

Passive Immunization of *Pasteurella multocida* Infected Rabbits

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Abstract.- We raised neutralizing antibodies against *Pasteurella multocida* (PM) in the serum of rabbits and used these antibodies as immunotherapy in PM-infected rabbits. For this purpose five rabbits were vaccinated three times at 21 days interval, using formalized oil base haemorrhagic septicaemia vaccine (OBHSV). A high titer (256 IHA units) of anti-*Pasteurella multocida*-antibodies in blood serum was determined on 56th day post boosting using indirect haemagglutination (IHA) test. This antibody titer was further enhanced to 1024 IHA units with three consecutive inoculations of virulent PM suspension at 21 days interval. A virulent PM with mean lethal dose (LD₅₀) of 10^{-6.749} was used for challenging the rabbits. The hyperimmune serum (1 ml, 256 IHA units) injected intravenously and intramuscularly to PM-challenged rabbits showed 100% protection and 66.66% protection, respectively, while antigen control group showed 0% protection. Moreover, it was found suitable to passively immunize the animals before exposure or simultaneously with the exposure of PM. Taken together, our experimental data shows that the hyperimmune serum can therapeutically be used as passive vaccine to prevent infections against PM.

Key words : Haemorrhagic septicaemia, haemorrhagic septicemia oil based vaccine, hyperimmune sera, indirect haemagglutination test, passive immunization.

INTRODUCTION

Haemorrhagic septicaemia (HS) is a septicaemia pasteurellosis of cattle and other ruminants and occurs predominantly in Asia and Africa. HS is placed amongst the most formidable bacterial diseases of cattle and buffaloes (Afzal and Muneer, 1988), responsible for gigantic economic losses, worth more than Rs 1.887 billion per annum (Chaudhry and Khan, 1978). All the healthy ruminants when exposed to HS-infected animals become sick especially before the rainy season. The clinical signs in large ruminants include swelling of neck, frothing, high rise in temperature and difficult breathing. The animal dies within 24-48 h post infection. To prevent the animals with risk of exposure to Pasteurellosis, the animals should be vaccinated to prevent any disease symptoms. There are two ways of doing prophylaxis against HS, *i.e.* active immunization done by vaccination, or passive immunization achieved by transfer of antibodies, which is further typed into natural arising from

maternal antibodies and artificial induced by inoculation of hyperimmune serum.

Passive immunization against HS could be achieved by using a hyper immune serum to provide immediate protection to animals that have been exposed to infectious organisms and are suspected to lack active immunity against that organism. Passive immunization is routinely used for protecting individuals exposed to botulism, tetanus, diphtheria, hepatitis, measles and rabies (Kuby, 1994), foot and mouth disease (FMD) and snakebite (Radostitis *et al.*, 1994).

In Pakistan, the majority of the large ruminants are vaccinated against HS by using an alum-precipitated vaccine. Poor antigenicity and improper storage of the vaccine, and immunosuppressing biological agents are incriminated to be the cause of the failure of immunoprophylaxis in these vaccinated animals (Ajmal *et al.*, 1985). Therefore, in spite of vaccination against HS, outbreaks of the disease in animals are not uncommon.

In the present study we achieved to produce a hyperimmune serum against *Pasteurella multocida* (PM) in rabbits and evaluated the efficacy of this hyper immune serum in PM-infected rabbits. We also optimized the effective time period for passive

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immunization against HS.

MATERIALS AND METHODS

Bacterial culture

For this study, a PM sample was obtained from AVICENA lab, Sheikhpura Road, Lahore, Pakistan. The bacteria were reconfirmed on the basis of morphology, cultural characteristics and biochemical reactions as described earlier (Sarma and Boro, 1980). The isolate was activated through its passage on blood agar prior to its pathogenicity test. The fresh cultures on blood agar were transferred to tryptose soya broth and incubated at 37°C, under aerobic conditions for 18 h.

LD₅₀ of PM

LD₅₀ of PM was calculated using the technique described by Reed and Muench (1938) using laboratory rabbits. The PM isolate was examined for its pathogenicity in rabbits.

Experimental animals

The rabbits were kept in the animal house of University of Veterinary and Animal Sciences, Lahore, providing standard conditions, and all experimental approaches were according to ethics as recommended by Canadian Council on animal care.

Pathogenic assessment of PM isolate

For pathogenicity one ml infectious dose per rabbit (n=5) was inoculated intraperitoneally keeping uninoculated as control (n=3). Postmortem examination of the dead animals, along with re-isolation of the pathogen from their heart blood was also carried out. Stained smears of heart, lungs, liver and blood were examined to confirm the presence of pathogen in biological tissues (Buxton and Fraser, 1977).

Bacterial growing conditions, vaccine formulation and raising of hyperimmune sera

In order to obtain mass culture of PM, the bacteria was cultured in the medium containing tryptose (20.0 g), glucose (1.0 g), sodium chloride (5.0 g), thiaminium dichloride (0.005 g) and distilled water (1000 ml). The pH was adjusted to 7.3 (Anonymous, 1992). The culture of the bacteria

was inactivated by addition of formalin at the final concentration of 0.12 percent and then this culture was incubated at 37°C for 24 h. The sterility and safety test of this inactivated culture was performed (Anonymous, 2004).

In order to produce oil adjuvant vaccine, one part of formalin inactivated dense culture was mixed with four parts of oil base (four ml of Span-80, 95 ml of liquid paraffin and one ml Tween-80). The mixture was blended for five minutes to have a homogeneous suspension. The vaccine was checked for its safety and sterility (Anonymous, 2004). To produce hyperimmune sera, the oil-based vaccine was injected in five rabbits on day zero (1 ml intramuscular injection at thigh muscular region). On the 21st day post priming, the rabbits were boosted with the same dose and by the same vaccination route. The rabbits were re-boosted with the same vaccine, dose and route on 21st day post boosting.

To further increase the level of antibodies and to check the existing antibodies in rabbits against PM vaccine, the rabbits were challenged with 0.1 ml virulent PM suspension intramuscularly on 21st day post re-boosting. The challenge dose was prepared by diluting one colony of 18 hours old culture of PM on tryptose-blood agar in 10 ml sterilized normal saline (0.85 % NaCl). On 21st day post challenge, the rabbits were again injected with the same route, same dose and same antigen. On 21st day post second challenge, the rabbits were challenged again by same route, same antigen but with a dose of 0.5 ml. On 14th day post 3rd challenge, blood was collected and sera were separated.

The indirect haemagglutination titer of antibodies in the blood serum against PM was determined as per standard protocol of Anonymous (2004). The data regarding IHA antibody titer was processed for calculation of GMT for comparative efficacy of the vaccine (Villegas and Purchase, 1980). To check the neutralizing titer (effective dilution) of antibodies, different dilutions of serum were injected in rabbits and then these rabbits were challenged with virulent PM. For this purpose 36 rabbits were divided into six groups A, B, C, D, E, and F (n=6/group). Rabbits of group A, B, C and D were inoculated with 1ml of 64, 128, 256 and 512

IHA units of hyper immune serum respectively through intravenous route. The rabbits of group E (serum control) were inoculated with 1ml of undiluted serum (1024 IHA units) intravenously. The rabbits of group F (antigen control) were inoculated with one ml of normal saline intravenously. Following immunotherapy, the rabbits of group A to F were challenged with suspension containing virulent PM having $LD_{50}10^{-6.6}$ (0.1ml intramuscularly). Indicators of Pasturellosis already mentioned (Buxton and Fraser, 1977; Leslie *et al.*, 1998) were used for observation, confirmation and response to our treatment.

The effective time through intravenous route and intra muscular route for passive immunization against PM were determined as explained later.

RESULTS

To produce hyperimmune sera we boosted experimental rabbits (n=5) with oil based formalized vaccine on day 21st of priming and reboosted on day 21st of the boosting day. On day 21st of the re-boosting, IHA GMT titer against PM was calculated as 256 units. After re-boosting we further enhanced the antibody titer (IHA GMT) to 1024 when challenged with 0.1 ml virulent PM suspension intramuscularly three times with 21 days interval.

LD_{50} of virulent PM was determined as 0.1 ml intramuscularly with $LD_{50}10^{-6.749}$ (Data not shown). The antibody titer of hyperimmune serum was calculated by using an IHA test. Hyperimmune sera should ideally neutralize the virulent effect of the specific pathogen providing that sufficient amount of antibodies are circulating in the body. We observed that one ml of undiluted hyperimmune serum (with 1024 units against PM inoculated to PM-infected rabbits offered 100% protection. Next, we determined the amount of antibody necessary to neutralize virulent PM infection in rabbits. For this purpose we injected different dilutions of hyperimmune sera of one ml to each group of the rabbits intravenously. Hyperimmune sera with 512 and 256 IHA units gave desired 100% protection (Table I).

To know the effective time for the intravenous administration route to establish passive

immunization of the PM-infected rabbits, the rabbits were randomly divided into four groups G-1, G-2, G-3 and G-4 having six rabbits in each group. Each rabbit of G-1 was inoculated with one ml of the effective dilution (as calculated in the previous experiment, 512 IHA units) of the serum intravenously and were challenged with 0.1ml of virulent PM ($LD_{50}10^{-6.749}$) intramuscularly after 15 min of serum inoculation. Each rabbit of G-2 was injected intravenously with one ml of effective dilution of serum simultaneously with the inoculation of virulent bacterial dose ($LD_{50}10^{-6.749}$) intramuscularly. Each rabbit of group G-3 was injected intramuscular with 1ml of the virulent bacteria and rabbits were examined for 6 h approximately to get rise 1 ° F in body temperature, after which these sick rabbits were passively immunized with the effective dilution of the serum intravenously. Group G-4 was inoculated intramuscular with 0.1 ml of the virulent PM and 1 ml normal saline was inoculated as negative control intravenously. The efficacy of the hyperimmune sera through intravenous route was 100% in G-1 and G-2 while 0% in G-3 and G-4, respectively (Table II).

Table I- Determination of neutralizing titer of hyper immune sera against *Pasteurella multocida* challenge.

Groups	Anti-PM IHA antibodies ^a (units)	No. of infected ^b rabbits	Recovery ^c (%)
D	512	6	6(100)
C	256	6	6(100)
B	128	6	4(66.66)
A	64	6	2(33.33)
E	Serum control undiluted serum (1024 IHA units)	6	6(100)
F	Antigen control serum with 0 IHA titre	6	0(0)

a, 1ml of hyper immune serum was injected intravenously to each rabbit; b, Each rabbit was injected intramuscularly with 0.1ml suspension of PM $LD_{50}10^{-6.749}$; c, percentage recovery

To determine the effective time for intramuscular route to establish passive immunization of the PM-infected animals, the rabbits were randomly

Table II.- Effective time for passive immunization of *Pasteurella multocida* infected rabbits through intravenous and intramuscular routes.

Groups	Total no. of rabbits	Rabbits recovered	Protection ^a (%)
Intravenous routes			
G1	6	6	100
G2	6	6	100
G3	6	0	0
G4	6	0	0
Intramuscular routes			
G1	6	6	100
G2	6	4	66.66
G3	6	0	0
G4	6	0	0

^aPercentage protection

G1, Rabbits were immunized intravenously with the hyper immune serum (1ml of 256-anti PM IHA units) 15 min before challenge infection with 0.1ml of virulent PM intramuscularly; G2, Rabbits were passively immunized intravenously (1ml of 256-anti PM IHA units) and challenged intramuscularly with 0.1ml of virulent PM simultaneously; G3, Rabbits were challenged intramuscularly with 0.1ml of virulent PM and after rise of body temperature to 1°F, were immunized with 256-anti PM IHA units intravenously; G4, Rabbits were challenged intramuscularly with 0.1ml of 0.1ml of virulent PM but were not immunized.

divided into four groups G-1 to G-4 having six rabbits in each group. Each rabbit of G-1 was inoculated with 1ml of the effective dilution of the serum intramuscular and was challenged with 0.1ml of virulent PM ($LD_{50}10^{-6.749}$) intramuscularly after 15 minutes of serum inoculation. Each rabbit of G-2 was injected intramuscular with 1 ml of effective dilution of serum simultaneously with the inoculation of virulent bacterial dose ($LD_{50}10^{-6.749}$) through intramuscular route. Each rabbit of group G-3 was injected intramuscular with 1ml of the virulent bacteria and rabbits were examined for rise in body temperature to 1°F (10 h approximately), after which these sick rabbits were passively immunized with the effective dilution (256IHA units) of the serum intramuscular. Group G-4 was inoculated intramuscular with 0.1ml of the virulent PM and 1ml normal saline was inoculated simultaneously as negative control intramuscularly. The efficacy of the hyper immune sera through intramuscular route was 100% in G-1, 66.6% in G-2 and 0% in G-3 and G-4, respectively (Table II). The

result shows that the efficacy of hyperimmune sera for passive immunization through intramuscular route is maximum (100%) in G-1, while minimum in G-3 and G-4 (Table II)

DISCUSSION

In this study, specific PM-induced hyperimmune serum was applied for therapy of PM infected rabbits. We observed 100% protection in experimental rabbits by inoculating undiluted hyperimmune sera in experimental rabbits while its protection level was declined with serial dilution. Rabbits inoculated with 1ml normal saline 0.85% (negative control groups) when infected with virulent PM offered no protection against challenge and positive control group (inoculated with serum only, 1024 IHA units) indicated no clinical signs and symptoms. The results of the present study are congruent with those of Tang *et al.* (1995), Yushen *et al.* (1997) and Schwarz *et al.* (1990). In the experiments of Tang *et al.* (1995) hens were hyperimmunized with 3 injections of an attenuated strain of avian viral arthritis (AVA) virus. At 20-50 days after the last injection, these authors observed a good correlation between the serum and egg yolk antibodies titers as determined in an ELISA. Hyperimmune egg yolk given intramuscularly to five day old broiler chicks protected them against challenge with virulent virus. They also observed that it also had a curative effect when given at the onset of clinical signs. Yushen *et al.* (1997) used yolk IgG and hyperimmunized yolk in their two experiments for the prevention and treatment of IBD in chickens. Both the IgG of yolk and the hyperimmunized yolk were effective for controlling of IBD. Schwarz *et al.* (1990) showed that IVIG (pooled human immunoglobulin for intravenous injection) was an excellent source of parvovirus B19 specific IgG. IVIG had been used successfully in a 24-week-old hydropic fetus by giving intravenous treatment to the mother. Fetal ascites and pericardial effusion both were successfully resolved.

Use of one ml of 256 IHA units of hyper immune serum through intravenous route gave better protection to rabbits infected with PM compared to the use of one ml of 256 IHA units of hyper immune serum injected intramuscularly. This

might be due to rapid multiplication of bacteria at the site of inoculation and delayed absorption of serum. When serum is injected intravenously, it might control the growth of bacteria by neutralizing them. Most probably the preformed serum in the blood is readily available to cover specifically the antigenic surface elements of bacterial body, thus would block its attachment to cell receptor on the host cells and would diminish its spread. In case of intramuscular inoculation of serum, antibodies could take time to reach to circulation or receptor organs and bind to bacterial epitopes. Thus intravenous inoculation of serum provides more and rapid protection as compared to intramuscular inoculation of serum.

For passive immunization schemes, the timing of the inoculation of serum is foremost important as animals without antibodies against a particular pathogen can be easily colonized during the risk time and hence easily infected, relatively to the animals having already enough specific antibodies in the blood circulation before exposure. The bioavailability of the serum in circulation to cope with incoming antigen is an important factor to consider. Rabbits inoculated with raised hyperimmune serum before or at the time of infection were safe and could resist infection probably because of the enough level of neutralizing antibodies in their circulation which, could prevent bacterial growth to enough numbers to establish infection or block the attachment of invading bacteria to the receptors on primary organs and thereby prevented them from colonization or prevented the growth of bacteria to sufficient numbers to establish infection. To determine the ideal time for effective immunotherapy, one ml of effective dilution (256 IHA units) of hyperimmune sera was inoculated to treatment groups by intravenous and intramuscular routes, while control group was inoculated only with the challenged antigen. It was observed that rabbits inoculated intravenously as well intramuscularly with hyperimmune serum (256 IHA units) 15 minutes before the inoculation of challenged bacteria, provided 100% protection. While the rabbits inoculated with hyperimmune serum intravenously and intramuscularly and challenge simultaneously showed 100% and 66.66% recovery respectively

and the group inoculated with hyperimmune serum at the rise of 1°F body temperature by either route showed 0% recovery. Whereas the entire rabbits in the control group died. The results of present study are in line with those of Anonymous (1985) and Anonymous (1997) who have reported that immunoglobulin (Ig) efficacy varied with the severity of exposure and the delay in administering Ig. Our results showed that hyperimmune serum against PM can be used to control the disease in rabbits at risk and it is better to immunize them as soon as possible before exposure of pasteurellosis. In our experiments, we used rabbit as a model ruminant for passive immunization against pasteurellosis under experimental conditions. Altogether we can conclude that our raised hyperimmune serum might be a promising candidate to be used as a prophylactic measure against PM in ruminants during the infection season *i.e.* the rainy season or to protect healthy ruminants when there is an outbreak of PM in an area, thus it could be used as passive vaccine to prevent infection. Further experimental studies are proposed to determine the longevity of the serum effectiveness in rabbits before and after it has been infected, the duration of the protective effect of hyperimmune serum, and the protection of healthy animals inoculated with hyper immune serum against PM when housed with PM infected animals under experimental and field conditions.

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