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An Acellular Anti-*Bordetella Bronchiseptica* Vaccine Efficiency Test in Wistar Rats

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Abstract. The researches were made between February and May 2009 within the Microbiology Laboratory of the Faculty of Veterinary Medicine Cluj-Napoca. A number of 6 *B. bronchiseptica* free adolescent female Wistar rats were infected intranazally with a field isolate of B. bronchiseptica isolated from a pneumonic pig and the same *B. bronchiseptica* strain was ultrasound disintegrated for the preparation of the acellular vaccine. Three rats were subcutaneously vaccinated in days 14 and 28 after infection and in day 60 after infection all rats were euthanized through atlo-occipital dislocation under ethylic ether anaesthezia. Blood samples, lung and trachea portions were collected for bacteriological and serological tests. Clinical observations, serological and bacteriological findings showed a good protection given by the vaccine.

Key words: *Bordetella bronchiseptica*, rats, serology, bacteriology, vaccine, efficiency;

INTRODUCTION

Bordetella bronchiseptica is a small, Gram-negative cocobacil that colonizes the upper respiratory tract of mammals. In healthy rats infection with *B. bronchiseptica* never leads to clinical manifestation but infected rats remains carriers of *B. bronchiseptica* for all their life (3).

Immunogenic structures of *B. bronchiseptica* mainly consist of adhesins and toxins. The majority of these virulence factors of *B. bronchiseptica* are expressed only in the virulent phase. Adhesins such as filamentous haemagglutinin (FHA), fimbriae (FIM) and pertactin (PRN) are expressed on the cell surface and they are highly immunogenic. These structures should be contained in a suspension obtained through ultrasound disintegration of *B. bronchiseptica.* Subcutaneous administration of this suspension in rats should lead to a protective systemic immune response against these immunogenic structures.

MATERIAL AND METHOD

The study was performed during February-May 2009 within the Microbiology Laboratory of Veterinary Medicine Faculty, Cluj-Napoca.

Preparation of the acelluler vaccine: a field isolate of *B. bronchiseptica* isolated from a pneumonic pig was used for vaccine preparation. The isolate was grown on five 90 mms diameter Petri dishes with 5% blood agar. Insemination of the plates was carried out by flooding them with the isolate suspended in distillated water. After 24 hours at 37°C, the cells were harvested with 5 ml of 0.85% NaCl per plate, recovering approximately 4 ml *B.*

bronchiseptica suspension from each plate. Suspended *B. bronchiseptica* cells were disintegrated with an ultrasound generator DUS P 150 (INCDFT Iaşi) at 21-22 Khz in 20 ml volume. The ultrasound disintegrator worked 90 minutes at a power level of 150 W. After the ultrasound treatment, the suspension was distributed in 5 ml volumes in 50/10 mm glass tubes and centrifuged at 6000 rpm for 30 minutes. Supernatants were collected and protein content of the antigen solutions was measured at the Clinical Laboratory of Veterinary Medicine Faculty, Cluj-Napoca. The protein content of the antigen solution was 2.3 mgs/ml. Antigen solution was diluted with 0.85% NaCl to a 2.0 mgs/ml protein concentration and 37% formalin was added to a final concentration of 0.5%. This antigen solution was kept at 4°C and was used for the immunization of the rats and also in the double diffusion test assays.

Infection of the rats: Six *B. bronchiseptica* free adolescent female Wistar rats were infected with the field isolate of *B. bronchiseptica* used for vaccine preparation. The rats were slightly anaesthetized with halothane. Using a monocanal automatic pipette a droplet of 15 µl of the *B. bronchiseptica* suspension containing $7x10^7$ CFU/ml was introduced in each nostril of the rats. Animals were monitored daily for signs of discomfort and respiratory distress.

Immunization of the rats and sample collection: Three rats were subcutaneously vaccinated in days 14 and 28 after infection with 0.2 ml of antigen solution. In day 60 after infection all rats were euthanized through atlo-occipital dislocation under ethylic ether anesthesia. Blood samples were collected during anesthesia from the retro orbital sinuses, lung and trachea portions were collected after euthanasia for bacteriological and serological tests. A 0.5 cm section of trachea and an apical pulmonary lobe were removed aseptically, triturated in 1 ml of sterile saline solutions. Twofold dilutions were made (1:2, 1:4 and 1:8) and 0.25 ml of these solutions were plated on Petri dishes containing MacConkey agar.

Test of the sera samples: Sera samples were tested using agar immune diffusion test and slow agglutination test.

Agar for immune diffusion was prepared according to the following formula: Medinal: 2.1g, Veronal: 0.37g, NaN_3: 0,02g, agar Noble 2.5g and distillated water 200 ml. The solution was heated in a water bath at 100°C until the solution became transparent. The solution was cooled to 60°C and distributed in Petri dishes of 50 mm in diameter (5 ml for a box). After the gel hardened, six peripherical equidistant wells and a central well with diameters of 5 mm were performed using a tube of glass provided with a pear.

To perform these immune diffusion tests, negative control and positive control sera were used. They were placed in two peripherical wells facing each other while sera obtained from the rats were placed in the remained peripherical wells using a 40 µl volume of sera. Antigen solution (the same solution used for vaccination) was placed in every central well (40 µl each). Petri dishes were incubated at 37°C for 48 hours in a thermostat, where humidity was kept high.

The antigen for the agglutination test was prepared by growing the field isolates of *B. bronchiseptica* on ten plates of 5% blood agar. Insemination of the plates was accomplished by flooding the plates with a suspension of the field isolate in distillated water. After 24 hours at 37°C, the cells were harvested with phosphate-buffered saline (PBS), pH 7,2. PBS contained two solutions: solution I was prepared with 0,907 g KH_2PO_4 + 100 ml distillate water and solution II was prepared with 1,187g Na_2HPO_4 dehydrate and distillated water up to 100 ml. 27.4 ml of solution I was mixed with 72.6 ml of solution II to obtain the final PBS solution. Prior cell harvesting from each plate rapid haemagglutination test was carried out successfully, making sure that *Bordetella bronchiseptica* cells are in phase I. Harvested organisms were killed with 1% formalin while being held at 4°C for 48 hours with occasional shaking. The preparation constituted the stock antigen for the test that was maintained under

refrigeration conditions. For preparation of antigen used in the test assay, stock antigen was added to PBS to a concentration of 60% transmission (%T) at 625 nm on a Spekol ZV Spectrofotometer.

Prior to testing, the undiluted sera were inactivated at 56°C for 30 minutes in a water bath. The sera were serially diluted in 50 µl volumes with PBS starting with a 1:5 dilution in micro titer plates with U type wells, where the first well for each sample contained 250 µl PBS and the following wells contained 150 µl PBS. Passing 150 µl diluted sera in the second wells, doubling dilution was performed. The antigen (150 µl) was added to every well and the plates were incubated in a thermostat for five hours at 55°C than the plates were kept 20 hours at 4°C.

To determine the agglutination endpoint, tests were read using a desk spot lamp placed under the plates at a distance of 30 cm, lighting up the bottom of the wells in a 45 degree angle. From above the bottom of the wells were examined with a magnifying glass and with a digital Canon camera set on Supermacro function. The agglutinin pattern was judged as positive when only definite granules of antigen were seen in a layer covering the bottom of the wells. The highest dilution of serum giving a positive reaction was taken as the titer (1, 2).

Bacteriological tests: After euthanasia the nasal cavities of the rats were washed with 10 µl of PBS to determine the presence or the absence of *B. bronchiseptica* at this anatomical site. PBS was recovered by aspiration and samples were inseminated on Petri dishes containing MacConkey agar. A 0.5 cm section of trachea and an apical pulmonary lobe were removed aseptically from every animal and triturated in 1 ml of sterile saline solutions. Twofold dilutions were made (1:2, 1:4 and 1:8) and 0.25 ml of these solutions were plated on Petri dishes containing MacConkey agar and incubated aerobically at 37°C for 48 hours. After incubation colonies of *B. bronchiseptica* were counted on every plate to determine the number of CFU for each trachea portion and each apical pulmonary lobe.

RESULTS AND DISCUTIONS

The immune diffusion test in unvaccinated rats did not revealed any immune response to *B. bronchiseptica* infection, while in vaccinated rats this test was positive for every animal. In the vaccinated group the immune diffusion assay showed a single precipitation line with antigenic community among these lines and the line obtained between the antigen and the positive control serum. It is obvious that these differences observed in the immune diffusion assays between vaccinated group and unvaccinated group are given by antibodies produced due to vaccine administration.

Regarding the slow agglutination test two of the unvaccinated rats hat an antibody titer of 1:5 and one of them had a higher titer (1:20). Vaccinated rats had higher antibody titers then the unvaccinated group, two of them had a titer of 1:40 and one rat had a titer of 1:80.

Bacteriological findings showed that all unvaccinated rats were *B. bronchiseptica* carriers, the bacteria was isolated in all cases both from the nasal cavities and the trachea, but not from the lung samples. In the vaccinated group only one rat was found to be *B. bronchiseptica* carrier. In this case the bacterium was isolated from the trachea of one of the rats with an antibody titer of 1:40.

Serological findings showed a clear systemic immune response after vaccine administration and bacteriological findings showed that immune response due to vaccine administration was a protective one.

CONCLUSIONS

- Double immune diffusion test and slow agglutination test showed a significant rise of antibody levels produced against *B. bronchiseptica* in infected and vaccinated rats group compared with the infected but unvaccinated rats group;
- Bacteriological findings showed that immune response due to vaccine administration was a protective one;

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