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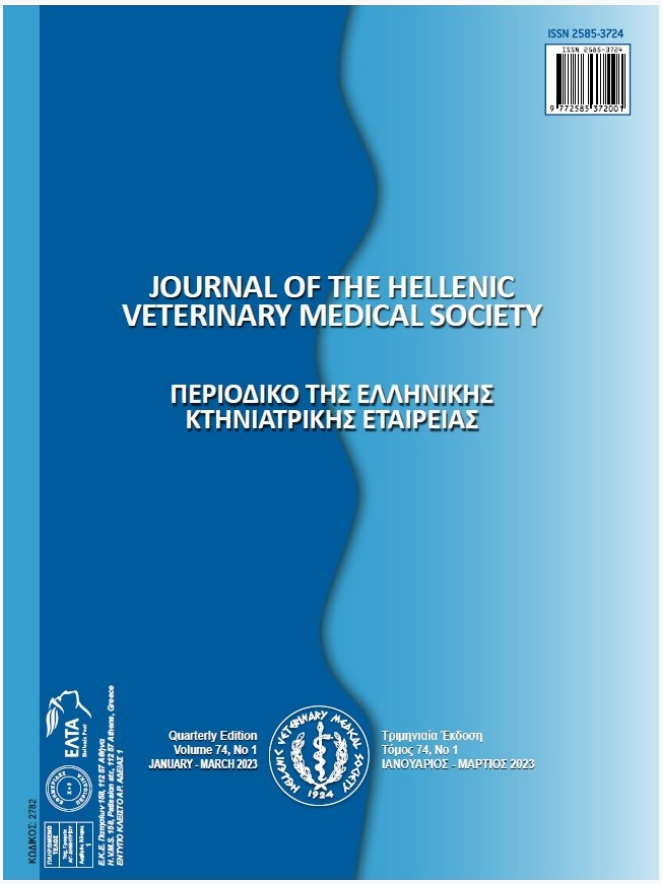

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Research article
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The pathogenicity and immunogenicity of *Pasteurella multocida* in low and rich iron media

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ABSTRACT: Pasteurella multocidahas the highest prevalence among animal populations and may cause hemorrhagicsepticemia in cattle and pneumonia in sheep and goats. It is known that the pathogenicity of this organism depends on the levels of iron.Present study attempts to evaluate any changes in blood parameters and visceral organs of mice by cultivation of P.multocida under iron sufficient and insufficient conditions. P.multocida was cultured on three media: BHI alone, supplemented with bipyridyl andiron. Bacteriawere injected into the mice by intra-peritoneal rout. At the same time inactivated antigens were prepared from all media, mice were immunized twoweeks apart andblood samples were collected until week 9. Visceral organs were collected from dead mice for pathological studies. In contrast to immunogenicity and antibody titers, there are significant differences in pathogenicity and blood parameters in rich and limited iron conditions.

***Keywords*:** *Pasteurellamultocida,* Iron, pathogenicity, Immunogenicity.

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INTRODUCTION

Pasteurella multocida(*P.multocida)* is highly prevalent among animal populations and found as part of the normal microbiota of the oral, nasopharyngeal, and upper respiratory tracts. This organism is the main cause of a variety of economically significant diseases including fowl cholera in chickens and turkeys, hemorrhagic septicemia in cattle and buffalo, pneumonia in sheep and goats, atrophic rhinitis in swine, and snuffles in rabbits (Morishita et al., 1996; Rimler et al., 1989).*P.multocida* strains can express a specific polysaccharide capsule on their surfaceand their isolatescan be differentiated by capsular antigens into serogroups A, B, D, E and F (Rimler et al., 1987). The outer membrane proteins (OMP), lipopolysaccharides (LPS), toxins (PMT) and iron uptake proteins play a significant role in virulence of *P. multocida* by facilitating invasion of organism (Markey et al., 2013).*P.multocida* strains can produce a surface antigen known as iron regulated outer membrane protein (IROMP) with high molecular weight in iron-restricted medium. IROMPs are considered as important factors of bacterial virulence and immunization and may have more immunogenicity than OMPsas the bacteria require them for scavenging iron from their host(Choi-Kim et al., 1991; Glisson et al., 1993). Numerous studieshave shownthat IROMPs are immunogenic against homologous and heterologous challenge in mice, bird, rabbit and calves (Chawak et al., 2000; Prado et al., 2005). In addition, the antibodies against IROMPs block receptors and inhibit bacteria from iron uptake (Ruffolo et al., 1998).Anyway, few studies have been conducted on the host blood factors that change during invasion and infection by the *P.multocida*. Monitoring of the host blood factors such as minerals and hormones is the useful method for the investigation of the pathogenesis of cultured *P. multocida* in rich or low iron media. In present study, we report the significant changes in blood factors and pathology of mice experimentally infected with *P.multocida* cultured in sufficient and restricted iron media to assess the role of iron in immunity against pasteurellosis.

METHOD AND MATERIALS

Animals

62 female Balb/C mice (weight 18-22 g) were received from the Animal Resource Center of Razi Vaccine and Serum Research Institute, Shiraz, Iran. After the acclimatization period, animals were randomly divided into nine groups, four groups for infection (3 mice per each group) and five groups for immunization (10 mice per each group).They were provided with sterilized food and water ad libitum. Animal care and experimental procedures were carried out according to the recommendation of the National Counsel of Animal Experimentation Control (table 1).

Bacterial strain and growth conditions

P. multocida serotype A (Genbank accession number: JF694003) used in the study was obtained from the Division of Bacteriology, RVSRI, Shiraz, Iran. It was cultured ontwo different BHI (brain heart infusion) broths (Merck).One containing iron-chelating agent 2,2ˈ- bipyridyl (20 mg/ml BP) to reduce the concentration of iron, and another supplemented by $FeCl_3$ as rich iron media, at 37°C overnight. To determine the concentration of bipyridyl and$FeCl_3$, the growth curvesdifferent concentrations were examined (100 ml BHI containing0.075, 0.0375, 0.0187, and 0.0937 mM bipyridyl and also 2.5, 0.5, 7.5 and 10 mM $FeCl_3$). As best conditions for the batch cultures 0.075 mM finalconcentration of bipyridyl and 10 mM $FeCl_3$were selected.

Preparation of bacterial inoculum

Bacterial colonies were culturedin BHI broth, BHI+$FeCl_3$, and BHI+bipyridyl and were incubated for 24 hours in shaking incubator at 37°C. Bacterial antigen was prepared by centrifugation at 10000 rpm for 10 min at 4°C and sediments washed twice. The cells were re-suspended in PBS. McFarlandStandards have been used to standardize the approximate number of bacteria and the bacteria were prepared at 9×10^9 CFU/ml.

Experimental infection and immunization

As mentioned above, four groups of mice were selected for the study of infection. The first group of mice was inoculated with *P. multocida*alonethe second group with the same dose of bacteria plus bipyridyl, thethird group was inoculated with a combination of bacteria and$FeCl_3$and the fourth group considered as control and injected by sterile PBS.Different four groups of mice (Table 1) were inoculated by intra peritoneal route at the dose of 500 µl of each groups.Blood samples from 12 mice were collected for hematological assay and their spleen, liver, heart, kidney and lungs were taken out for pathological assay.

The immunization study was done on 5 groups of mice. Positive control group was inoculated by bac-

teria adjuvanted with Frond's.The second group received the bacteria adjuvanted with aluminum potassium hydroxide (Alum).The third and fourth groups were receivedbacteria supplemented with bipyridyl and bacteria supplementedwith $FeCl_3$respectively, adjuvanted with Alum. The fifth group received PBS to serve as control. All mice were received 200 µl of inoculum by subcutaneous route on day 0 and then 14. Before immunization, the bacteria were inactivated with formaldehyde (0.3%).

Serum analysis

All five groups of mice (Table 1) were inoculated subcutaneously twice,on days 0 and 14. Nine weeks post inoculation, blood samples were collectedfrom the facial vein of mice andwere kept in a sterile micro centrifuge tube without anticoagulant for serum separation. After centrifugation at 3000 rpm for 10 minutes,the serum supernatant was collected carefully. Serum samples were stored frozen at -20 ºC until used.

Antibodies against *P. multocida* were determined by indirect immunoassay ELISA test (Enzyme-Linked Immunosorbent Assay) using 96-well ELISA micro plates as described previously (Roier et al., 2012).

Blood analysis

Hematological analysis was performed using CBC test with sysmex instrument and Man Company Solutions according to manufacture instructions. The plasma was separated and total proteinsand liver enzymes were analyzed with Pars Azmoon Kit (Pars Azmoon Co.-IRAN).

Pathological analysis

Samples from spleen, liver, heart, kidney and lungs were taken and send to the laboratory for preparation of section and pathology slide.

Statistical analysis

All data were analyzed using SPSS software version 16. The data were considered significant at $P < 0.05$.

RESULTS

Hematology and blood chemistry

The results of RBC count and hemoglobin showed noticeabledecrease in group 2 that inoculated with bacteria plusbipyridyl($P<0.05$).Also total protein, albumin and glucose showed the same results in this group.On the contrary, there was a significant increase in liver enzymes,urea and creatinine in groups 1 and 2($P<0.05$). Similarly, neutrophil, monocyte and lymphocyte count increased in these groups and considerable difference was observed with control group. Besides, eosinophil count showed a rise in groups 1, 2 and 3 with no significant differences ($P> 0.05$) (table 2).

Humoral immune responses

Test serafrom immunized mice were used to assay for titer of antibody. Fronds control was used as positive control for Elisa. Elisa was performed to investigate the immunogenicity of inactivated *P. multocida* in two media. Diagram.1 shows the humeral antibody responses in sera for all groups (1 until 9 weeks). Also, *P. multocida* adjuvanted with Alum showed higher immunization than the other groups. *P. multocida* cells grown in iron replete media ($FeCl_3$) and iron limit media (bipyridyl) are in the next categories. Control group did not shown immunization. There were considerable significant differences between vaccinated groups ($P< 0.05$).

Clinical and necropsy finding

Mice immunized with inactivated *P. multocida*-didn't show clinical signs of infection until the end of trial. In contrast, mice inoculated with live *P. multocida* derived from different media showed noticeable lesions in all organs. No marked lesions could be found in control group (Fig. 1A). The most sever necrosis have been shown for the bipyridyl group.

The infected organs for the bipyridyl group have been shown in Fig. 1B.Hemorrhagia and congestion were the most severely affected in lung tissue for the

Table 1. Studied groups for infection and immunization

	Infection	Immunization
1	*P. multocida* (*P.m*)	*P. m* + Fronds (C^+)
2	*P.m* + bipyridyl (bp)	*P. m* +Alum (C^+)
3	*P.m* + $FeCl_3$	*P.m* + Alum + bp
4	PBS (C^-)	*P.m* + Alum + $FeCl_3$
5		PBS (C^-)

Table 2. Blood chemistry and hematology of host after infected by P.multocida cultured in low and rich iron media

Blood parameter	Groups*			
	1	2	3	Control
RBC	9.1	8.7	10.2	10.6
Hemoglobin	12	10	14	15
Total protein	4.4	4.1	5.1	5.5
Albumin	1.8	1.5	2.1	2.4
Glucose	47	36	117	143
Alkaline phosphatase	416	440	330	260
Aspartate amino transferase	236	270	200	162
Alanine amino transferase	141	171	105	71
Urea	20	25	17	15
Creatinine	6	8	4	3
Neutrophil	40	48	30	15
Monocyte	9	11	6	4
Lymphocyte	80	88	66	56
Eosinophil	4	4	3	2

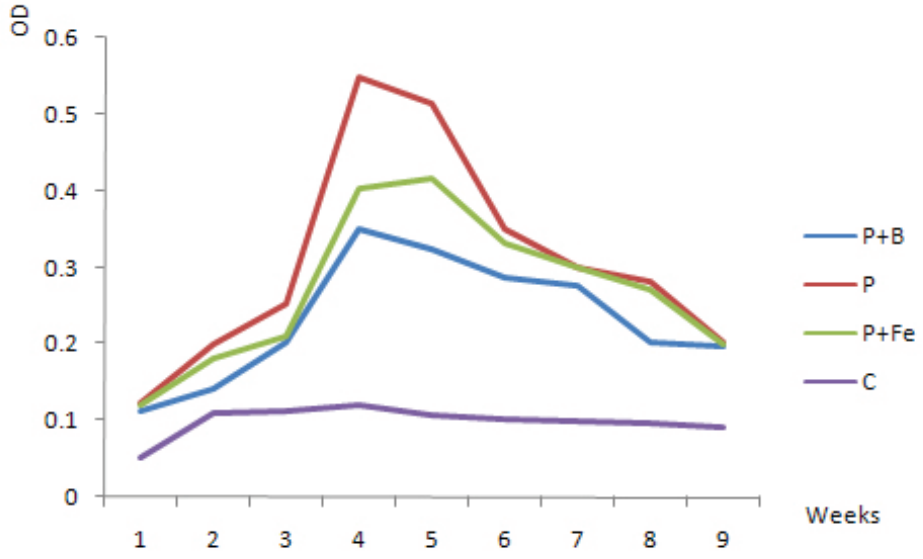

Diagram. 1. Immune responses to *P. multocida* antigen in different media. P: *P. multocida* with Alum, P+B: *P .multocida* with bipyridyl, P+Fe: *P. multocida* with ferric chloride, C: Negative control.

bipyridyl group (Fig. 1B, I). Hearts from all infected groups showed erosion and accumulation of blood in cardiac cells (Fig. 1B, III). Liver and spleen sections showed positive reaction to *P. multocida* infection and degenerative changes were observed in all infected organs (Fig. 1B, II and V). Also the inflammation in renal cells was monitored in the kidney (Fig. 1B, IV).

DISCUSSION

Active cross-protection studies, using Balb/c mice as an animal model, wereemployed to assess the role of FeCl3 and bipyridylin infection and immunity against pasteurellosis.Current studies confirm that P. multocida can interact in host tissue and blood to cause disease (Boyce et al., 2012).This work showed mild to severe fluctuation in the hematology and biochemistry factors and tissue lesion of mice infected with P. multocida grown in low and rich iron media.

The results of this study demonstrated a drop in RBC and hemoglobin count in consequence of infection by growing the bacteria in iron limitedmedia. This organism is a commonly causative of inflammation which therefore anemia will occur.It seems that limitediron media has an effect on *P.multocida*virulence (Kaneko et al., 2008; Weiser et al., 2003). Besides the infection and increase in lymphocyte, monocyte, eo-

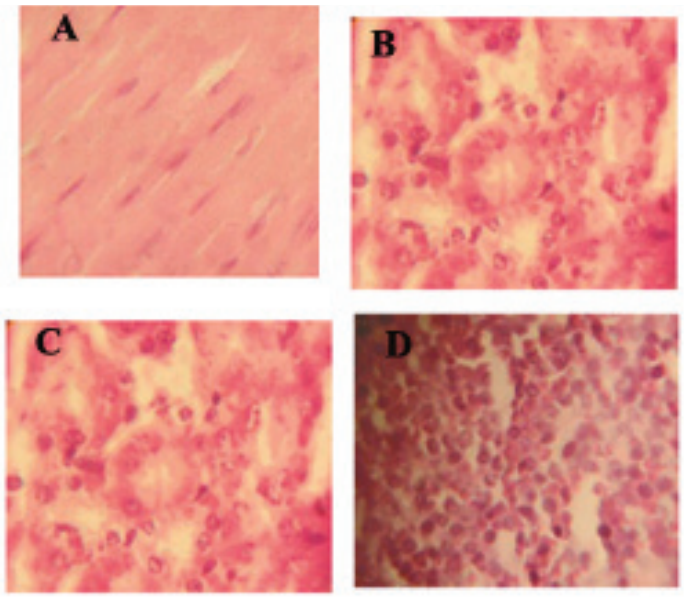

Fig. 1. The uninfected organs in control group,a) heart, b) kidney, c) spleen and d) lung . There is no necrosis or hemorrhagic observed in organs

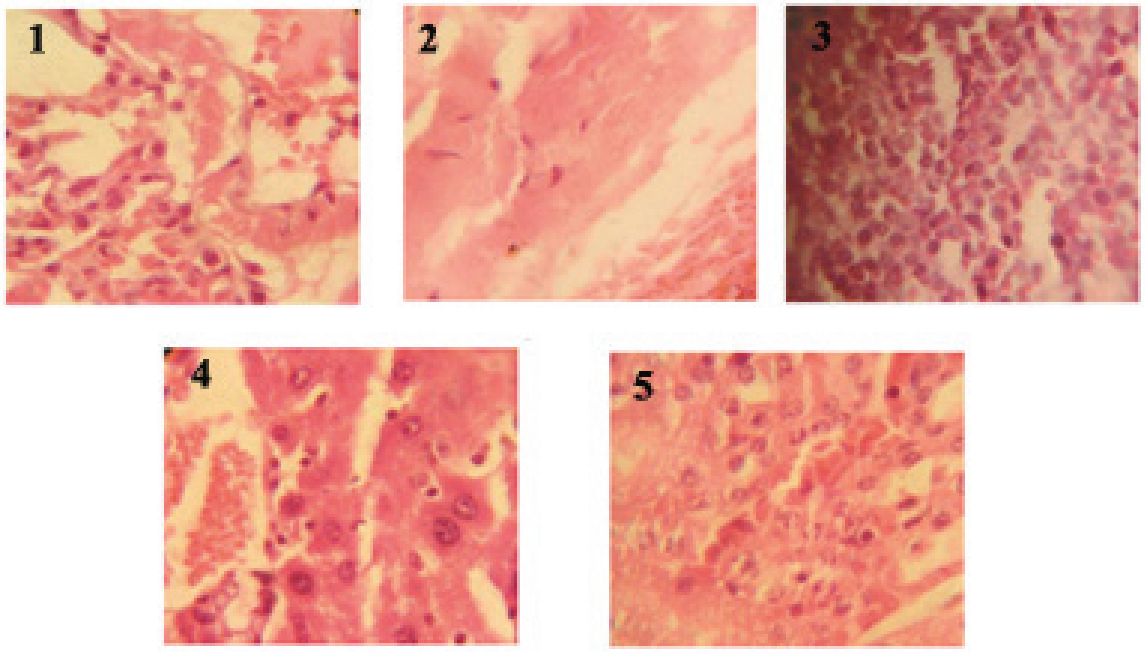

Fig. 2. The infected organs for bipyridyl group, 1) lung, 2)heart, 3) spleen, 4) liver and 5) kidney.

sinophil and neutrophil level, the blood inflammation may be indicated.*P. multocida* infection changes the RBC count and immune cells in rabbit and goats (Sadeghian et al., 2011). Our results are similar to the finding of Katoch et.al (2015)(Katoch et al., 2015) in goats, who reported an increase in monocyte count.

Our results showed that after infection, albumin decreased and liver enzymes increased in the low iron media. The increase of liver enzymes may be due to severe lesions in liver tissue which can produce a high level of enzymes (Ringler et al., 2014).Moreover, significant increase in urea and creatinine levels was observedin infected group with bacteria in iron limited media. This significant rise can be considered as kidney necrosis and inflammation (Dunn et al., 2004; Muriel 2006).This results are similar to the Abdelhady et.al (2015) (Abdelhady et al., 2015)studies in rabbit and chicken infected by *P. multocida*.

The histological analysis showed necrosis, inflammation, hemorrhagic and congestion in all tissues of mice inoculated by bacteria in iron limit media. These findings demonstrate the role of iron for thegrowth and virulenceof*P.multocida*. Genetic analysis has claimed that a major part of genome is designed for iron uptake in *P.multocida* (Boyce et al., 2012; May et al., 2001).Our study also showed that the lung was the most severely affected of the organs. Since the respiratory system is the main route of *P.multocida* infection, lung necrosis is observed in multiple animal species such as goat, buffalo and cattle (Abubakar et al., 2011; Dowling et al., 2002; Sadeghian et al., 2011).

Acute form of infection was observed in mice infected by bacteria cultured in iron limitedmedia. Glisson et.al (2013) (Glisson J.R. 2013; Suarez 2013) reported that *P.multocida* can be isolated from spleen, lung, liver and heart bloodof infected animals during the acute form of the disease.

P.multocida in the absence of iron have a noticeable change in biochemistry factors and tissue necrosis, but it did not have any significant effect on antibodies levels in mice. Similarly, Prado et.al (2005) (Prado et al., 2005)reported no differences in antibody levels against *P.multocida* cultured in sufficient and restricted iron media.

Consequently, our results showed that inoculation by *P.multocida* cultured in iron limitedmedia can change the bacterial virulence and growth potential, which in turn will affect the severity of biochemical and tissue response. However, this condition showed no considerable modification in antibodies levels against *P.multocida* infection.

CONFLICT OF INTEREST

None declared.

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