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Test of Cross Reaction Between the Two Local Sudanese Strains of *P. mulocida* (B and E)

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Abstract: The present study was carried out on the two local Sudanese *Pasteurella multocida* strain B and E which cause Hemorrhagic Septicemia (HS) disease in the Sudan and used for the production of the vaccine against this disease. This study was done to test the cross reaction between the two strains. The study showed that there was a cross reaction between the two strains in the rabbit sera when tested by ELISA. The active cross protection test in rabbits showed that vaccine of strain E protected 50% of the rabbits against challenge with strain B, while the vaccine of B could not protect the rabbits against the challenge with strain E 0%. Each vaccine of B and E could protect the rabbits 100% against the challenge with the same strain (homologously).

Key words: *Pasteurella multocida* (*P. multocida*), local Sudanese strains B and E, cross reaction, vaccine, rabbits, ELISA

INTRODUCTION

The gram-negative bacterium *Pasteurella multocida* (*P. multocida*) is the etiological agent of Hemorrhagic Septicemia (HS) in cattle, fowl cholera in birds and atrophic rhinitis in pigs (Mannheim, 1984). *P. multocida* can be separated into 16 serotypes based on the characteristics of their lipopolysaccharide (LPS) antigens (Heddleston *et al*., 1972). Also it can be separated into 5 serogroups based on the antigenicity of their capsule (A, B, D, E and F) (Carter, 1967; Rimler, 1987).

Many authors have demonstrate the cross reaction or cross protection between *Pasteurella* species. Previous investigations have reported cross reactivity between different strains of *P. multocida* as judged by Ouchterlony gel diffusion precipitation analysis (Prince and Smith, 1966a, b). Mukkur (1977) said that there is a cross protection between *P. multocida* type A and *P. haemolytica* serotype 1 because they possess common immunogenic antigens(s). Rimler (1996) reported that different serotypes of *P. multocida* associated with HS and FC of domesticated and wild animals might share antigens that are cross-protective. Ibrahim *et al.* (2000) reported that, *P. multocida* grown *in vivo* provided protection against heterologous serotype challenge. Furthermore, passive cross protection was produced with antiserum raised against *P. multocida* grown *in vivo*. Similar results were obtained by others (Heddleston and Rebers, 1974; Rimler *et al*., 1979; Rimler and Rhoades, 1981; Rimler, 1987).

According to above we suggested to study the cross reaction between the strains. So, the aims of this study were to test the cross reaction in vaccinated rabbit serum by ELISA then test the active cross protection in rabbits by vaccination and then challenge.

MATERIALS AND METHODS

Bacterial strains and growth conditions: The two strains of *P. multocida* (B and E) of this study were obtained from the Department of Bacterial Vaccines, CVRL. They had been isolated from different outbreaks of HS in Sudan (Shigidi and Mustafa, 1979). They were propagated and kept lyophilized at -20°C for the production of the vaccine against HS disease. One ampoule of each strain was aseptically opened and reconstituted in 5 ml Brain Heart Infusion Broth (BHIB) and incubated at 37°C overnight. Growth was checked and tested for purity by examination of wet smear and stained smear with Gram's stain.

Preparation of the seed: One milliliter of each culture was injected s/c in a rabbit. Immediately after the death of the rabbits, blood was collected aseptically from the heart and cultured in tubes containing 5 mL of BHIB and incubated for 24 h at 37°C. After check of purity the seeds were kept at 4°C till used.

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Preparation of the vaccine: The seed of each strain was cultured in 100 mL BHIB, incubated at 37°C for 24 h with gentle shaking (100 rpm). The viable bacteria count was determined by the standard total plate count method Colony Forming Unit (CFU) in Brain Heart Infusion Agar (BHIA). To kill the bacteria 0.5% formaldehyde (37%) was added to the cultures and incubated with gentle shaking at 37°C for 24 h, blood agar plates were cultured for sterility. The broth culture was centrifuged at 5000 rpm/10 min at room temperature to pellet down the bacterial cells. The pellet was washed with normal saline by centrifugation at 5000 rpm/10 min 3 times. For vaccine production the bacterial suspensions were estimated to have a minimum bacterial content of 1.5 g L^{-1} dry weight for volume (OIE 2009). $Al(OH)_3$ adjuvant was added (1 mg mL^{-1}). The sterility test of the final vaccine was done by inoculate a sample from the vaccine in BHIA.

Experiment 1: Test of cross reaction between the two strains: This experiment was done so as to the cross reaction between the two strains was tested by determination of antibody (Ab) levels in the serum samples. Every serum sample was subjected to react with antigens of B and E homologously and heterologously. Serum samples were subjected to indirect ELISA to determine the serum IgG levels against *P. multocida* strain B and E. This experiment was used as a guide for the second one.

Experimental animals: Four rabbits were used in this test. Rabbit 1 was vaccinated with strain B, rabbit 2 was vaccinated with strain E and rabbit 3 was vaccinated with both B and E and rabbit 4 as a negative control. One week post vaccination, serum samples were collected from the heart of each rabbit. The above mentioned vaccine was used in this test.

Preparation of antigen for ELISA: Stock culture of *P. multocida* strain B and E were used. The bacteria were cultured onto Blood Agar (BA) (10% sheep blood) and incubated at 37°C for 24 h. The same-sized colonies were selected and then inoculated into 100 mL BHIB and incubated at 37°C for 18 h., the bacterial Colony-Forming Unit (CFU) of the grown *P. multocida* strain B and E in the BHIB was estimated using standard total plate count method. The inoculums were washed 3 times with PBS to get rid of the BHIB. In between each washing the inoculums were centrifuged at 5000 rpm for 30 min. The pellets were then resuspended in carbonate-bicarbonate coating buffer (pH 9.6) and boiled in water bath at 97°C for 20 min. The suspension was cooled and dispersed into 1 mL vials and kept frozen at -20°C until used in ELISA.

Serology: Blood was collected from the heart. Blood was centrifuged (5000 rpm/5 min), serum harvested and frozen at -20°C until assayed by ELISA. The microtiter plates were coated duplicate with either *P. multocida* B or E to the final concentration of 10^{-8} suspension present in 100 μL per well in carbonate-bicarbonate buffer pH 9.6. The plates were dried overnight at 4°C. Serum samples were diluted at 1:800 then introduced into the wells and incubated for 1 h at 37°C. After washing with PBS-T20, anti-rabbit IgG-horseradish peroxidase conjugate (Whole molecule- Sigma), diluted at 1:8000, was added into each well and incubated for further 1 h. After washing, the substrate containing OPD (Sigma), citrate buffer and 30% hydrogen peroxide (Sigma) was added. 100 μL of this solution was added into each well and incubated 10 min at room temperature. Adding of sulphuric acid stopped the reaction and the plates were read at 492 nm (filter) using a Multiskan MS, labsystems, Finland.

Experiment 2: Determination of active cross protection: This experiment was designed to study the active cross protection between the two strains of *P. multocida* strain B and E. The experiment was conducted on rabbits.

Experimental animals: Twenty four clinically healthy locally breed rabbits weighting between 1.5-2 kg were used in this experiment. These rabbits were purchased from the local market of Kassala town. They have been kept and colonized at animal experimental house at Kassala Veterinary Research Regional Laboratory. They were fed cut grass supplemented with pellets and drinking water *ad labium*. The rabbits were divided randomly into 6 groups. Group I (5 rabbits) vaccinated with strain B and challenged with strain B. Group II (5 rabbits) vaccinated with strain E and challenged with strain E. Group III (4 rabbits) vaccinated with strain B and challenged with strain E. Group IV (4 rabbits) vaccinated with strain E and challenged with strain B. Group V and VI (each of 3 rabbits) were negative control groups for strain B and E respectively.

Vaccination and challenge: Rabbits were vaccinated s/c with 1 mL of the above mentioned vaccine. Unvaccinated rabbits were included to serve as controls in all the experiment. After two weeks rabbits were challenged s/c with a 0.5 mL of a 10^{-7} dilution of 18-20 h broth culture. The challenge dose representing approximately 100 mouse lethal dose (Thomas and Saroja, 1972). All rabbits were observed for a period up to 7 days following challenge and only those survived for 7 days after challenge were considered immune.

RESULTS

Test of cross reaction between the two strains in rabbits' sera: Table 1 shows cross reaction was detected in sera of rabbits 1, 2 and 3. The ODs is shown in Table 1. Bivalent vaccine revealed high AB levels in case of both Ags (2251 for B and 2689 for E).

Determination of active cross protection: In Table 2 the results of active protection were shown. From the table we noticed that the rabbits in group (1) which vaccinated with B were protected against the challenge with B and the same thing in case of group (2). Group (3) which vaccinated with B, all of them dead after challenged with E. The statistical analysis showed that the difference in survival was significant ($p = 0.003$) that mean that there was no protection. In group (4) which vaccinated with E two of four rabbits (50% protection) were survived. The statistical analysis showed that the difference in survival was not significant ($p = 0.073$) that mean E partially protect rabbits from B. All controls were dead post challenge.

Table 1: Determination of antibodies (Abs) level in the serum of rabbits vaccinated with monovalent and bivalent HS vaccine

	B	E
Rabbit (1) vaccinated with B	1596	775
Rabbit (2) vaccinated with E	638	699
Rabbit (3) vaccinated with B +E	2251	2689
Rabbit (4) non-vaccinated	432	404

Table 2: Results of active cross protection in rabbits between the two strains *P. multocida* (B and E)

Group No.	Vaccinated	Challenged	No. of rabbits	No. of Survived	Survival (%)
1	Strain B	Strain B	5	5	100
2	Strain E	Strain E	5	5	100
3	Strain B	Strain E	4	0	0
4	Strain E	Strain B	4	2	50
5	Control	Strain B	3	0	0
6	Control	Strain E	3	0	0

DISCUSSION

The compounding confusion between the serotyping systems and the inability to definitive type HS-causing strains has hindered both the understanding of the disease process and the determination of its true incidence. No virulence determinants of HS-causing isolates of *P. multocida* have been identified and characterized at the molecular level, although it has been suggested that heat-stable protein endotoxins, detectable in tissues and fluids by indirect serological tests, may be important in disease progression (Rebers *et al*., 1967; Rhoades *et al*., 1967).

Current *P. multocida* vaccines contain either inactivated bacteria (bacterins) or live attenuated bacteria (Derieux, 1984; Myint *et al*., 1987; Verma and Jaiswal 1998). Bacterins are inexpensive to produce but must be injected often that cause severe tissue reactions and gives very limited protection against heterologous serotypes (Davis, 1987; Rebers and Heddleston, 1977). Although the final criteria in evaluating HS vaccine is best carried out in cattle it would be advantageous if laboratory animals such as rabbits could be substituted for similar study as the later are cheap and readily available. Many authors have reported that rabbits could be substituted for cattle to test the potency of HS vaccine (Thomas and Saroja, 1972).

The cross reaction experiment (experiment 1) showed both strain B and E, cross react with each other (heterologous cross reaction).

The cross protection experiment (experiment 2) was designed and formulated based on the results of experiment 1. One hundred percent protections against homologous challenge in case of both vaccines B and E were observed. In heterologous challenge, it was found that the vaccine of strain B could not protect against E (0% survived), while the vaccine of E could partially protect against challenge with B (50% survived). These findings is agreed with the findings of Elbashir (1993) who found that there was a high protection degree against homologous challenge 83.3% for B and 91.3% for strain E, while high mortalities in heterologously challenged with strain E 08.3% and with strain B 00.0%. This experiment may be needs to be repeated with more numbers of animals.

As seen in Table 1 strain B eluted high Ab level to E more the reaction of E against B, accordingly it was suggested that the vaccine of B can protect against both B and E, but unexpectedly, rabbits that vaccinated with strain E partially protected when challenge with strain B, while those vaccinated with strain B were totally unprotected when subjected to strain E. However, the reaction *in vitro* conditions is different from those *in vivo* conditions. That means it is not always possible to establish a positive correlation between the antibody titer and protection in animals. Moreover, the binding of bacterial protein-polysaccharide complex was affected by formalin which used during vaccine preparation. The binding effect of formalin on the protein-polysaccharide complex is interesting. Formalin is used almost universally in the preparation of pasteurella vaccine and is believed in most laboratories to be superior to other bactericidal agents. This fixative effect may be the rationale behind a more or less traditional method (Bain, 1954).

Whole cell bacterins can provide some degree of protection, but only against the homologous LPS serotype. There is good evidence that cross-protective antigens are expressed only under *in vivo* conditions.

Empirically derived, live, attenuated vaccines can protect against heterologous serotypes, but because the basis for attenuation is undefined, reversion to virulence is not uncommon (Adler *et al*., 1999). Although in previous studies we found that strain B is more virulent than strain E (Sarah *et al*., 2007) Sarah Abusalab (unpublished data), but this study showed that strain E is more protective than strain B, that is because strain B failed to protect the rabbits from challenge with strain E, while the later induced partial protection against B challenge (50%).

In conclusion this study revealed that; although there was cross reactions between the two strains, but that was not enough to achieve active cross protection in rabbits. That means there is need for more studies to determine the immunogenic properties of the two strains. This study need to be repeated with more number of animals.

The most important finding in this study, that both vaccines B and E could protect the rabbits 100% against the challenge with the homologous strain. This means that the control of HS disease in Sudan can still be practiced with the conventional bivalent vaccine currently produced by Veterinary Research Institute VRL.

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