



Research Article

Anti-inflammatory, immunomodulatory and antioxidant activities of Allicin, Vitamin C and Doxycycline, or their combination against *Pasteurella multocida* infection in Rabbits

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ABSTRACT

Pasteurella multocida (*P. multocida*) is a Gram-negative rod-shaped coccobacilli with non-lactose fermenting property. This study examined the potential efficacy of allicin, vitamin C, and doxycycline in reducing the intensity of *P. multocida* infection in rabbits. 20 locally bred, adult rabbits were divided into five equal groups. Different treatments such as allicin, vitamin C and doxycycline were applied in all groups. Rabbits in group 1 and 2 were kept as control and did not receive any treatment. Group 3 was treated with 500mg allicin and similarly, group 4 and group 5 treated with 100mg doxycycline and combined treatment like allicin 500mg, 100mg, doxycycline and 100mg Vitamin C respectively. Challenge and protection were given after one week in all groups. Except group 1, all remaining groups of rabbits were infected (intranasal) with *P. multocida* 2×10^4 CFU/ml. Antioxidative activity, biochemical assay, hematological and immunological assays were performed. The results showed significant decrease in count of lymphocytes, hemoglobin and RBCs in infected group with increased phagocytic percentage. Serum biochemical enzyme including ALT, ALP and LDH level were significantly increased in infected group. SOD level showed significant increase in combined treated group and significant decrease in infected group. Sera were collected and titrated for antibody titers through indirect hemagglutination assay (IHA). Results were subjected and data was analyzed by 2 factorial CRD. The results depicted significantly higher IHA titers in group 5 followed by group 3 and 4 with short term sustainability. Rabbits in group 3 treated with 500mg allicin presented promising results than the group 4 rabbits treated with doxycycline. Hence, concluded that the use of antibiotics like doxycycline did not provide maximum protection against *P. multocida*. While the synergistic effect of allicin, vitamin C and doxycycline showed maximum protection providing evidence for promising remedy for HS.

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Introduction

Pasteurella multocida is a Gram-negative, non-motile, capsulated, and non-spore forming bacteria (Boulianne et al. 2020). These facultative anaerobic bacteria from coccobacillus family are opportunistic pathogens that cause various clinical manifestations including increase in body temperature, redness of the mucous membrane of the eye, nasal discharge,

hypersalivation, diarrhea, inflammation, increase in breath rate with difficulty in breathing, and inflammation in submandibular regions, in cattle, buffaloes, cats, minkes, lions, camels, elephants, deer, and horses (Almoheer et al. 2022). The potential route of entry for the pathogen into animal body is through inhalation or ingestion of contaminated water and grass (Puspitasari et al. 2022). Pakistan and India

collectively bear an approximate loss of \$1.5 billion per annual due to respiratory diseases in animals. Whereas only in Punjab, province of Pakistan, approximately 12.4 million USD losses annually due to these diseases (Muhammad et al. 2022).

P. multocida and *Mannheimia (M.) haemolytica* are the most common bacteria that are isolated from calves that are infected with bovine respiratory disease (Elsayed et al. 2021). These bacteria penetrate into the lower respiratory tract and vital organs due to the many stress factors like transportation, poor management, diet changes, weather changes, and animals that are already suffering from respiratory diseases. Introducing infected animals in herds can cause the development of severe diseases in herds' animals (Gatie et al. 2021).

These bacteria contain eight LPS genotypes (L1-L8) five capsular genotypes and sixteen serogroups (Christensen et al. 2021). These capsular antigen-based serotypes are A, B, D, E, and F. These serotypes are very important because with the help of these antigens different strains of *P. multocida* can be easily identified (Cuevas et al. 2021). Serogroup type A and F causes fowl cholera and septicemia. Type B causes hemorrhagic fever in cattle, sheep, goat, pig, buffalo, horse, deer, camel, and elephants. Type D causes atrophic rhinitis in swine. Type E is an African serotype that causes respiratory disorders in buffalo and cattle. Type F causes Pasteurellosis in rabbits. *P. multocida* causes snuffles disease in rabbits (Massacci et al. 2018). Hemorrhagic septicemia (HS) is caused by a B:2 and E:2 strain of *P. multocida*. Detection of specific serotype of *P. multocida* can be done by immunological Assay. Indirect hemagglutination assay (IHA) and slide agglutination test are used for capsular typing and agar gel precipitation test for both somatic and capsular typing (Zhao et al., 2021). Allicin has numerous useful physiological purposes, for instance anti-inflammatory, antiviral, immunomodulatory, anti-fatty liver effects by its bioactive components. Allicin has useful effects on oxidative stress and inflammatory reaction in vitro as well as in vivo. It is a potential natural antioxidant with a property of inhibiting unwanted oxidation processes (Alrumaihi 2020).

Vitamin C is water soluble vitamin. It is present in the extracellular fluid and cytosolic compartments of cell. Primary aim of Vitamin C is to immunomodulate and immunostimulate the response of host immune system against the pathogens (Morsli et al. 2022). During bacterial or viral replication, the level of pro-inflammatory cytokine increases causing inflammatory response. Vitamin C is used as an immunomodulatory agent and used to control immunodeficiency diseases. Vitamin C has also contained immunostimulant activity to increase the level of T-lymphocytes and to inhibit the death of T-cells by signaling pathway (Salehi et al. 2019). Vitamin C inhibits the replication of *P. multocida* by restricting the expression of virulence factors of the bacteria. Vitamin C is also used as food supplement to overcome the bacterial load in host cell and to increase the survival rate of animals that are already suffering from *P. multocida* infection (Rizk et al. 2022). Doxycycline belongs to tetracycline antibiotics group. Doxycycline is inexpensive broad-spectrum antibiotics. It is used to treat upper and lower

respiratory tract infection. Doxycycline gave a better result than other tetracycline antibiotics group like oxytetracycline and chlortetracycline. Doxycycline contained higher antimicrobial activity due to ability to penetrate into tissue, higher affinity of plasma protein and high half shelf life (Majewski et al. 2014). Doxycycline has a high level of activity against *P. multocida* but few isolates of *P. multocida* were resistant to doxycycline in buffaloes (Naz et al. 2012).

Materials and Methods

Bacterial Strain

The culture of *P. multocida* type B was obtained from the Cell Culture Laboratory, Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan. The final concentration of *P. multocida* type B culture 2×10^4 CFU/ml was used for inoculation.

Drugs

Allicin was purchased from nutrifactor Pharma (500mg/ml, oral suspension). Doxycycline (100mg/ml, oral suspension) was taken from ATCO laboratories, Karachi Pakistan.

Vitamin

Vitamin C 100mg/ml oral suspension was purchased from nutrifactor Pharma Pakistan.

Experimental Animals

Adult male rabbits, (weight 1500-2000 grams) were obtained from the Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan. All rabbits were housed in pathogen-free area (3% solution of glutaraldehyde) and acclimatized for two weeks before starting the trial. All the rabbits were kept and maintained at 22-28°C with a 35-50% relative humidity. All the rabbits were endowed with light and dark cycle of 12 hours each in a well-ventilated, spacious and temperature regulated (22-28°C) room. Rabbits were fed on fresh green fodder Lucerne twice a day and the availability of fresh water was ensured the whole day ad-libitum. This study was performed according to the guideline for laboratory animal. Approval from the institutional Biosafety Committee (IBC), University of Agriculture Faisalabad was taken to conduct the current study. All efforts were done to reduce the severity of hemorrhagic septicemia.

Experimental design

After acclimatization, total 20 rabbits were divided into 5 groups, each group contained 4 rabbits. Except group 1 all remaining groups of rabbits were infected (intranasal) with *P. multocida*. Rabbits in group 1 was a control group, all the rabbits in this group received only normal saline (1ml). Rabbits in group 2 was infected with 2×10^4 CFU/ml of inoculum. Similarly, Group 3 and Group 4 rabbits received two doses (500mg/ml, oral suspension) of allicin and two doses of doxycycline (100mg/ml, oral suspension) on regular basis. Group 5 rabbits also received combined treatment like 2 doses (500mg/ml, 100mg/ml, 100mg/ml, oral suspension) of allicin, vitamin C, and doxycycline respectively on daily basis.

Blood sampling

Blood was collected on a weekly basis from the Juglar vein, 3ml of blood in EDTA tube for the hematological studies and for the evaluation of Phagocytic percent and 5ml of blood in gel clotted tube for serum separation to assess indirect hemagglutination assay and biochemical parameter (Tahir et al. 2013).

Hematological assay

Blood samples, collected in EDTA containing solution tube were used for the determination of complete blood count including, lymphocytes, monocytes, red blood cell, hemoglobin, differential leukocytes count and white blood cells according to Kanda et al. (2020).

Biochemical assay

Serum samples were separated to analyze the biochemical parameter like alkaline phosphatase (ALP), aminotransferase (ALT) and lactate dehydrogenase according to the Bazzano et al. (2022) and Ha et al. (2022) methods respectively.

Oxidative stress marker

Serum samples were analyzed for the determination of superoxide dismutase (SOD) activity according to Dworzański et al. (2020).

Indirect hemagglutination test

An indirect hemagglutination test (IHA) was conducted to determine the antibodies titer of *Pasteurella multocida* (Ibrahim et al. 2021).

Statistical analysis

All the data regarding the search was interpreted under two factor factorials under CRD.

Results

Clinical signs and Mortality rates: on 5th day of starting the animals trail, the only infected group 2 (without any treatment) showed acute signs and symptom of disease including increase in body temperature, redness of the mucous membrane of the eye, hypersalivation, diarrhea, difficulty in swelling, nasal discharge, an increase in breath rate with difficulty in breathing, with mortality rate 70%. Allicin, doxycycline and combined treatment (allicin, doxycycline and vitamin C) groups 3, 4, 5 respectively showed less severe signs of disease than no treatment group 2. The mortality rate of group 3, 4 and 5 was 35, 40 and 12%, respectively.

Complete blood count (CBC)

Blood sample was collected on 0, 21, 42 days. RBC's count, hemoglobin level were significantly increased in control group and significant decrease was observed in infected group on 0, 21, 42 days. There were non-significant changes in RBC's count and hemoglobin level in allicin, doxycycline and combined treated group 3, 4 and 5, respectively. But the hemoglobin level was increased in group 5 as compared to group 2. Total leucocytes count, eosinophile, monocytes, lymphocytes and neutrophile count of group 2 were compared with group 1. The results showed total leucocytes, monocytes count and neutrophile level were significantly increased, non-significant changes in eosinophile but decreased lymphocytes count in infected group as compared to

control group. The comparison of infected group was done with treated group 3, 4, 5. Group 4 showed increased eosinophile, total leucocytes count and neutrophile level but no changes in the level of monocytes and lymphocytes as compared to group 2. Group 3 and 5 showed decrease neutrophile level, decrease total leucocytes count in group 5 as compared to group 2 but non-significant changes in monocytes, eosinophile and lymphocytes. Phagocytic activity was significantly increased in group 2,3,4,5 as compared to the control group and also increased in group 3, 4, 5 as compared to infected group. Comparison was done among the treatment groups, results showed the increased phagocytic activity in group 5. Activity of group 4 was significantly high as compared to group 3. This can be summarized as: Combine treatment > Doxycycline treatment > Allicin treatment.

Hepatic enzyme

ALT, ALP and LDH level were significantly increased in infected group and significantly decreased in group 1, 3, 4, 5. The comparison was performed among the treated group 3, 4, 5. ALP and LDH levels were significantly increased in allicin treated group and significantly decreased in group 4, 5 and between 4,5 the level in group 5 decreased. ALT level was significantly increased in doxycycline treated group and significantly decreased in group 3, 5 and between 3,5 the level in group 5 decreased.

Oxidative stress marker

Superoxide dismutase (SOD) levels were significantly increased in control group and significantly decreased in group 2. The comparison was performed with group 2 to the treated group 3, 4, 5. Results showed treatment groups were continuously increasing antioxidative stress stage in group 5 and group 3 but doxycycline treated group did not give a better result.

Indirect hemagglutination Assay

Serum samples of total 4 rabbits from group 3, 4, and 5 were collected for IHA on days 1, 7, 14, 21 and 28. The geometric mean antibody titers was high in combined treatment group. The antibody titers were lower in doxycycline treatment group as shown in Table 1.

Discussion

The most important clinical signs demonstrated by the Rabbits suffering from Pasteurellosis include dyspnea, cough, tachycardia, tachypnea, mucoid nasal discharge, anorexia, listlessness, depression, and death of animals. Lungs are mostly affected organs and 20% of lungs are completely damaged, reduced lung capacity, chronic breathing problems, and reduced weight gain. Those rabbits' groups whose treated with allicin showed less severe clinical signs and symptoms. Allicin releases free radicals that inhabit the growth of bacteria, the motility level in allicin-treated groups was low. Allicin-treated groups contained anti-inflammatory antioxidant and antibacterial activity in their structure (Dkhil et al. 2011). Bacteria contained the thiol protein in their cell and also contained cysteine protease. Doxycycline belongs to the board spectrum antibiotics, infected groups treated with it had lower morbidity and mortality.

Because antimicrobial activity was high, infected rabbits displayed less severe signs and symptoms. Infected groups treated with the combination of allicin, vitamin C and doxycycline showed remarkably reduction in morbidity and mortality as compared to groups only treated with allicin and doxycycline. All rabbits infected with *P. multocida* developed reticulocytosis because trachea pulmonary hemorrhagic septicemia occurs as a result of increased blood and bone marrow response. The results demonstrated that we can reduce the level of RBCs while increasing the level of WBCs (Puspitasari et al. 2022). The reason for this result was that during infection, WBC levels increased in order to control the severity of the disease because the maximum number of first line of defence mechanism cells reached the site of infection. Infected rabbits' groups treated with the combination of allicin, vitamin C and doxycycline showed decrease severity of HS and increase level of RBC especially HB. The leukogram result showed an increase in leucocyte levels due to an increase in heterophile body immune inflammatory response to that disease.

Infected rabbits' groups increase the level of WBC because infection all monocyte, lymphocyte phagocyte reached at the site of infection and make a granuloma formation around the infected tissue site due to minimize the replication of bacteria to another site or organ for spreading of disease. Lymphocytopenia occurred in this study to decrease the level of WBCs due to the transfer of lymphocyte into the bacterial infected tissues. The phagocytic percentage was increased due to the cellular immune response inside the body against the infection to minimize the spread of infection our result agrees with the (Alam et al. 2018). During the inflammatory response the transition of monocyte and neutrophils activity was increased in the blood due to increase in the response of IL-6. The phagocytic activity was increased in allicin treated group due to the elevation in the activity of lysozyme, oxidative stress, CD4+ T cell (Cuevas et al. 2021). The *P. multocida* infection in rabbits were led to in the loss of the level of protein because due to the fever and anorexia. There was increased the level of liver renal enzyme like ALP, ALT and LDH our result agrees with (Jaglic et al. 2011). Those groups treated with the allicin doxycycline or their combination also decreased the level of liver enzyme. This result showed that liver was recovered and minimum damage of liver was occurred. Our results showed that oxidative enzyme like superoxide dismutase was gradually increased (El Hammed et al. 2016) when we combine our treatment with Allicin, Doxycycline or their combination with Vitamin C. This was concluded that treatment with Allicin, Doxycycline or their combination with Vitamin C reduced the infection- induced oxidative stress and improved the antioxidant state.

In the esteemed study, we prepared different formulations of Allicin, Doxycycline, and Vitamin C and their combination and conducted a laboratory trial to evaluate their efficacy in rabbits against *P. multocida* infection. A total of 20 rabbits were divided into 5 groups of 4 each and were given 2 tablets of Allicin, Doxycycline, and Vitamin C and also their combination of respective treatments 7 days. On day 1 of trial, blood samples collected from all 3 groups

did not show any Ab titers. Depicting that animal were completely free of Abs against *P. multocida*. The geometric mean titers of Group 3 were recorded 4, 9.5, 54.5, and 256 on days 7, 14, 21, and 28, respectively. Similarly group 4 and 5 recorded their GMT as 2, 6.4, 54.5, 64 and 2.2, 16, 95.00, 294.06 respectively. Upon comparison of Indirect Hemagglutination Test of group 3, 4, 3, 5 and 4,5. The results showed that GMTs group 3 and 5 were significantly higher than those of group 4. Geometric antibody mean titer was high in group then the group 3.

Conclusion

Hemorrhagic septicemia (HS) is an important bacterial infection of domesticated animals, which is spawned by *Pasteurella multocida* and gives severe economic losses for the farmers. The use of therapeutics against hemorrhagic septicemia is not efficient due to its acute nature and short duration of the disease. The results of this study showed that rabbits are suffering from this disease. Rabbits' organs are damaged but the administration of allicin gave protection because the allicin contained anti-inflammatory antioxidants activity. It was concluded that Allicin provided more protection than the usage of antibiotics like Doxycycline. Maximum protection was provided by combining Doxycycline, vitamin C, and allicin rather to just utilizing these medications individually. However, this protection does not continue for an extended length of time.

Table 1: Effect of Allicin, Doxycycline and Vitamin C or their combination against *P. multocida* infection in rabbits on geometric mean titer

Rabbits	Group 1	Group 3	Group 4	Group 5
Day 1	0	0	0	0
Day 7	0	4	2	2.2
Day 14	0	9.5	6.4	16
Day 21	0	54.5	54.4	96
Day 28	0	256	64	294.06

Values are represented as mean \pm standard error of mean n=30 ALT=Alanine transferase, ALP=Alkaline Phosphatase Different subscript (a, b, c, d, e) are for significant

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