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Comparative efficacy of Chitosan and oil adjuvanted *Pasteurella multocida* antigen in rabbits

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ABSTRACT

Hemorrhagic septicemia (HS) is highly acute, fatal and bacterial disease mainly affecting water buffaloes and cattle, caused by *Pasteurella multocida* having higher incidence in younger animals, manifested by high fever, respiratory distress, salivation, nasal discharge, tongue protrusion, hot and painful swelling around throat region and edema in brisket. The successful prophylaxis regime includes the adjuvant-based vaccination of the animals. The present study was, therefore, planned to prepare and evaluate the comparative efficacy of two formulations of chitin derived chitosan adjuvanted hemorrhagic septicemia (HS) antigen and comparing its efficacy against oil adjuvanted HS antigen. Chitosan adjuvanted antigen was prepared by diluting 0.2% (W/V) chitosan in 25mM sodium acetate solution (pH 5.0) and adding suspension culture of *Pasteurella multocida* at 1:2 and 1:4 chitosan and similarly 1:2 mineral oil adjuvanted antigen was prepared. Sixty adult male rabbits randomly divided into four groups of 15 each with Group 1 kept as un-inoculated control. Group 2 rabbits were inoculated with 1:2 Chitosan-adjuvanted antigen through subcutaneous route and Group 3 were given 1:4 chitosan-adjuvanted antigen while Group 4 received 1:2 oil-adjuvanted antigen. Sera were collected at weekly interval post vaccination for seven weeks and titrated for antibody titers through indirect hemagglutination test. The results indicated that maximum IHA titers on day 28 post inoculation in Group 4 with the highest GMT of 294.06, followed by Group 3 (GMT= 256.0) and Group 2 (GMT=222.86). Moreover, the rabbits of Group 3 showed maximum antibody titer of GMT= 128.0 compared to the other two inoculated groups. The antibody titers remained higher (GMT=84.4) in Group 3, followed by Groups 4 and Group 2, respectively. Therefore, it was concluded that initially Group 4 oil-adjuvanted antigen imparted the highest antibody titer earlier than the Groups 3 and Group 2 and it dropped down rapidly till 42nd day post inoculation while Group 3 gained persistently higher antibody titer till 42nd day post inoculation. However, the protection against virulent *Pasteurella multocida* challenge, after four weeks post inoculation, was found equally better among all inoculated groups with 90% compared to un-inoculated control group.

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Introduction

The use of young hemorrhagic septicemia is an acute, highly fatal and septicemic bacterial disease, mainly affecting cattle and buffalo is one of the dominant sources of mortality in tropical regions of Africa, Asia and the Middle East. Younger animals are more prone to endemic regions, particularly during the rainy season (Shivachandra et al. 2011). Hemorrhagic septicemia is caused by *Pasteurella multocida* subsp *P. multocida.* Microscopically, it is

a coccobacillus having a Gram-negative cell wall, belonging to the family Pasteurellaceae. *Pasteurella multocida* is a notorious G-ve bacterium, a coccobacillus, normally residing on/in the nasopharynx of domestic animals (Harhay et al. 2018). This microorganism has also been separated in the gastrointestinal specimens and urinary tract extracts (Annas et al. 2014). According to the Carter's serotyping system, this *P. multocida* is broadly dissected into five serotypes based on capsular antigen, *viz.,* A, B, D, E, and F. But on the other side, with reference to the Heddleston system using gel diffusion precipitation, this bacterium is classified into 16 different somatic antigen serotypes, *viz.,* serotype 1 to 16 (Narcana et al. 2020).

Most frequently hemorrhagic septicemia has been recorded in water buffalo (*Bubalus bubalis*) and cattle. These are also the paramount reservoir hosts for *P. multocida*. This condition has also- been narrated in many other ungulates *viz.,* African buffalo, goat, sheep, elephants and saiga antelopes, bison, dromedary camels (*Camelus dromedarius*) and pigs (Marza et al. 2016). Route of transmission for hemorrhagic septicemia can be ingestion or inhalation. It can also be transmitted during direct contact to infected animals or via fomites such as contaminated cloth/clothes, feed, water, and workers (Sugun et al. 2016). In most clinical cases, the incubation period is observed around 3-5 days, although it can be reported as short as just a few hours without showing any clear symptoms (Aiello and Moses 2016).

Clinically, the disease is demonstrated by sudden onset of febrile condition (104-108°F), with moderate to severe respiratory anguish, salivation, fibrinous discharge from nostrils, protrusion of tongue, unable to walk properly, edematous, hot and painful swelling on / or around brisket and throat, occasionally between the forelegs (Marza et al. 2016). A marked increase in the sprouting of multidrug-resistant (MDR) microbial strains has been reported across the last decade and a halfa. Yet the antibiotics are the first line of treatment to prevent and contain the infections caused by *P. multocida*. However, it has been also stated that the imprudent ploy of drugs has fostered the sprouting of drug-resistant bacterial strains (Oh et al. 2018; Oh et al. 2019).

The prophylactic regime includes the adjuvant-based vaccination of the animals. Adjuvants are immunostimulatory ingredients that are present in the vaccine preparations in order to help in directing and amplifying adaptive immune responses from the host body. Chitosan, which is derived from chitin, has been found to show potential as an effective adjuvant for inactivated antigens. It is a high-profile cationic carbohydrate (polysaccharide) investigated for its use as an adjuvant, owing to its biocompatibility and biodegradability (Moran et al. 2018). This study comprises of the chitosan adjuvant-based antigen and its field trials to determine the efficacy of this antigen in comparison with the commercially available vaccine. Although there are several vaccine methodologies available commercially, but HS outbreaks still transpire every year with varying extent of damage to the economies. Vaccination campaigns started after the outset of the infection are not methodical to prevent high mortalities and losses. Therefore, identifying the areas endemic for HS to prioritize obligatory vaccination is very necessary to prevent such devastating outbreaks (Farooq et al. 2011).

Trimethyl Chitosan (TMC), derived from chitosan, encompasses all of these properties. Apart from that, it additionally has the features of high-water solubility, with high charge density, permeation enhancing ability, mucoadhesive property and caliber to withstand a broad range of ionic conditions, making its applicability spectrum much broader than other candidates. Chitosan has also been known to work analogously with several popular adjuvants *viz.,* alum, Cyclic Guanosine Monophosphate (cGMP), complete Freund's adjuvant and incomplete Freund's adjuvant, which rationalizes its character as a very dynamic adjuvant (Malik et al. 2018). Present work revealed the immunological efficacy of chitosan adjuvanted *Pasteurella multocida* in rabbits.

Materials and Methods

All bioethical and biosafety parameters were strictly followed throughout the experimental phase of laboratory animal trails as advised by institutional biosafety committee (IBC) of University of Agriculture, Faisalabad Pakistan.

Procurement of *P. multocida*

Pure culture of *P. multocida* was obtained from Institute of Microbiology, University of Agriculture Faisalabad, Pakistan. The culture was further re-characterized for confirmation.

Identification of *P. multocida*

Cultural Identification

A single isolated colony from pure culture was selected and grown on Tryptone Yeast Extract Agar (TYE) (Ashraf et al. 2011) and Blood agar plates. (Wessmann and Hilker, 1968). Pure stock culture was subjected to microscopic examination and its bipolar nature was confirmed by Giemsa-staining (Orynbayev et al. 2019). The culture was subjected to biochemical analyses by API- 20 NE (Biomeriux**,** France) kit. All the instructions of the manufacturer were followed by biochemical characterization of the pure culture (Ashraf et al. 2009).

The polymerase chain reaction (PCR) system was carried out for complete confirmation of *P. multocida*. PCR amplification was done using the given primers (Hunt et al. 2000). Both the primers *viz.,* KTT72 and KTSP61 were obtained from Gene Link company, USA.

Preparation of Mass Culture

To prepare mass culture, a single colony from the stock culture was inoculated aseptically into the large glass flask containing Tryptose Yeast Extract (TYE) broth and incubate for 24 hours at 37°C with slight shaking at 20 rpm/ minute as described by Nandani-Peiris and Alwis (1991).

Standardized mass culture having $1X10^6$/ml was then subjected to Formalin inactivation by adding 0.3% Formalin in culture suspension under continuous stirring for 6 hours. Formalin added culture was then tested for formalin inactivation through streaking onto TYE agar plates and incubating at 37℃ for confirmation of inactivation.

Preparation of Chitosan Adjuvanted Antigen

Two different concentrations of chitosan adjuvanted antigen were prepared with 1 part of formalin inactivated culture added into 1 part of 0.2% chitosan in phosphate buffer saline (PBS) to maintain its pH at 7.2. Homogenize the resultant mixture completely for 10 minutes and 1:2 Chitosan adjuvanted antigen was ready to use after storage in refrigerator for at least 10 days before animal inoculation (Chang et al., 2010)

On the other hand, 1:4 chitosan adjuvanted antigen was prepared through mixing of 1 part of formalin inactivated culture into 3 parts 0.2% chitosan in PBS at 7.2 and was homogenized thoroughly. The resultant 1:4 chitosan adjuvanted antigen was stored for at least 10 days in refrigerator prior to use in experimental animals (Chang et al., 2010).

Preparation of Oil Adjuvanted Antigen

A 0.9-part formalin inactivated Ag from culture suspension was added into 0.1 part of Tween-80 (water surfactant) and homogenized, while 0.8 part of mineral oil was mixed with 0.2 part of Span-80 (oil surfactant) separately. Finally, one part of Antigen suspension and one part of Oil base was homogenized to prepare 1:2 oil adjuvanted antigen carrying Hydrophile-Lipophile Balance (HLB) at 7.0. The antigen was stored under refrigeration temperature.

Table 1: Biochemical tests, sugar fermentation and enzyme production characteristics of laboratory culture isolate

Culture No.	**Nitrate Reduction**	**Hydrogen Sulfide production**	**Methyl Red**	**Indole production**	**Catalase production**	**Voges-Proskauer**
JA20195BC	+ve	+ve	-ve	+ve	+ve	-ve
	Sorbitol	**Dulcitol**	**Lactose with gas**	**Trehalose**	**API 20NE ID #**	**Species ID** 96 %
	+ve	-ve	-ve	+ve	3000004	*Pastuerella multocida*

Table 2: Immune response of Chitosan and oil adjuvanted *Pasteurella multocida* antigens rabbits using geomean antibody titer through indirect hemagglutination test

	GMT ± SD			
Sera collected at	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Day 1	0	0	0	0
Day 7	0	2	4	2.29
Day 14	0	6.06	10.55	16
Day 21	0	55.71	55.71	97.00
Day 28	0	222.86	256	294.06
Day 35	0	64	128	84.44
Day 42	0	55.71	84.44	73.51

Quality Checks

All three types of prepared antigens were subjected to sterility and safety tests through inoculation into Thioglycolate broth and subcutaneous inoculation in rats, respectively as described by OIE (2018).

Experimental Inoculation in Rabbits

Sixty local breed, healthy and fit adult male rabbits, ranging weight 1500 - 2000 grams were obtained from market. These rabbits were maintained in the Laboratory Animal House facility in the Institute of Microbiology. These rabbits were divided into four groups, with 15 rabbits in each. G1 was maintained as un-inoculated; G2 was inoculated with 1:2 Chitosan adjuvanted antigen, G3 inoculated with 1:4 Chitosan adjuvanted antigen and G4 inoculated with 1:2 Oil adjuvanted antigen.

Vaccine Protocol

Rabbits in Group 2 received two doses of 1:2 Chitosan adjuvanted antigen subcutaneously 7 days apart from the first dose. Similarly, Group 3 and Group 4 rabbits were given 2 doses each of 1:4 chitosan adjuvanted antigen and 1:2 oil adjuvanted antigen respectively through subcutaneous route in

a space of 7 days from each other. Rabbits in Group 1 were kept as placebo and did not receive any antigen at all. Instead, Group 1 animals were given 0.25ml of normal saline sub-cutaneous.

Blood samples of 2ml from 5 rabbits at random from each group were collected on day 1st till 42nd post inoculation of the trial. The sera were separated and stored at -20°C. The specific antibody titer was determined through indirect hemagglutination (IHA) test, as described by Sajjad et al (1988).

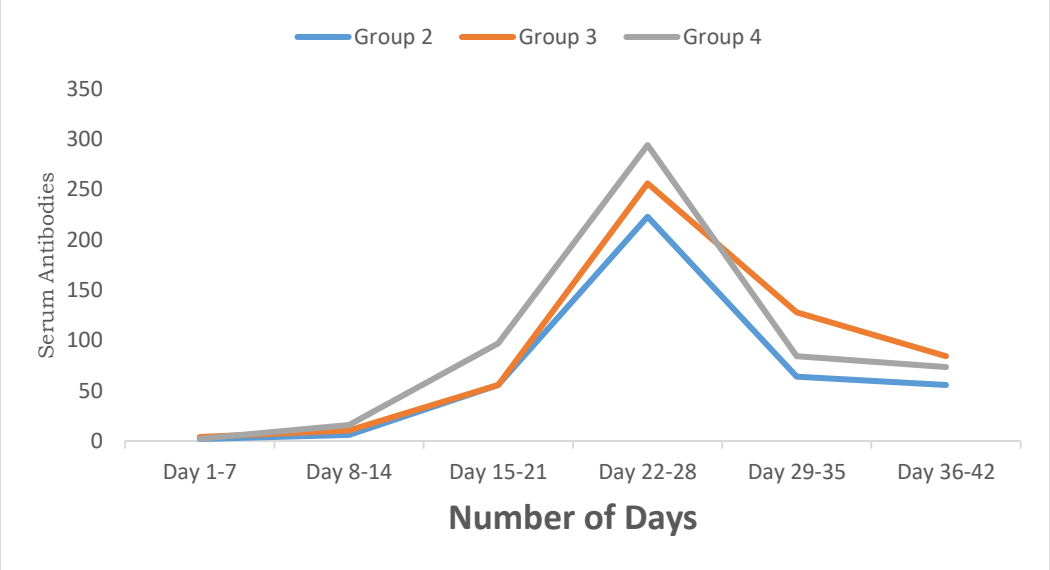

Fig. 1: Line Graph showing IHA Titers of Rabbit Serum

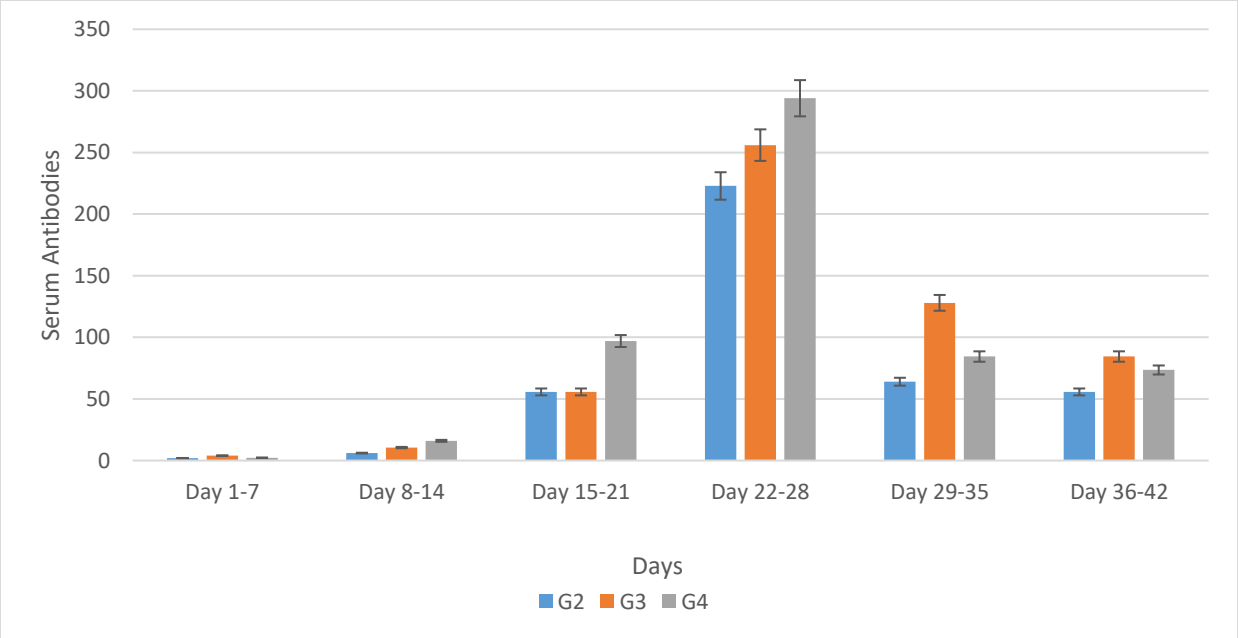

Fig. 2: Comparative antibody titer (GMT+-SD) in rabbits inoculated and un-inoculated with different ratio of chitosan and oil adjuvanted *Pasteurella multocida* using IHA test. ($p<0.05\%$)

Challenge/Protection Test

After 28 days of antigen inoculation, five rabbits from each inoculted and non-inoculated groups were separated and challenged with the active viable culture of *P. multocida* (LD50 = $1X10^{4.6}$/ml) through sub-cutaneous route @ 0.50 ml (Chang et al., 2010).

Results

Confirmation and Re-Characterization of *P. multocida*

a) Cultural Identification

Round, sticky, mucoid, slightly elevated colonies from the center, having 2-3mm diameter, were observed on Tryptone Yeast Extract Agar and it produced non-hemolytic colonies on blood agar.

b) Morphological Identification

Upon Gram staining, reddish-pink coccobacilli were observed confirming the presence of Gram–ve organism. Upon Giemsa staining, the bipolar nature of the organism was confirmed.

c) Biochemical Identification

Biochemical characterization by API 20 NE showed that the organism belonged to *Pasteurella* genus. The result showed the culture as positive for urea hydrolysis test, H_2S production, Catalase production as detailed in Table 1. The PCR test showed the confirmation of 618bp product, thus indicated Robbert Type B specific serotype of *Pasteurella multocida* (Khloo et al. 2017).

Comparative Immune Response of Chitosan Adjuvanted antigen

The Group 1 rabbits showed no antibody titer while serum samples of Group 2 rabbits showed the IHA geomean antibody titer (GMT) against *P. multocida* ranging from 2.0 to 222.86. Initial GMT was 2.0 on the 7th day then increased to 6.06, 55.71, 222.86, 64.0 and 55.71 on 14, 21, 28, 35 and 42nd day post inoculation. The GMT antibody response in Group 3 showed a minimum of 4.0 on 7th day post inoculation and it reached to 256.0 on day 28th maintained at GMT of 84.44 at 42nd day as described in table 2. Till day 28, GMT of Group 4 was highest at 294.86 among the other groups, but after challenge protection test, its GMT showed decreasing trend, measuring 84.44 and 73.51 respectively.

Discussion

In the esteemed study, we prepared two different formulations of chitosan adjuvanted antigen (1:2 and 1:4) and conducted a laboratory trial to evaluate their efficacy in rabbits against 1:2 oil adjuvanted antigen, also prepared during the current study. A total of 60 rabbits were divided into 4 groups of 15 each and were given 2 doses of respective antigen 7 days apart from each other and Group 1 was kept control and did not receive any treatment. The blood samples of 5 rabbits from each group were drawn on weekly basis post-inoculation and were subjected to IHA Ab titration for comparative evaluation.

On day 1 of trial, blood samples collected from all 4 groups did not show any Ab titers, depicting that animals were completely free of Abs against *P. multocida*. The geometric mean titers of Group 2 were recorded 2, 6.06, 55.71, 222.86, 64 and 55.71 on day 7, 14, 21, 28, 35 and 42, respectively. Similarly, Group 3 and 4 recorded their GMT as 4, 10.55, 55.71, 256, 128, 84.44 and 2.29, 16, 97.00, 294.06, 84.44, 73.51, respectively.

From the above data, it can be concluded that in all three formulations of antigen, peak Ab titers were recorded at 4 weeks post inoculation, and then a decline in Ab titers were recorded in all inoculated groups. The rate of decline of Ab titers after 28 days was recorded different for all groups receiving different antigen formulations, which was a very notable thing as briefed in Fig. 1.

Upon comparison of Indirect Hemagglutination Test of Group 2 (1:2 chitosan adjuvanted antigen) and 3 (1:4 chitosan adjuvanted antigen), GMTs of Group 3 were significantly higher than those of Group 2, depicting that 1:4 chitosan adjuvanted antigen showed better results in protection against hemorrhagic septicemia in laboratory animals than 1:2 chitosan adjuvanted antigen (Chowdhury et al., 2014).

Indirect Hemagglutination Test of Group 2 (1:2 chitosan adjuvanted antigen) and 4 (1:2 oil adjuvanted antigen), GMTs of Group 4 were found statistically significantly higher than those of Group 2, depicting that 1:2 oil adjuvanted antigen showed much higher degree of protection against hemorrhagic septicemia in laboratory animals than 1:2 chitosan adjuvanted antigen.

Upon comparison of Indirect Hemagglutination Test of Group 3 (1:4 chitosan adjuvanted antigen) and 4 (1:2 oil adjuvanted antigen), GMTs of Group 4 were recorded significantly higher than those of Group 3, depicting that 1:2 oil adjuvanted antigen showed much higher degree of protection against hemorrhagic septicemia in laboratory animals than 1:4 chitosan adjuvanted antigen. But surprisingly, IHA titers of Group 4 were not sustained for longer duration and after 28 days of inoculation, IHA titers of Group 3 receiving 1:4 chitosan adjuvanted antigen were significantly higher than 1:2 oil adjuvanted antigen as shown in Fig. 2.

Conclusion

The presentation was conducted to prepare and evaluate the comparative efficacy of different formulations of chitosan adjuvanted antigen to oil adjuvanted antigen of hemorrhagic septicemia. A laboratory trial was conducted in rabbits and two doses of antigen were given 7 days apart in their respective groups. Serum samples were isolated every week after inoculations and subjected to Antibody titration through IHA. Upon statistical analyses, the geometric mean titers of IHA were highest for Group 4 (1:2 oil adjuvanted antigen), followed by Group 3 (1:4 chitosan adjuvanted antigen) and Group 2 (1:2 chitosan adjuvanted antigen) in the first 4 weeks post-inoculation. Later, the titers were reduced and after 4 weeks, GMT of IHA was highest for Group 3, followed by Group 4 and 2 respectively.

Therefore, we can summarize that oil adjuvanted antigen imparted highest protection during early stages post inocultion, but its results did not sustain well for longer duration. Whereas 1:4 chitosan adjuvanted antigen imparted best and sustained protection against HS in laboratory animals.

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Ethical Statement
No Ethical permissions were required for this article.

Availability of Data and Material
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Consent for Publication
All the authors gave their consent for publication.

Competing Interest
The authors declare that they have no relevant financial or non-financial interests to disclose.

Author Contribution
ZS, wrote the manuscript, MSM managed references, tables and figures, SM revised the article.

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