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RESEARCH ARTICLE

A Novel Phage Based Marker Vaccine and DIVA Assay for Hemorrhagic Septicemia in Bovines

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ABSTRACT

Hemorrhagic septicemia (HS) is an acute, fatal disease of bovines caused by *Pasteurella multocida* serotypes B:2 and E:2. Presently used oil emulsion and/or alum precipitated vaccines suffer from adverse effects, short term immunity, need for repeated vaccinations and reports of vaccination failures. We report here development of a *Pasteurella* bacteriophage based marker vaccine (containing an iron restricted protein and an adjuvant) and an immunoblot based DIVA assay for HS in bovines. *P. multocida* (B:2) grown under iron restricted conditions using 2', 2' dipyridyl followed by lysis using a lytic Pasteurella bacteriophage to prepare the marker vaccine. Animals in Phage lysate vaccine (PLV) group showed higher antibody titers in comparison to conventional alum precipitated vaccine (CAPV) group at all stages of vaccination. The peak titers induced by the PLV group was 2.34±0.21 at 90 days post vaccination (dpv) as revealed by ELISA. In the CAPV group the peak antibody titers were observed at 60 dpv (2.13±0.07) by MAT. The detection of "novel iron restriction protein (137KDa) was done by western blot and ELISA. The serum of marker vaccinated cattle revealed presence of antibody to the 137KDa IROMPn in comparison to conventional alum precipitated HS vaccine group in which it was absent. The new marker vaccine with improved antigenicity and DIVA assays reported here would help in effective vaccination and thus the prevention, control and eradication of this disease, which is of great importance to farmers engaged in the rearing of bovines around the globe.

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INTRODUCTION

Hemorrhagic septicemia (HS) caused by *Pasteurella multocida* is an acute, fatal, septicemic disease of cattle and buffaloes (Annas *et al*., 2015). Catastrophic epizootics with high morbidity and mortality rates in Asian cattle and buffaloes are caused primarily by B: 2 strains and in Central Africa usually by serogroup E (Ranjan, 2011). Currently used alum and oil adjuvanted whole cell vaccines, provide some protection but suffer from short term immunity drawback. Alum precipitated *P. multocida* P_{52} killed bacterin confers immunity for four to six months only with reports of vaccination failure (Qureshi and Saxena, 2014).

Bacteriophage mediated bacteria lysis and subsequently generated proteins have been proved to be very good immunogens with stronger protection than conventionally inactivated bacteria especially in case of antibiotic resistant bacteria (Yosef *et al.,* 2015). The conventional methods (heat, chemical, and irradiation) of inactivating/killing bacteria for vaccine production have been reported to damage antigens and reduce their immunogenicity (Lauvau *et al.,* 2001). The idea of using natural bacterial pathogens such as bacteriophages is well known Bacteriophage mediated lysis releases bacterial antigens that are suited to act as immunobiological agents and. High antibacterial efficacy of lytic phages tested in animal models and clinics has been well established (Sekankova *et al.*, 1996; Drulis-Kawa *et al*., 2015). Phage lysates can be used as vehicles for vaccine antigens and promising results have revealed that such vaccines can also be used in animals as well as and humans (Clark and March, 2004). *Pasteurella* organisms when grown in vitro under iron restricted conditions reveal a different outer membrane/ whole cell lysates protein profile in comparison to those obtained under normal conditions. Outer membrane protein (OMP) extracts obtained by growing organism under iron restricted conditions are

more immunogenic than the corresponding outer membrane protein of the organism grown under normal conditions due to a proteinaceous material produced under iron restricted condition but not under normal growth conditions (Gilmour *et al.,* 1991; Kharab and ShivCharan, 2011). These proteins have been named as iron restricted outer membrane proteins (IROMPS) or siderophores.

In view of the economic importance and endemicity of HS in the livestock in Asia and Africa, and recurrent vaccination failures, the present study was undertaken to combine the beneficial effects of phage lysates and iron restricted growth conditions for the development of a novel phage lysate *P multocida* B:2 marker vaccine as well as a companion DIVA assay against hemorrhagic septicemia. The immunoblot assay (DIVA) targeted a predominant iron restricted protein (137KDa) present in the sera of the marker vaccinated cattle.

MATERIALS AND METHODS

Animals: Ten healthy male Zebu calves (*Bos indicus*) aged 6-8 months and adult Swiss Albino mice and Soviet Chinchilla rabbits were maintained at animal isolation unit and small animal house, respectively of GADVASU, Ludhiana. All the animal protocols were reviewed for IAEC guidelines and approved by the Institutional Animal Ethics Committee (IAEC).

Bacteriophage isolation and transmission electron microscopy (TEM): *Pasteurella* phage was isolated from sewage and liquid manure samples of animal sheds using agar over lay technique of Santos *et al.* (2009). The phage was amplified to 200ml master lot using the liquid culture method by Rawat and Verma (2007). The phage was tested for its lytic activity against *Pasteurella multocida* (B:2), *Pasteurella multocida type A* and other organism like *Salmonella dublin, S enteritidis, S typhimurium, Bordettela brochiseptica* and *Escherichia coli.* Purified phage suspension was subjected to TEM. Phage suspension (10µl) was spotted on top of a hydrophilic formvar-carbon-coated copper grid (Nissin, EM Corporation) and allowed to adsorb for 5min. Phages were stained with 2% aqueous phosphotunstic acid (pH 6.8) prepared in 1% ammonium acetate. TEM of the *Pasteurella* phage was done using Morgagni 268D, Fei Electron Optics, Electron Microscope, (200KV, 29000x magnification) at Department of Anatomy, AIIMS, New Delhi.

Preparation of marker vaccine: *P. multocida* B:2 field strain was used for preparation of a phage lysate vaccine after cultural, morphological, biochemical characterization (Cheesbrough, 2006) and capsular-PCR typing (Townsend *et al.,* 1998). *Pasteurella* phage was added in the culture (phage-bacteria ratio of 1:100) as per the multiplicity of infection of phage (Verma *et al*., 2013). The lysate was adjuvanted using sterile alum (10%w/v). The protein concentration of the phage lysate was determined by nanodrop spectrophotometer and lowry assay.

Sterility, safety testing and challenge experiments of lysate vaccine: The sterility and safety testing in mice of phage lysate marker vaccine was done as per Indian Pharmacopoeia Section 2.2.11 and OIE for testing of HS vaccines.

Vaccine experiment with the candidate marker vaccine: Animals (n=5 each) were categorized as PLM (phage lysate marker vaccine) and CAPV (conventional alum precipitated vaccine) group. Animals in PLM group were vaccinated with 3ml of phage lysate marker vaccine s/c, and CAPV group were vaccinated as per the recommended dosage (5ml, s/c). Clinical condition scoring was carried out daily for 10 days.

Sampling: Sera sample were collected pre- and after vaccination (0, 30, 60, 90, 120, 210 days post immunization) using vacutainer system. Sera were heat-inactivated at 56ºC for 30 min and stored at -20ºC until use.

Serological assays: Microplate agglutination test (MAT), Indirect Hemagglutination Assay (IHA) and indirect ELISA were used for estimation of antibody titers in sera sample. MAT was done as per the protocol of Kimura *et al.* (2008). The whole cell killed antigen for MAT was prepared from *P. multocida* (B:2) vaccine strain P_{52} at Punjab Veterinary Vaccine Institute. IHA was done as per the method of Sawada *et al.* (1982). Monoclonal antibody ELISA kit of Department of Veterinary Microbiology, COVS, LUVAS, Hisar, India was used to estimate HS specific IgG antibodies in the sera. The plates were read on a micro-plate ELISA reader (Tecan) at 450 nm.

HS DIVA: The outer membrane proteins/ IROMP enriched fractions of *P. multocida* P_{52} were subjected to one dimensional SDS-PAGE in 12.5% resolving gel and 5% stacking gel in a vertical slab gel electrophoresis system (Biorad) as per the method of Laemmli (1970). Immunoblotting was done as per the procedure of Towbin *et al*. (1979) using sera samples (1:200 dilution) from PLV, CAPV groups as well as with sera of animals from suspected field cases of HS.

Statistical analysis: Antibody responses were analyzed by ANOVA and 't'-test using SPSS-16 software.

RESULTS

The phage isolated and used in the present study was found to be *Pasteurella* genus specific and exhibited lytic activity against the vaccine strain P52 (B:2) as well as the multidrug resistant field isolates of*P. multocida* serotype (B:2) and fowl cholera agent (*P. multocida* type A:1) as well. The phage exhibited no lytic activity against *Staphyloccocus aureus, Escherichia coli, Brucella, Salmonella dublin, S. enteritidis, S. typhimurium* and *B. bronchiseptica* isolates indicating its specificity for genus *Pasteurella*. The negative stain transmission electron microscopy of the phage revealed an isometric head and a well-marked long non-contractile tail characteristic of the order Caudovirales, family Siphoviridae suggesting its placement to Group B. The icosahedral head of the phage measured approximately 27 x 24nm (Fig. 1). The lysate was found to be safe in mice. Mice inoculated with the lysate did not reveal any untoward reaction or death during an observation period of 7 days prior to immunization of the experimental cattle.

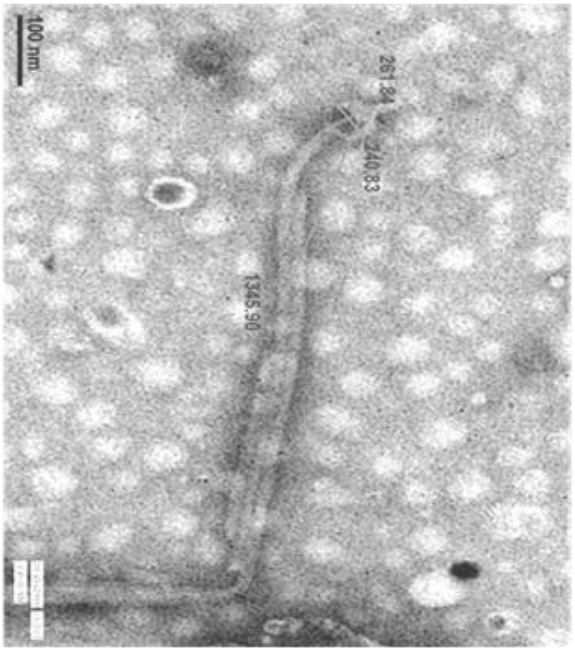

Fig. 1: Electron micrograph of *Pasteurella* phage (100nm scale)

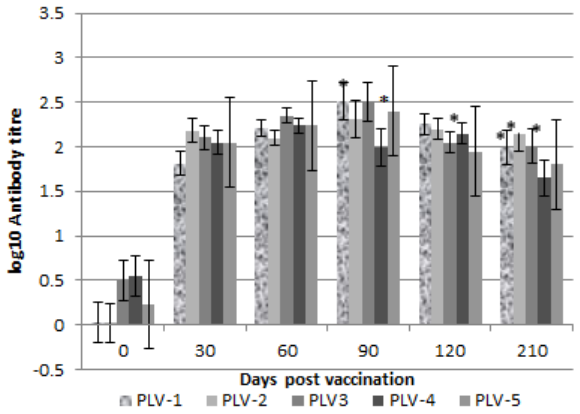

Fig. 2: Antibody responses of cattle in phage lysate vaccinated (PLV) group by ELISA

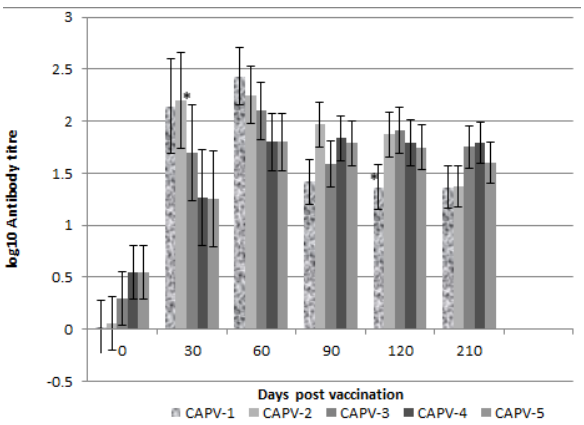

Fig. 3: Antibody responses of cattle Conventional alum precipitated vaccine (CAPV) group by ELISA.

Challenge response: All the unvaccinated mice in control group succumbed to challenge infection within 24 post challenge. Mice administered 90 DPI sera (15 mouse PD100) from cattle were found to have antibody titers that provided 100% protection to mice against challenge with virulent *P. multocida* P_{52}. Mice PD100/ml (minimum dose affording protection against challenge) of the lysate for P_{52} was estimated to be 0.1ml. However, 120 DPI sera from PLV group gave 70% protection to mice.

Antibody response to the vaccines: The antibody responses were measured prior (0day) and post vaccination in both PLV and CAPV groups at 30, 60, 90, 120 and 210 DPI. All the animals in PLV group showed a significant rise in antibody titers following vaccination peaking between 60-90 dpv by MAT, IHA and or ELISA. The PLV group showed higher titers as compared to CAPV group at all stages of vaccination though not always statistically significant (Fig. 2). The mean titers observed at earliest time point (30dpv) was 2.04±0.13 by ELISA in PLV group. The peak titers induced by PLV group were highest on 90 dpv (2.34±0.21). In the CAPV group the peak antibody titers were observed at 60 dpv (2.13±0.07) by MAT as well as by ELISA (2.07±0.27). ELISA revealed significantly higher titers ($P<0.05$, t test) at 120 (2.12±0.12) and 210 dpv (1.92±0.19) in the PLV group in comparison to the CAPV group on similar days post vaccination (1.73±0.21, 1.57±0.2, respectively). Comparison of titers between the two vaccine groups by't' test revealed that the vaccination titers were significantly higher in PLV group at 90 DPI ($P<0.05$) by MAT, 90, 120DPI ($P<0.05$) and 210 DPI ($P<0.01$) by IHA and 90 DPI ($P<0.05$) by ELISA, than those at the corresponding days in the CAPV group.

At 60 dpv in case of PLV group, antibody titers (log 10) peaked to 2.204 and 2.279 by MAT and IHA, respectively whereas in CAPV group of 2.13 and 2.05 were observed at 60DPI by MATand IHA, respectively. In comparison to PLV group the decrease in antibody titers in CAPV group was significant at 120 dpv with continuation of the trend upto 210 dpv (Fig. 3).

The present study revealed a novel iron restriction protein (137KDa) in the immunoblot with sera of PLV group of animals which was absent in the CAPV group as well in sera from naturally infected animals. Besides the major immunogenic iron restricted protein (IROMP) band of 137kDa immunoblot analysis revealed that some major immunodominant proteins like 32, 38, and 48kDa indicative of normal P_{52} protein profile present in sera from the PLV group were missing in the CAPV group (Fig. 4). Addition 100 and 70 kDa proteins were observed in PLV group immunoblot.

DISCUSSION

Bacteriophages are specific for their target bacterium and hence create no negative effects on the surrounding tissues or the environment (Basdew and Laing, 2014). Phage lysate bacterin contains bacterial fragments produced by the burst caused by lytic bacteriophage in combination with bacteriophage particles released by the burst leading to enhanced protective immune response. In case of some attenuated bacterial vaccines prepared through chemical fixation with aldehyde cross-linkers denaturation of macromolecules occurs. Similar changes are observed during heat treatment to kill bacteria (Lee *et*

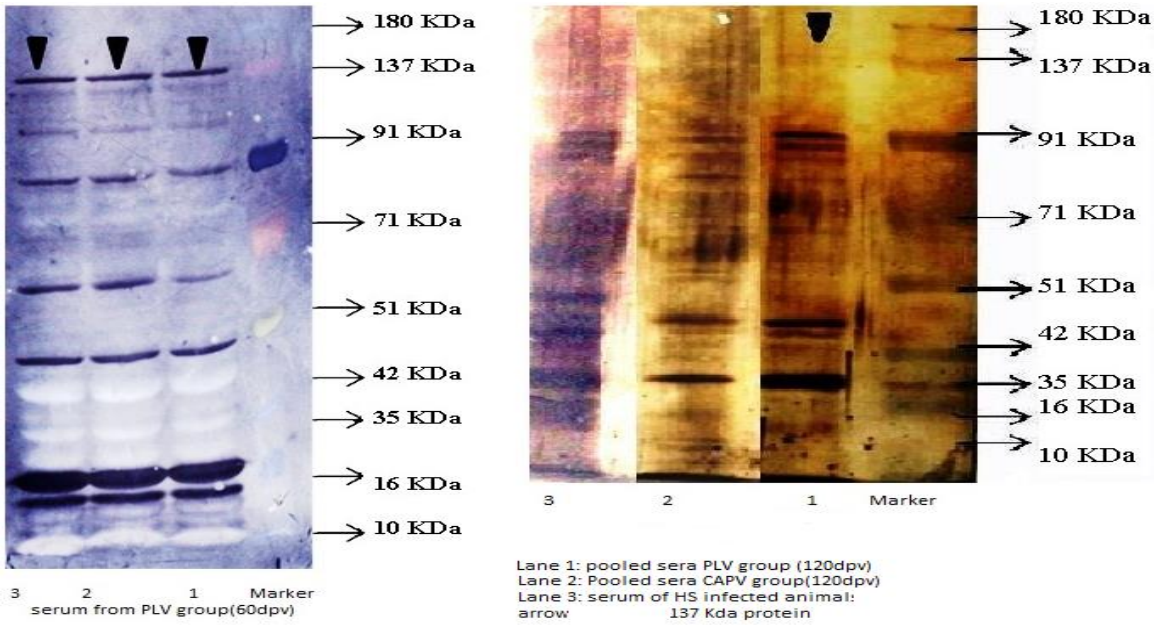

Fig. 4: DIVA assay (60 and 120days post vaccination)

al., 2012). Phage generated bacterial lysates and/or ghost preparation can be promising in veterinary medicine being owing to safety, effectiveness and relatively cost efficient. Phage lysates being simpler to prepare have an added advantage over ghost vaccines that require genetic engineering and optimal expression of e-gene containing constructs for bacterial lysis. The relative simplicity of preparation of phage lysates is advantageous when vaccine against multiple serotypes of a given species of bacteria have to be prepared. Bacteriophages represent a more economical and environment-friendly alternative to the environmentally-damaging use of chemical bactericidal (Balogh *et al.*, 2010).

Pasteurella phage isolated in present study had a icosahedral head and a non-contractile tail suggesting its placement in family *Siphoviridae* suggesting its placement to Group B Similar morphologies of bacteriophages of *Pasteurella multocida* have been reported earlier also. Similar morphologies of bacteriophages of *Pasteurella multocida* have been reported earlier also (Davies and Lee, 2006).

Gilmour *et al.* (1991) showed that vaccine containing iron regulated protein *of P. haemolytica* A2 enhanced protection against experimental pasteurellosis in lambs. Immunoblotting of sera from specific pathogen free lambs against whole cell antigens of *P. haemolytica* A2 grown under iron-restricted conditions demonstrated that antibodies to IRPs were present only in the sera of animals immunized with sodium salicylate extract of iron regulated proteins. Tesfaw *et al.* (2014) revealed that *Mannheimia haemolytica* A2 and A7 serotype combination expressing iron regulated outer membrane protein as a vaccine against intratracheal challenge exposure to *Pasteurella* in sheep expressed significant protection against homologous strains.

The 137kDa IROMP visible in immunoblot of marker vaccinated group of animals appears to be a promising antigen for development of a diagnostic DIVA assay in association with the novel marker vaccine. Monoclonal antibody based ELISA revealed more sensitivity in comparison to MAT and or IHA in detecting antibody titers at one or other stage post vaccination in PLV as well as in CAPV group. The phage lysate marker vaccine demonstrated sustained antibody titers in the animals even at 210 days post vaccination

Marker vaccines and their companion DIVA tests have been developed for very few bacterial infections (Mass *et al.*, 2006), but none is available till date against *Pasteurella multocida*. The novel phage lysate marker vaccine used in our study generated prolonged and higher magnitude of antibody response than the conventional alum precipitated HS vaccine. The new companion DIVA blot could also successfully differentiate between the immune responses of phage lysate vaccinated and conventional vaccinated or naturally infected group of animals.

Conclusions: The development of a field applicable DIVA assay is feasible as indicated with the results of DIVA immunoblot obtained in the present study. Such as DIVA blot in combination with the vaccine could go a long way towards better control and eradication of HS. However, recombinant IROMPs would be required for developing a commercial penside DIVA kit. HS phage lysate vaccine can also prove to be a better alternative to the currently available vaccines suffering from short term immunity or other drawbacks.

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Authors contribution: HMS conceived the project and SQ executed the plan. Both the authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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