MUCOSAL VERSUS SYSTEMIC IMMUNITY AGAINST *P. MULTOCIDA* INFECTION IN RABBITS

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Abstract: In comparative protection against Pasteurella multocida infection in rabbits evaluated by compare the efficacy of mucosal immune response to systemic immune response. Rabbit groups were immunized intranasally and subcutaneously with a bacterin vaccine and cell lysate of P. multocida serotype B: 2. Mucosal protection in rabbit groups immunized intranasally with the bacterin and cell lysate was measured by the indirect haemagglutination testing of nasal lavages. Subcutaneous immunization of rabbits with the two vaccines induced protection in rabbits. The protection was demonstrated in serum samples collected from immunized animals and examined for antibody titer by the indirect haemagglutination test IHA. Protection of immunized rabbits was further proved by challenge inoculation of the animals with viable P. multocida. The survival rate in all subcutaneously vaccinated group was 100%. While the survival rate in intranasally immunized groups was 100%, and 75% with bacterin and cell lysate respectively. This study concluded that immunization of rabbits intranasally with P. multocida induces an effective protective immune response against P. multocida

Keywords: P. multocida; immunity; rabbits; vaccine; adjuvant.

I. INTRODUCTION

Pasteurella multocida is an important bacterial pathogen of domestic rabbits. Although infection may be subclinical, disease characterized by rhinitis, pneumonia, abscessation of viscera and subcutaneous sites, metritis, orchitis, septicemia, and otitis media may occur [3]. In most cases, the likely site of initial infection is the upper respiratory tract. Transmission occurs readily through direct contact of susceptible rabbits with carrier animals, and airborne transmission does not occur after exposure periods of three week [6]. Stress or such as crowding, transportation, and high ammonia concentrations in the air often stimulate latent *P. multocida* to proliferate and cause disease [7].

In attempts to protect rabbits from infection with *P. multocida*, a variety of vaccines have been examined, including those composed of inactivated whole bacteria [1], streptomycindependent live *P. multocida* [5], outer membrane proteins of the organism, [4], and *P. multocida* potassium thiocyanate extract (PTE) [10], by intramuscular, subcutaneous, or intranasal routes of administration.

Based on the previously mentioned facts, the present study was delineated (i) to investigate the effectiveness of vaccination in rabbits with (HS) bacterin, and cell lysate and to test the safety of *Nigella sativa* oil, pea-nut oil and freund´s incomplete adjuvant as mucosal adjuvant for delivery of vaccines preparations. (ii) to evaluate the efficacy of intranasal (I/N) versus subcutaneous (S/C) administration of the vaccine in stimulating protective immunity against *P. multocida*.

II.   MATERIALS AND METHODS

Bacteria

The freeze-dried isolate of *P. multocida* type B was obtained from the Department of Microbiology, faculty of Veterinary Science, University of Khartoum, isolated from outbreak of haemorrhagic septicaemia [14]. Freeze-dried strain was reconstituted in nutrient broth and incubated at 37°C for 24 hours; growth was checked and tested for purity.

Pathogenicity studies

The freeze-dried strain was reconstituted in nutrient broth of the *P. multocida* was subjected to pathogenicity finding. Two rabbits for isolate were inoculated intranasally with 100 ul of broth culture and observed for 48 hours. The dead rabbits were subjected to post mortem examination and heart blood, lung, spleen and liver were collected and reisolation of *P. multocida*. The smears prepared from heart, liver, spleen and lung from the succumbed rabbits were stained with leishman´s stain and examined microscopically.

Haemorrhagic septicaemia vaccine

The vaccine was obtained from the vaccine bank, which was developed at the bacterial vaccines production unit of the Central Veterinary Research Laboratories. They were all propagated in the Gottingen (fermenter) under the optimized physical growth conditions, temperature 37.5^{o}C, pH 7.4 and stirring 300 rpm, for vaccine production, as a rule, all production steps of vaccine were done under strict sterile condition, determinated by [8].

Cell lysate antigen

Bacteria *P. multocida* serotype B: 2, was grown in nutrient broth for 48 hours at 37°C. 100 ml culture were harvested by centrifugation at 3000 rpm for 15 minutes at 4°C and the packed cellular mass was washed three times with phosphate buffer saline pH 7.0. the cells were then resuspended in the same buffer and subjected to ultrasonic disruption (Sonicator) (MSE-England) 18000 rpm for 10 minutes at 4°C [11]. Cell debris and unbroken cells were removed by centrifugation at 3000 rpm for 15 minutes and the supernatant was stored frozen until use.

Mucosa adjuvant

The safety of *Nigella sativa* seed oil as mucosal adjuvants was initially tried in rabbits. Two rabbits were used and each received 100 and 250 ul intranasally and subcutaneously respectively of crude oil. Pea-nut oil mixed (I:1 v/v) with Freund´s incomplete adjuvant was experimented as safe mucosal adjuvant to deliver the vaccine.

Animal used

Twenty four rabbits which were used in this study were purchased from the local market. Animals housing of the Department of Microbiology of Faculty of Veterinary Medicine, University of Khartoum, Rabbits were not previously vaccinated against pasteurellosis, and allowed to adapt for 2 weeks. Food and water were supplied and later divided into 4 subgroups each of six rabbits.

Rabbits immunization

The protective efficiency of the bacterin vaccine and cell lysate of *P. multocida* were evaluated in rabbits. Briefly, twenty four 4 to 6 months old rabbits were randomly assigned into 4 groups. Before immunization, blood and nasal washes samples were collected to check the presence of anti *P. multocida* antibodies. Rabbits in groups 1 and 2 were immunized intranasally and subcutaneously with bacterin with dose of 100 ul of bacterin and pea-nut-incomplete Freund's adjuvant (1:1 v/v), while groups 3 and 4 were immunized intranasally and subcutaneously with a 100 ul of equal volume of cell lysate and mucosal adjuvant. This groups were followed by booster injection of two vaccines and mucosal adjuvant on day 24. Control group of two rabbits of four groups were received the normal saline via the same route.

Blood and nasal washing samples were collected on day 0, 7, 14 and 21 and day 31 after the booster inoculation.

Challenge inoculation of rabbits

After immunization, rabbits in all groups were challenged on day 35 post immunization by intranasal administration of dose of 2.5×10^6 CFU/ml. this dose was chosen based on results of a previous study [9].

Laboratory Assay

Antibodies against the *Pasteruella multocida* were measured by using indirect haemagglutination test (IHA) using human blood "O" (RBC's) [2] , but sheep red blood cells have been used according to [14].

Test Procedure

The test was carried out in microtitre plates of Flow Laboratories, each having 96 U shaped wells, arranged in 8 rows and 12 columns designated as A-H and 1-12, respectively. Two fold dilutions of the test sera starting from 1: 5 to 1: 640 were made in normal saline solution and added in 25μl amounts to all the wells of plate except those of column 11 and 12 which were maintained as controls. First four wells (A-D) of column 11 were added with known negative serum

and last four wells (E-H) with the known positive serum. All the wells of the column 12 were added with normal saline solution. Sensitized RBC's (1%) were added in equal amounts (25μl) to all the wells of the plate, so that column 12 served as control for the RBC's. The plates were incubated at room temperature for two hours and the observations were recorded. Thereafter the plates were kept under refrigeration for overnight shake lightly, allowed to resettle and read again. Results were interpreted as under:

Positive: No button formation, clumping occurring in an unordered and ragged pattern.

Negative: Button formation, RBC's clumping in an organized and regular pattern.

III.   RESULTS

Bacterial isolation and identification

On post mortem examination, severe congestion of trachea with accumulation of frothy fluid and congestion of lung with dark depressed areas were observed. On leishman´s staining, Heart blood smears, tissue smears prepared from liver, spleen and lung revealed characteristic bipolar organisms of *P. multocida.* The heart blood and tissue samples from rabbits were subjected to bacterial isolation. Grams staining of the smears revealed characteristic gram negative occobacillary organisms. These findings are in accordance with [12].

Pathogenicity studies

The isolates subjected to rabbits inoculation tests killed the rabbits in 24 – 48 hours. Similar results are obtained with the *P. multocida* isolates of avian origin [3].

Immune response of rabbits Bacterin

Nasal lavages and serum from immunized rabbits and controls were tested for local and systemic antibody by the indirect haemagglutination test. The highest titers were recorded in samples taken on day 21. The antibody titer increased after booster dose (Table1).

Cell lysate

The results of nasal washes and serum samples measured by indirect haemagglutination test for mucosal and systemic antibodies taken from vaccinated and control rabbits. High level of titers were reported in samples taken on day 21 and after boosting the animals. Results are shown in (Table 1).

Table 1. Average results of indirect haemagglutination test of four rabbit groups immunized with bacterin and cell lysate.

Vaccines route of inoculation	No. of Rabbits	Day 0	Day 7	Day 14	Day 21	day 31
Intranasal Bacterin subcutaneous	6	0	5	10	17.5	35
	6	0	5	17.5	50	100
Intranasal Cell lysate subcutaneous	6	0	0	7.5	15	30
	6	0	7.5	12.5	25	90
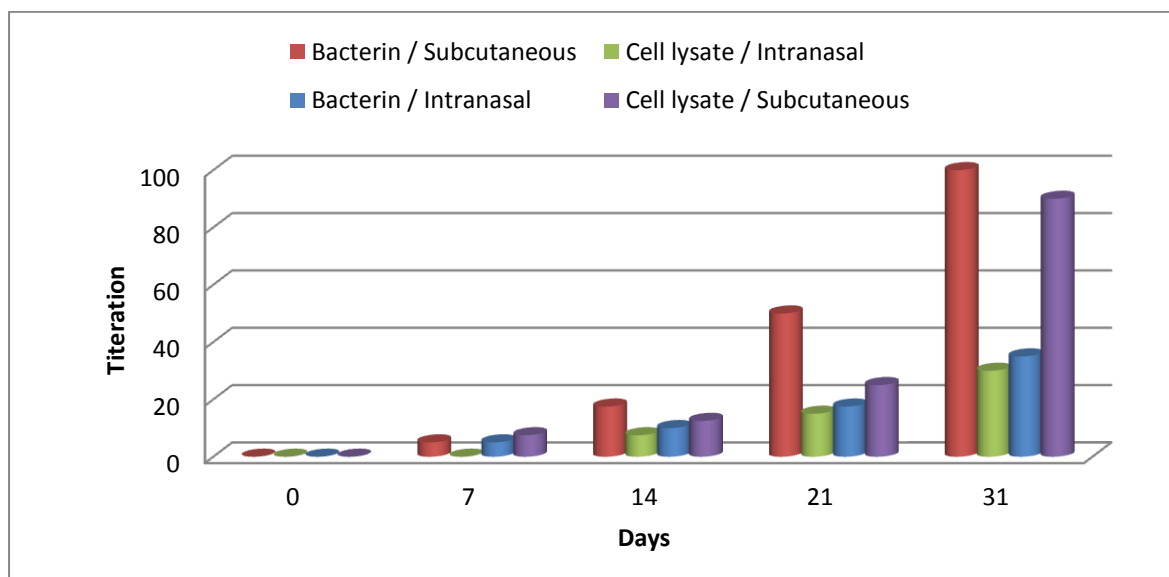
Figure 1. Average of results of laboratory assay of four rabbits groups immunized with the HS bacterin and cell lysate

Challenge exposure of vaccinated rabbits Bacterin

The survival rate of intranasally and subcutaneously bacterin immunized groups of rabbits was a 100% while the entire control group killed within 24 hours post challenge dose.

Cell lysate

100% and 75% were the survival rate of rabbits vaccinated subcutaneously and intranasally respectively with cell lysate. All animals in the control group succumbed after 24 hours post challenged.

IV.   DISCUSSION

In the current study, protective immunity developed in rabbits after either intranasal and subcutaneous vaccination with bacterin and cell lysate that contains a mixture of soluble cellular components, some of which are presumed to be of significance for bacterial virulence [10].

The present study was conducted to compare between efficacy of intranasal vaccination and subcutaneous inoculated in protecting rabbits against pneumonic pasteurellosis with haemorrhagic septicaemia vaccine and cell lysate preparation from *P. multocida* B:2.

Mucosal adjuvant is importance in the delivery of vaccines to mucosal surface, this adjuvant should be non irritant and have the minimal local side effect. *The Nigella sativa* oil used in this study as mucosal adjuvant was found toxic and lethal to rabbits when applied intranasally or subcutaneously. The exact mechanism of toxicity in rabbits remains to be clarified and no previous studies were reported in literature describing the toxicity of *N. sativa* oil in rabbits. The oil was selected and experimented as a mucosal adjuvant due to its immunopotentialing activity.

In this study Freund´s incomplete adjuvant and pea-nut oil (1:1 v/v) were also tried as a mucosal adjuvant in rabbits and no adverse reactions were reported, hence it was used in the present study to deliver the different vaccine preparations that included a bacterin and cell lysate each of which was used to vaccinate groups of rabbits via intranasal and subcutaneous route.

Immunity in vaccinated animals was measured indirectly by determination of the antibody titer using the indirect haemagglutination test and directly by challenge of vaccinated animal using viable *P. multocida.*

The bacterin vaccine was experimented in two groups of rabbits (6 rabbits each) and it was administered intranasally and subcutaneously, using pea-nut freund's incomplete adjuvant in both routes. The indirect haemagglutination titers were greater in the subcutaneously inoculated group, when compared with the titer of intranasally inoculated group. The two vaccinated groups showed 100% survival rate when challenged with 2.5×10^6 CFU/ml of *P. multocida*. The results of bacterin vaccination in rabbits of this study was in agreement with that reported by [8] who experimented the immunogenic quality of HS bacterin vaccine in group of rabbits administered through the subcutaneous route.

The cell lysate vaccine was inoculated in two groups of rabbits (6 rabbits each) and it was administered with the mucosal adjuvant via intranasally and subcutaneously routes. The antibody titer of the cell lysate vaccinated groups was higher in the subcutaneously inoculated group after compared with the intranasally inoculated group. The survival rate of the two groups following challenge with 2.5×10^6 CFU/ml was 100% for subcutaneous vaccinated group and 75% for intranasal vaccinated group. Results of this study clearly demonstrated that cell lysate vaccine of *P. multocida* administered subcutaneous into rabbits were more efficient in eliciting antibody immune response and more protective against challenge exposure when compared to the same vaccine inoculated via the intranasal route.

The finding of the bacterin vaccine gave a better protection when injected subcutaneously is in consistency with work of [8], who used a bacterin vaccine produced in a continuous cultivation system. Her work concluded that vaccines produced in fermenter were antigenically superior to static culture vaccines.

In this study mucosal immunity to *P. multocida* resulted by vaccination with a bacterin was in general less protective than subcutaneous route but bearing in mind that we used adjuvant might necessitate the

experimentation of other mucosal adjuvant which might confer higher protection than the pea-nut

freund´s incomplete adjuvant used in the present study. Even though intranasal immunization with bacterin is not an effective way to control infection, the method of vaccine delivery is not necessarily practical, especially when vaccinating a large number of animals. However, the efficacy of mucosal vaccination suggests that, it may eventually by possible to deliver vaccine by alternative routes, such as orally, to induce mucosal immunity in respiratory tracts.

V. CONCLUSION

1-The study demonstrated intranasal immunization versus subcutaneous immunization in rabbits. 2- The immunity done by inoculated the animals with the HS bacterin and cell lysate using the pea-nut-freund´s incomplete adjuvant, to induced protective immunity against *P. multocida* infection. 3- Immunity in animals done by both two routes of administrations was measured by determination of the antibody titers using indirect haemagglutination test and direct challenge of vaccinated animals using viable P. multocida. 4- The high level of antibody titers were reported in groups that received the HS bacterin. 5- The highest level of antibody titers against *P. multocida* were obtained after booster dose.

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