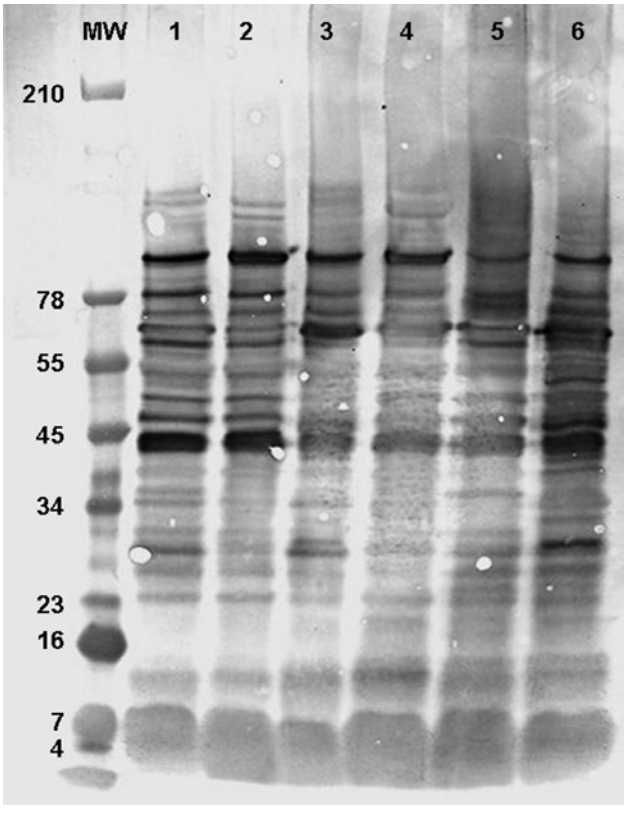

Fig. 2: Western blot with antibodies against *G*7440.
1) *AH*1; 2) *AH2*; 3) *BY*; 4) *Vlk*; 5) *Shvl*; 6) *G*7440.

cows is yet to be assessed, we hypothesize that increased titers of anti-*P. levii* antibodies could be achieved using a vaccine and may prove useful in reducing the incidence or severity of BNVV in affected herds.

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