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Development of experimental oil based inactivated HS vaccine from field isolates of pasteurella multocida from cattle in bangladesh

Abstract

The present research work was conducted to developed oil based inactivated HS vaccine from fields isolates of *Pasteurella multocida* from cattle in Bangladesh and determination of antibody titers following vaccination in bovines. For this purpose, a total of 26 samples were collected where 16 blood samples were from Bogra and 6 blood samples from Mymensingh in clinical cases and 4 morbid materials such aslung, liver, and heart were collected from suspected cases of hemorrhagic septicemia (HS) in cattle of Rajshahi. The samples were processed for demonstration of bipolar organisms and isolation of *Pasteurella multocida.* There were 9 samples were isolated as *P. multocida* in which 3 samples from Bogra, 2 samples from Mymensingh and 4 samples from Rajshahi. The isolates were identified by cultural and morphological characteristics, biochemical characteristics, serological and pathogenicity tests. The dense culture of the organism was achieved in a blood agar by incubation. The formalin inactivated *HS*vaccines (oil adjuvanated) was prepared and administered in cattle at the dose rate of 2ml (1.5 OD600 = ≥7.5×108 CFU/ml) through subcutaneous (SC) route in each selected Group (A, and B) in the field of Gouripur Upazilla, Mymensingh and Nokla Upazilla, Sherpur respectively. Blood was collected from each group A and B before and after 28 days of primary vaccination and pre-vaccinated sera was kept as control. The mean PHA titers were 134.86, 169.14 and <4±0.00 in Group A and B and control group respectively. The PHA titers were analyzed by *t*-test to determine the immune level of vaccinated cattle. The vaccine produced better immune response in post vaccinated groups of cattle as compared to pre-vaccinated groups of cattle. So, it may be concluded that oil based inactivated HS vaccine induced high level of passive haemagglutinating (PHA) antibodies in the vaccinated cattle.

Keywords: *pasteurella multocida*, isolation, HS vaccine, oil adjuvant, passive haemagglutination, cattle

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Abbreviations: PHA, passive haemagglutinating; HS, hemorrhagic septicemia; SC, subcutaneous; MDR, multi drug resistant

Introduction

Hemorrhagic septicemia is an economically important bacterial disease of cattle and buffaloes.[6] The disease remains a significant obstacle to sustainable livestock production in most parts of tropical Asia and Africa. It is caused by *Pasteurella multocida*a natural inhabitant of the mucosal surfaces of upper part of the respiratory tract of ruminants, and under predisposing environmental or management conditions which constitute stress for the animals such as transport (shipping fever), marketing, change of feed, climate or ventilation.[1] The disease is per acute, having a short clinical course, involving severe depression, pyrexia, submandibular edema, and dyspnea, followed by recumbency and responsible for 70% above bovine mortality.[2]

Diagnosis of the Pasteurellosis has been traditionally based on history, clinical signs, necropsy findings and isolation of the causative organism. The organism can be identified by classical microbiological tests particularly biochemical tests.[3] Blood smears and impression smears of the organs stained by Leishman's stain can be used for diagnosis by demonstration of bipolar organisms. The infected animals are treated with a variety of therapeutic agents such as antibiotics are used to a large extent for treatment of hemorrhagic septicemia. However, the prolonged and indiscriminate use of antibiotics has resulted in organism and even multi drug resistant (MDR) forms of *P. multocida*have emerged.[4] Therefore, an effective control of disease could only be achieved by vaccination. Formalin-killed, alum-precipitated vaccine of *Pasteurella multocida*is presently the most widely used vaccine for preventing HS. However, immunity lasts for 4 to 5months and protective efficacy is only 60%. Water-in-oil adjuvant vaccines of *P. multocida*using mineral oil and lanoline have been developed and used in some countries. The oil based vaccine which persisted for more than six months due to viscosity of the adjuvants was low (easily injectable) and the oil phase did not separate upon centrifugation at 1,000g for 30minutes or standing at room temperature for 30days.[5–7] Keeping in view the above facts, the present study has been undertaken to developed oil based inactivated HS vaccine from the field isolates of*Pasteurella multocida* in cattle in Bangladesh and determination of PHA titres of the experimentally prepared oil based inactivated HS vaccine in bovines.

Materials and methods

Collection of samples

Samples (blood, heart, liver, lung) were collected from cattle of different ages and sex group suffering from clinically suspected cases of HS and apparently healthy cattle suspected to be infected with *P. multocida*from different selected areas of Mymensingh, Rajshahi and Bogra. The samples were processed for isolation and identification of suspected pathogen by standard method.[8]

Cultural examination of the samples

The collected samples were cultured according to the standard method described by Cowan.[9] The collected organisms were inoculated in blood agar, nutrient agar, MacConkey agar, eosine methylene blue agar, salmonella shigella agar and nutrient broth enriched with yeast extract and beef extract for better growth. The inoculating media was incubated at 37○C in bacteriological incubator for characteristic colony formation. Biochemical tests were also performed for confirmation of the organism according to the methods described by Chessbrough & Cown.[9,10]

Preparation of stock culture

The two different isolate of *P. multocida* organisms were inoculated separately in blood agar, nutrient agar media and nutrient broth enriched with yeast extract and beef extract. The inoculating media were incubated at 37°C in bacteriological incubator for characteristic colony formation. Subsequent subcultures were done for getting pure culture.

Maintenance of stock culture

Blood agar slants were used to maintain the stock culture. The *P. multocida*organisms were inoculated in slant by streaking and were incubated at 37°C for 24hours. Finally, sterile mineral oil was overlaid and kept the slant at 4°C or room temperature. Equal volume (0.5ml) of overnight culture of *P. multocida in* nutrient both and 80% sterile glycerin are taken in eppendorf tubes, mixed well and stored at -80°C.

Inoculation of bacterial isolates into mice

P. multocida isolates (0.5ml) were inoculated in mice through subcutaneous route. The mice were observed at 2hours interval up to 24hours post inoculation and those died or sick were necropsed. The heart blood was smeared, stained by Leishman's stain and finally observed under microscope.

Staining method

The smears were prepared from representative colonies and microbes were characterized microscopically by using the Gram's staining, Leishman's staining and Methylene blue staining technique or method. These staining were performed according to the method described by Merchant & Packer.[11] Motility of bacteria was performed by hanging drop slide preparation as described by Merchant & Packer.[11] The organism can be identified by classical microbiological tests particularly biochemical tests.[3]

Biochemical tests

Biochemical tests for all the isolates were performed. Peptone water grown culture of each isolate was inoculated in 1% glucose, sucrose, sorbitol, manitol, fructose, dulcitol, lactose, silicin, and arabinose, incubated aerobically at 37°C for 72hours. Indol, oxidase, catalase, urease production and nitrate reduction tests were carried out according to their standard bacteriological procedure.[12]

Preparation of oil based inactivated HS vaccine

HS vaccine was prepared in the laboratory at the dose rate of 2ml (1.5OD600 = ≥7.5×108 CFU/ml) and kept in the laboratory. For this, *P. multocida* isolates were cultured in BA media and kept in bacteriological incubator at 37 °C for 24hours. The purity of culture was examined and subsequently sub-cultured in the same media for 24hours. The isolated colonies were then inoculated in 500ml NB containing yeast extract and beef extract and incubated at 37°C in bacteriological shaker incubator for 24hours for massive growth. Later on formalin was added in the broth culture (1:125 ratio) and then sterility test was done according to the procedure described by Choudhury et al.,[13] One milliliter of formalin added NB was inoculated in separate BA plates and kept overnight at 37°C in bacteriological incubator. The plate showing no growth indicated complete inactivation of *P. multocida*organisms and negative for any other contaminating organisms. After 24hours, precipitated potash alum (1:30.30 ratio), autoclaved Paraffin oil (1:1.7 ratio) and Arlacel (1:6.7 ratio) mixed with formalin killed broth culture by stirring for 30minutes and simultaneously Tween-80 (1:50 ratio) was added and mixed. All these activities were performed in the Hemorrhagic Septicemia vaccine production division of Livestock Research Institute (LRI), Mohakhali, Dhaka, Bangladesh. Finally, it was dispensed in crew capped vials and stored at room temperature for further use. Thus the vaccine is formalin inactivated oil adjuvant type.

Experimental immunization

A total of 32 cattle with the history of vaccination against Hemorrhagic Septicaemia disease were selected for this experiment, of which 10 cattle (group A) selected from the Muktijoddha Bazar, Gouripur Upazila under the district of Mymensingh and 22 cattle (group B) were selected from Nokla Upazila under the district of Sherpur and prevaccinated sera were considered as control group. Experimental prepared HS vaccine was administered at the dose rate of 2ml (1.5OD600 = ≥7.5×108 CFU/ml) through subcutaneous route at the neck region in each group of cattle. Sera were collected at 28days after primary vaccination.

Collection of serum from cattle blood

Blood was collected prior to vaccination, 28days after primary vaccination using sterile syringe and needle. Syringes were then held in slanted position and blood was allowed to clot at room temperature for an hour. Blood clots were detached from the wall of the syringe by pressing the piston and were kept overnight in the refrigerator at 4 °C for separation of the serum. Then serum was carefully removed and centrifuged at 2000rpm for 10minutes for clarification and then stored at -20 °C in screw capped vials until used.

Inactivation of cattle serum

The serum samples were inactivated at 56 °C for 30minutes in water bath before PHA test.

Passive haemagglutination (PHA) test

Humoral immune response was determined by the application of passive hemagglutination test (PHA). Antibody titres of the bovine animals that immununized with experimental prepared oil based inactivated HS vaccine was determined by modified passive hemagglutination (PHA) assay according to the method described by Tripathy et al.,[14] with slight modification. The modification for the tests was as follows in Table 1.

Results and discussion

The*P. multocida*organism was characterized by using various microbiological techniques among the morphology, staining and cultural characteristics of the organism in different culture media were studied according to the procedure described by Carter & Rundell[12] reported that some strains of *P. multocida*might not grow in media without blood or blood serum. In the present investigation, the selected isolates were found to grow well in bovine blood and

nutrient agar media producing more or less characteristic colonies of *P. multocida*likesmall colonies (Figure 1 & 2). No hemolysis was noticed on BA media (Figure 2). The other characteristics of these colonies included whitish, discrete, opaque, circular and translucent in appearance. Shigidi & Lariviere et al.,[12,15–17] used bovine and sheep blood agar for the culturing of *P. multocida.* Culture on MacConkey agar, EMB agar, and SS agar plates yielded no colonies (Figure 3 & 4). The growth of*P. multocida*in NB was characterized by diffused turbidity and in few occasions pellicle was formed.

Table 1 Reagents/parameters for Passive haemagglutination (PHA) test

Reagents/Parameters	Tripathy et al.[14]	Present Investigation
PBS	PH 6.4	7.2
Tannic acid solution	1:25000	1:20000
Strength of Na2HP04. 12H2O	0.15 M	0.2 M
Strength of KH2PO4. 2H2O	0.15 M	0.2 M

Table 2 Biochemical tests and its results

Tests		Results
Fermentation reaction with five basic sugars	Dextrose/Glucose	+
	Maltose	-
	Lactose	-
	Sucrose	+
	Mannitol	+
	Control	-
Indole		+
Catalase		+
MR		-
VP		-

(+) = Fermentation with production of acid, (-) = No fermentation / negative, MR = Methyl red, VP = VogesProskauer.

Table 3 Mean PHA titers of sera of cattle pre-vaccinated and post-vaccinated with HS vaccine through SC route as determined by *t*-test

Groups	Route of vaccination	Sera collection interval (days)	PHA titer of pre-vaccinated and control (Mean±SD)	PHA titer (Mean± SD)	P-value by t test
				at 28 DPV	
A	SC	28	<4±0.00	134.86± 114.582	
B	SC	28	<4±0.00	169.14± 88.052	0.095

PHA: Passive Haemagglutination; DPV: Days post vaccination; SC: Subcutaneous route; Mean: Geometric mean of cattle; SD: Standard Deviation; NS: Non significant Level of significance: NS (P>0.01)

*P. multocida*organisms were Gram negative, cocco-bacillary or short rod shaped and generally arranged single or in pairs (Figure 5). Rod shaped bacteria with bipolar appearance was observed after Leishman's staining (Figure 6) of fresh culture of the organisms, which was indicative of *P. multocida.* Methylene blue staining technique revealed blue, cocco- bacillary shaped organisms (Figure 7). The above findings were also shown by De Alwis.[18] Bergey et al.,[19] also recorded similar staining characteristics of*P. multocida.* All the organisms were found to be non-motile when examined under microscope by hanging drop technique.

From the Table 2 it was revealed that the *P. multocida*organism fermented dextrose, sucrose and mannitol but not maltose and lactose. These fermented sugars produced acid (indicated by the change of yellow colour of the medium) without gas (Figure 8). The *P. multocida*organism also gave positive indole test and negative methyl red (MR), voges-proskauer (VP) test (Figure 9) and also gave positive catalase test (Figure 10). These biochemical reactions were closely correlated with Anupama & Topley et al.,[3,17,20,21]

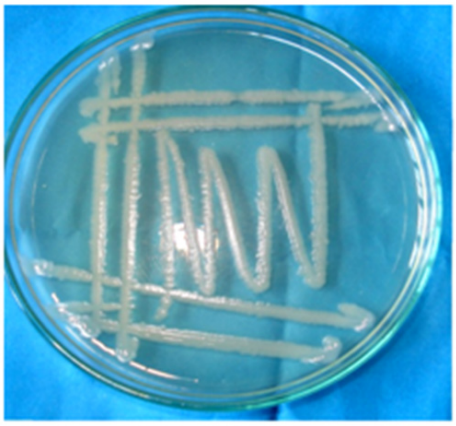

Figure 1 Colony in NA.

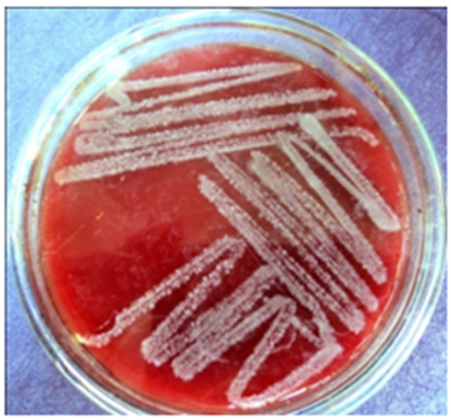

Figure 2 Colony in NA.

Jones et al.,[22] found that calf and mice died of acute septicemia after periocular and subcutaneous infection with *P. multocida*isolated from acute case of hemorrhagic septicemia. In experimental inoculation of *P. multocida*isolates in mice produced characteristic changes in different visceral organs. Following inoculation, mice died within 24hours and there was a marked septicaemic lesions consisting of white necrotic foci and hemorrhages in lungs, trachea, liver and spleen.

Passive haemagglutination test was conducted to determine the humoral immune response of the serum of cattle as per the method described by Tabatabaei & Muneer et al.,[5,23] The pre-vaccination PHA titers of sera samples of all vaccinated and control cattle was found with a mean of <4.00±0.00. After 28days of primary vaccination the mean PHA titers were 134.86 and 169.14 in group A and B respectively. In this present study, it was observed that group B produced comparatively better immune response than group A which was similar with the result of De Alwis & Tasneem et al.,[24,25] Vaccinated cattle produced the mean PHA titers were 134.86 and 169.14 in Group A and B respectively (Table 3). According to the findings of Mondal et al.,[26] after vaccination sera possessing IHA titre 1: 128 or above could protect challenge but sera having IHA titre 1:64 or below failed to protect infection with *P. multocida.* In this present experiment, it

was observed that sera from vaccinated cattle possessed higher titer than the findings of Mondal et al.,[26] Vaccinated cattle did not show any clinical signs such as transient rise in rectal temperature, dullness, depression and adverse reactions at the site of inoculation which were similar to the findings of De alwis& De lafaire et al.,[27–29] . From the above study it can be assumed that PHA titers from vaccinated cattle may be protectable against hemorrhagic septicemia disease.

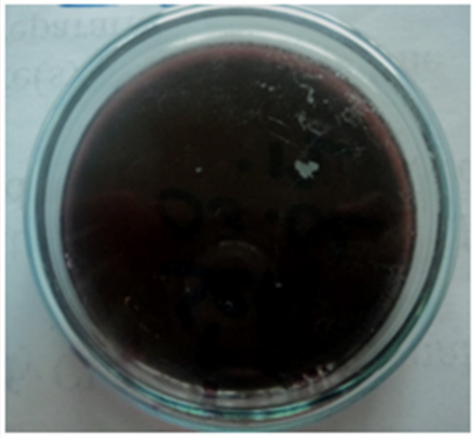

Figure 3 No growth in McConkey agar.

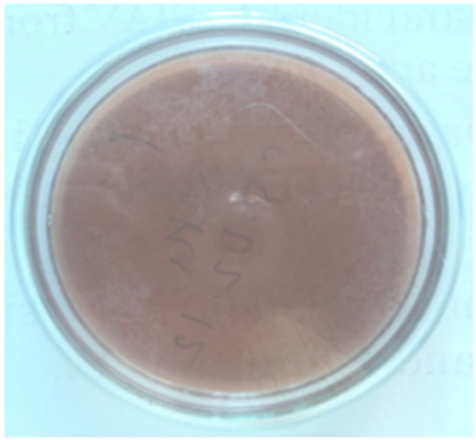

Figure 4 No growth in SS agar.

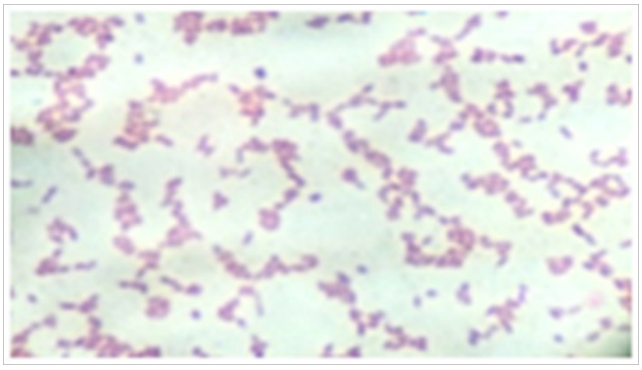

Figure 5 Gram`s Staining.

Conclusion

The present research work was carried out with a view to isolation and characterization of P. multocida from cattle, preparation of vaccine and determination of its efficacy. The isolates were identified by cultural and staining characteristics, morphological and biochemical characters, pathogenicity test. Demonstration and isolation of bipolar organisms (P. multocida) from blood, heart, liver, spleen and lungs of cattle were quite good. The experimentally produced oil-based inactivated haemorrhagic septicaemia (HS) vaccine induced high level of passive haemagglutinating (PHA) antibodies in the vaccinated animals. From the above study it can be assumed that PHA titers from vaccinated cattle may be protectable against hemorrhagic septicemia disease.

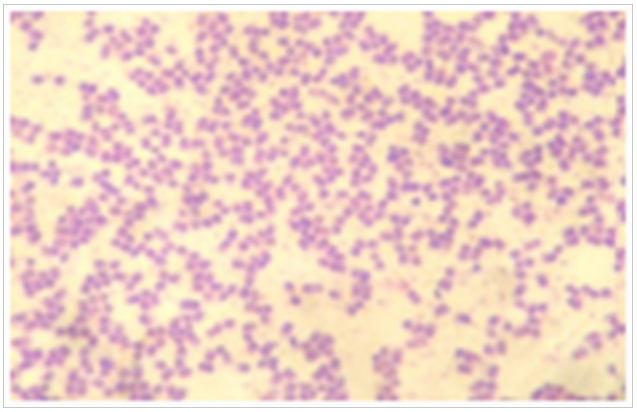

Figure 6 Leishman's staining.

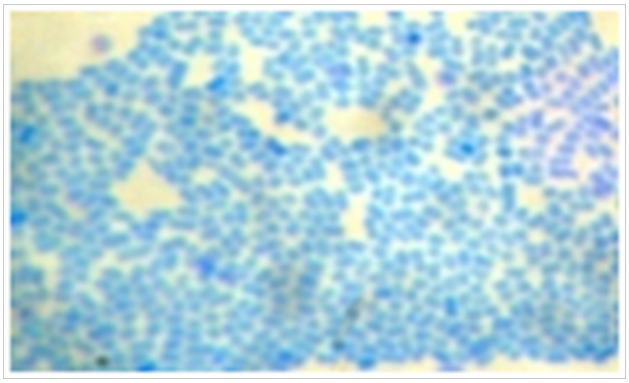

Figure 7 Methylene blue staining.

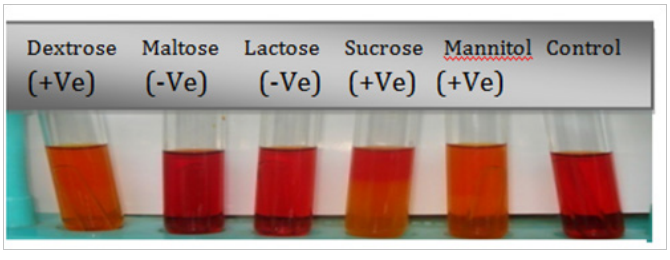

Figure 8 Sugar fermentation test *of P. multocida.*

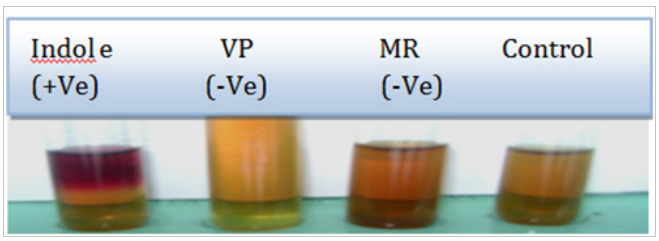

Figure 9 Indole, VP, MR tests of *P. multocida.*

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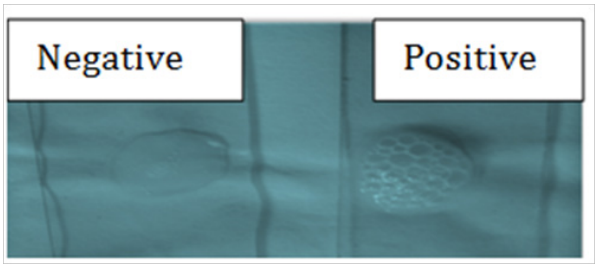

Figure 10 Catalase test of *P. multocida.*

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Conflicts of interest

Author declares there are no conflicts of interest.

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