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HYPERIMMUNISATION OF RABBITS WITH ULTRASOUND DISINTEGRATED *BORDETELLA BRONCHISEPTICA* AND CONTROL OF ANTIBODY SYNTESIS TROUGH DOUBLE DIFFUSION AGAR GEL

Răpuntean Gh., Dénes A. L.

University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine 3-5 Mănăştur Street, 400372, Cluj-Napoca, România,
e-mail: attila25@email.ro

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Abstract. Two rabbits were hyperimmunisated with an ultrasound lysate of a pig origin *B. bronchiseptica* and one rabbit was hyperimmunisated with an ultrasound lysate of *B. bronchiseptica* dog strain origin. Each rabbit was inoculated three times. Blood samples were collected before the hyperimmunisation begun and after each inoculation. Antibody production during immunization was controlled using the method of double diffusion in agar gel. After the first inoculation only 2 of 3 rabbits responded with antibody production detectable with this method, but after the third inoculation all the rabbits responded with antibody production at least against three antigenic compounds of the ultrasound disintegrated *B.bronchiseptica*. Serum samples obtained from each rabbit after the third antigen inoculation were tested against both antigen solutions. These tests proved that all rabbits produced antibodies against the same three antigenic compounds of *B. bronchiseptica*.

INTRODUCTION

Immunogenic structures of *B. bronchiseptica* mainly consists 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.

Filamentous haemagglutinin (FHA) is a 220 kDa filamentous protein monomer. During *B. bronchiseptica* infection, FHA induces a powerful systemic and local immune response materialized in a massive antibody production directed against FHA. Fimbriae (FIM) are made of 21-24 kDa major fimbrial subunits (Fim2, Fim3 and FimA). Because of their repetitive polimer structure, fimbriae can stimulate IgM production in a T independent manner. Pertactin (PRN) is a 68 kDa protein expressed on the surface of the bacterial cell. These adhesins are highly immunogenic; therefore they are included in the composition of acelular *B. bronchiseptica* vaccines.

B. bronchiseptica main toxins are adenylate-cyclase toxin (Acy-Hly), the termolabile dermonecrotic toxin (DNT/HLT), tracheal cytotoxin (TCT) and lipopolysaccharides (LPS). Adenilate-cyclase toxin (Acy-Hly) is a bifunctional cytotoxin. It has adenylate-cyclase and hemolysin activity. During infection adenylate-cyclase is secreted and released from the bacterial cell. Dermonecrotic toxin (DNT/HLT) is a 160 kDa polipeptide found in the cytoplasm of the cells. It is released after the disintegration of the cells. Tracheal cytotoxin is a disaccharide-tetrapeptide monomer. It is a peptidoglycan component secreted during the

infection. Lipopolysaccharides (LPS) of *B. bronchiseptica* are termostabile and they are considered to be typical endotoxines.

MATERIAL AND METHOD

Preparation of the antigen. Two strains of *B. bronchiseptica,* one of pig origin and one of dog origin, were used for antigen preparation. Each strain was grown on ten 90 mm diameter Petri dishes with 5% blood agar. Insemination of the plates was carried out by flooding them with the isolates suspended in distillated water. After 24 hours at 37°C, the cells were harvested with 5 ml of physiological saline solution per plate, recovering approximately 4 ml *B. bronchiseptica* suspension from each plate. Isolates were disintegrated with an ultrasound generator DUS P 150 (INCDFT Iaşi) at 21-22 Khz in 40 ml volume for each isolate. After the sonication, suspensions were distributed in 5 ml volumes in 50/10 mm glass tubes and centrifuged at 6000 rpm for 15 minutes. Supernatants were collected and protein content of the antigen solution was measured. The protein content of the antigen solutions were 2.2 mg/ml (pig strain) and 2.1 mg/ml (dog strain). Antigen solutions were diluted with physiological saline solution to a 2.0 mg/ml protein concentration and 37% formalin was added to a final concentration of 0.5%. These antigen solutions were kept at 4°C and were used for the immunization of the rabbits and also in the double diffusion test assays.

The immunization of the rabbits. The rabbits were 8 months old at the time of the first inoculation of the antigen solutions. Two rabbits (rabbit number 1 and 2) were inoculated with the antigen solution obtained from the dog strain, and one rabbit (rabbit number 3) was inoculated with the antigen solution prepared from the pig strain. Each time the antigen solutions were injected subcutaneously in the upper toracal area. Three inoculations were performed on each rabbit.The first inoculation consisted of injecting 1 ml antigen solutions, followed in 14 days by the second inoculation of 2 ml antigen solution. Third inoculation was carried out with 3 ml antigen solution, 14 days after the second administration. Post-inoculation local or systemic reactions were not observed. Blood samples were taken from the marginal auricular vein in 3 ml volume from each rabbit, seven days before the first inoculation, seven days after the first inoculation, seven days after the second inoculation and 14 days after the third inoculation. Blood samples were processed in sterile conditions to obtain the sera used in the test assay.

Test of the sera samples. Immunodiffusion agar was prepared according to the following formula: NaCl: 0.8 g, NaH_2PO_4, $2H_2O$: 0,4 g, Na_2HPO_4, $12H_2O$: 2,7 g , 0,02g NaN_3, agar Noble 1,25g and distillated water 100 ml. The solution was heated in a water bath at 100°C until the solution become transparent. The solution was cooled to 60°C and distributed in Petri dishes of 50 mm in diameter (5 ml for each dish). After the gel hardened, four peripheral equidistant wells and a central well with diameters of 5 mm were cut using a tube of glass provided with a pear.

The sera collected from the rabbits included in the experiment were distributed in the following manner: well nr. 1 serum collected before the inoculation, and wells nr. 2, 3 and 4 serum samples collected after the antigen inoculation. To perform these tests, one Petri dish was used for each rabbit, a 50 µl volume of antigen solution was placed in each central well and the 4 sera samples (50 µl each) were placed in 4 consecutive peripheral wells (wells number 1, 2, 3 and 4), respecting the order of collection. Petri dishes were incubated at 37°C for 48 hours in a thermostat, where humidity was kept high.

Following individual tests serum samples obtained from each rabbit after the third antigen inoculation were tested against both antigen solutions. For these tests two Petri dishes

were used. Antigen solutions of 50 µl volumes were placed in the central wells and serum samples of 50 µl volumes were placed in three consecutive peripheral wells. Petri dishes were incubated at 37°C for 48 hours in a thermostat in a high humidity atmosphere.

RESULTS AND DISCUTIONS

Individual tests for observing antibody production during immunization had the same results in case of rabbit number 1 and rabbit number 2. No line of precipitation could be observed between the central wells and the wells containing the serum samples obtained from the blood samples collected before beginning of the immunization (wells nr. 1). A single thin line of precipitation was observed between the central well and the wells containing the serums obtained from blood samples collected after the first inoculation. These lines of precipitation were very close to the wells containing the serums, indicating an antigen excess. After a curve (reaction of identity) these lines of precipitation were present between the wells containing the serums obtained from blood samples collected after the second inoculation. Here the lines of precipitation become thicker and they were positioned half way between the wells. After an another curve (reaction of identity) these lines of precipitation become very thick, but they remained at half way between the central well and the wells containing the serum samples obtained from blood samples collected after the third inoculation. A second thin line of precipitation was observed in both tests between the central well and wells number 3, situated very close to these wells, indicating an antigen excess in their case. After a curve (reaction of identity) these lines of precipitation could be observed between the central well and the wells containing the serum samples obtained from blood samples collected after the third inoculation (well number 4). Here these lines of precipitation were thicker and they were at a greater distance from well number 4 than in case of well number 3. Between the central well and well number 4 a third thick line of precipitation appeared in both cases and they were situated closer to the central well (fig. 1).

In case of rabbit number 3 line of precipitation could not be observed between the center well and well number 1 and 2. Instead three lines of precipitation were present between the central well and the well containing the serum obtained from blood sample collected after the second inoculation (well number 3). The three lines were very close to each other. The middle line was thick, while the others were very thin. After a curve (reaction of identity), each line of precipitation was present between the central well and the well containing the serum sample obtained from blood sample collected after the third inoculation (well number 4). Here all the three lines of precipitation were thick, and the distance between them slightly increased (fig. 2).

When serum samples obtained from each rabbit after the third antigen inoculation were tested against the two antigen solution the obtained results were similar. In both case three identical lines were observed between the central well containing the antigen solution and the peripheral wells with the sera samples (fig. 3).

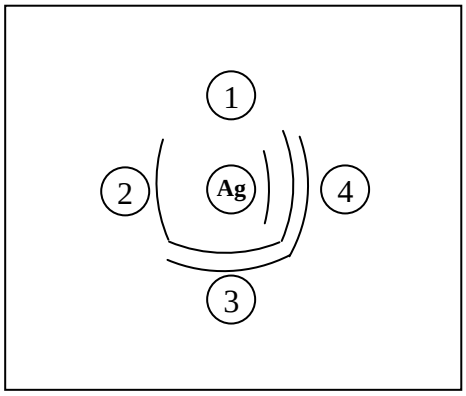

Fig. 1 - Immune response after antigen inoculation obtained from *B. bronchiseptica* of dog strain origin (serum rabbits 1 and 2).

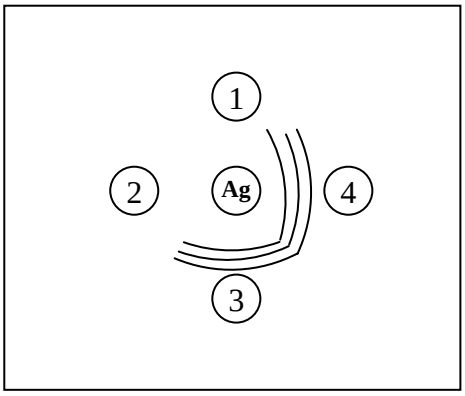

Fig. 2 - Immune response after antigen inoculation obtained from *B. bronchiseptica* of pig strain origin (serum rabbit 3).

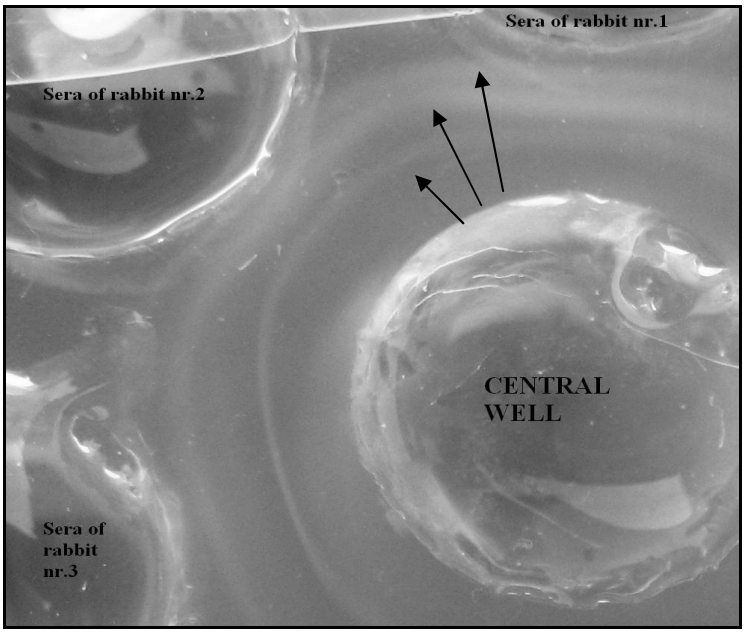

Fig. 3. The three lines of precipitation between the central well filled with the antigen *B. bronchiseptica* (dog origin) and the three peripheral wells filled with rabbit serum samples obtained after the third inoculation of the antigen solution.

CONCLUSIONS

After firs inoculation of the ultrasound disintegrated *B. bronchiseptica* only 2 of 3 rabbits responded with antibody production detectable with the method of double diffusion in agar gel.

After the third inoculation all the rabbits responded with antibody production at least against three antigenic compounds of the ultrasound disintegrated *B. bronchiseptica*.

Even if two rabbits were inoculated with an antigen solution prepared from a dog isolate of *B. bronchiseptica* and one rabbit was inoculated with an antigen solution prepared from a pig isolate of *B.bronchiseptica,* they produced antibodyes against the same three antigenic compound.

BIBLIOGRAPHY

1. MATTOO S., AMY K. FOREMAN-WYKERT, PEGGY A. COTTER, MILLER J.F., (2001), Mecanisms of *Bordetella* Pathogenesis: *Frontiers in bioscience*, 6, e168-e186;
2. MATOO S., CHERRY J.D., (2005), Molecular Pathogenesis, Epidemiology, and Clinical Manifestations of Respiratory Infections Due to *Bordetella pertussis* to Other *Bordetella* Subspecies: *Clinical Microbiology Reviews*, Vol. 18, No. 2 , p. 326-382;
3. ***http://www.alzforum.org/res/com/ant/glossary.asp