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Active Immunization with *Pasteurella multocida* Lysate Elicits Antibody that Protects Rabbits against Virulent *Pasteurella multocida* and Protects Mice by Passive Immunization

R. Durairajan[1], Harshit Verma[2], Awadhesh Prajapati[3], Mohammed Abbas[4], Mayank Rawat[5], Rishendra Verma[6]

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ABSTRACT

Background: Pasteurellosis is an economically important disease of livestock worldwide and vaccination is effective mean to control the disease outbreaks. One of alternative method for vaccine preparation could be the use of lytic phages as bacterial inactivating agents and employing “phage lysate immunogen” as an immunizing agent. Therefore, the study was undertaken to explore the protective immune response of novel phage lysate vaccine against *Pasteurella multocida*.

Methods: A phage lysate vaccine of *Pasteurella multocida* was prepared using isolated lytic phage and subjected to vaccine response study in rabbits. In this experiment, two mouse fixed-dose of lysate was used and the prophylactic efficacies of lysates were evaluated in rabbits by passive mouse protection test (PMPT), western blotting and direct virulent challenge.

Result: Passive transfer of antibodies to mice using post-immunization sera of rabbits protected the animals against the challenge with A: 1 strain (75%) and B: 2 strain (50%). In western blotting, a total number of 4 bands were observed in the region between 130 kDa to 25 kDa in the protein of the phage lysate. The study suggested the significance increase in humoral immunity by phage lysate in murine Pasteurellosis.

Key words: Humoral response, Passive mouse protection assay, Phage lysate, Protective efficacy.

INTRODUCTION

Pasteurellosis is an important disease of livestock and poultry caused by gram negative coco bacillus *Pasteurella multocida*. Various efforts has been made towards development of different recombinant or synthetic immunogenic antigens using different adjuvant formulations, defined live mutants and DNA vaccines for prevention and control of Pasteurellosis in animals (Hodgson *et al*., 2005). However, none were found effective vaccine candidate to control the disease. The widely used alum precipitated heat-killed vaccine for Hemorrhagic Septicemia and Fowl cholera induces only a short duration of immunity (Qureshi and Saxena, 2014). Lytic phage as bacterial inactivating agents and employing “phage lysate bacterin” as an immunizing agent is an unexplored feasible method may be the use of generation of vaccine. Phage lysate preparation contains structurally unaltered antigenic moieties of the bacterial cell is not only expected to mimic a non-replicating live organism in the host and induce the desirable protective response at significantly lower doses that can be adjusted but also circumvent most of the drawbacks of killed, subunit or live attenuated vaccines. This can also be used to develop a cross-protective vaccine preparation. The phage lysis does not denature bacterial macromolecules, which is common during the heat inactivation of bacteria. Thus, phage lysis is a means of effectively killing bacteria with minimally alterations in antigenicity (Sulakvelidze *et al*., 2009) and lysate have potential candidates for vaccine development and improvement (Durairajan *et al*., 2012) since bacterins confer only homologous protection against the homologous strain of the organism. Moreover, a very large amount of biomass is needed for each dose of the vaccine which makes their products extremely difficult. Although live experimental

[1]Veterinary University Training and Research Centre, Tamil Nadu Veterinary and Animal Sciences University, Melmaruvathur-603 319, Tamil Nadu, India.
[2]College of Veterinary Sciences, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut-250 110, Uttar Pradesh, India.
[3]ICAR-National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru- 560 064, Karnataka, India.
[4]Department of Sheep Breeding, Union Trerritory-Ladakh, India.
[5]Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar-243 122, Bareilly, Uttar Pradesh, India.
[6]Mycobacterium Lab, Indian Veterinary Research Institute, Izatnagar-243 122, Bareilly, Uttar Pradesh, India.

Corresponding Author: R. Durairajan, Veterinary University Training and Research Centre, Tamil Nadu Veterinary and Animal Sciences University, Melmaruvathur-603 319, Tamil Nadu, India. Email: duraivet2006@gmail.com

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vaccines have been shown to promote heterologous and homologous protections, they carry unknown attenuation markers and in some cases, have been associated with disease outbreaks (Ashraf, 2014; Ahmad *et al.,* 2014). Cross protection has been achieved with bacterins (Glisson *et al.,* 1993; Sarangi *et al*., 2014) as well as with OMP's extracts from *P. multocida* grown in iron-depleted mediums. OMP's present in the phage lysate may induce cross-protection against a wide range of *P. multocida*. Therefore, the present study was conducted with aim to evaluate heterologous protection conferred by bacteriophage lysed *P. multocida* cells.

MATERIALS AND METHODS

Bacterial strains and production of homogenous phage stocks

The study was conducted during July 2010 to June 2012 at Indian Veterinary Research Institute (Deemed University), Izatnagar, Bareilly, Uttar Pradesh, India. The lyophilized culture of *Pasteurella multocida* P52 and A: 1 strain were revived by standard methods. Bulk stocks of phages were prepared by conventional liquid culture methods as described by Rawat and Verma (2007). 1 liter sterilized NZCYM broth in a flat bottom flask (3-liter capacity Hafkins flask) was inoculated with 50 µl of 18-hour pure broth culture of *Pasteurella multocida* serotype A: 1 and incubated at 37°C for 2 hours. To each flask, which contains the young culture sufficient quantity of a previously available stock suspension of phage was inoculated to attain a 1:100 final phage bacterial ratio. The phage bacteria mixture was incubated at 37°C with vigorous intermittent shaking until complete lysis obtained (approximately 6 hours was observed). The bacterial lysate was filtered through a 0.22-micron membrane filter and collected aseptically in sterile bottles. The stocks were stored at 40°C for 1 month to eliminate residual lytic activity attributed to phage-induced enzymes. The sterility of phage lysate stock was tested by standard methods (Indian Pharmacophoea, 2010).

Ethical statement

All the experimental protocols carried out on laboratory animals were approved by (Letter no:25 dated 2 Dec 2011, IVRI IAEC proceedings) the Institute Animals Ethics Committee (IAEC) of Indian Veterinary Research Institute (IVRI), Izatnagar-243122 (India). Animals were kept in IAEC-approved facilities and provided water and food ad-lib. Blood was collected through cardiac puncture of animal.

Virulence and protection tests

Six rabbits (1-1.5 kg) have been tested for anti-*P. multocida* antibodies by Passive Mouse Protection Test (PMPT) and free of antibodies were selected for further study. Four rabbits, were immunized subcutaneously with 250 µl of phage lysate and two rabbits were kept as unvaccinated control. On days 21 post immunization four vaccinated and two unvaccinated rabbits were given s/c injection of *P. multocida* P52 (10^6 CFU/ml).

Confirmation of *P. multocida* by PCR tests

Serogroup-specific primers used in PM-PCR and Cap-PCR assay for confirmation of *P. multocida* (Townsend *et al.,* 2001; Harshit *et al.,* 2014). The organism was revived in NZCYM broth by 18-24 h incubation at 37°C and plated subsequently onto blood agar to study cultural characteristics. The culture was then tested for purity by biochemical tests as per standard techniques. The genomic DNA of the isolate was extracted by CTAB method and the isolate was reconfirmed as *P. multocida* by PM-PCR followed by determination of capsular type by multiplex PCR.

Passive mouse protection test (PMPT)

Serum samples were collected from all the vaccinated rabbits on 0, 7th, 21st, 28th, 35th, 42nd days of post-vaccination. Serum samples were also collected from controls rabbits. Two groups of six mice with 6 weeks old were given 0.3 ml of pooled sera via the intraperitoneal route. After 24 hrs they were a challenge with 0.25 ml of 10^{-6} dilution of P52 and A: 1 strain that had been grown to early log phase in BHI broth. The infected mice were observed for 5 days and deaths were recorded twice daily.

Western blotting

Pooled sera of each subgroup were blotted against the P52 and *P. multocida* (A: 1) strain. The proteins were prepared by scrapping continuous lawn culture of P52 into 3 ml PBS. This suspension, as well as phage lysate components, were mixed with an appropriate proportion of sample buffer and placed in boiling water bath for 5 min., centrifuged and 20 µl of supernatant loaded per well on 1 mm, 12% polyacrylamide gel transferred to nitrocellulose membrane using 250 mAmp for 1.5 hrs in a standard transblot apparatus. Primary sera was applied at a dilution of 1/200 and incubated overnight at room temp. Rabbit anti-Goat IgG was applied at 1/1000 dilution and incubated for 2 hrs at 37°C blot were developed with 4-chloro 1-naphthol.

RESULTS AND DISCUSSION

This study helps to identify new approaches for the development of a nearly ideal vaccine against Pasteurellosis. The cell wall fractions including LPS, proteins and polysaccharides that contributes towards the development of 'clinical protection' in experimental or target host/hosts. Therefore, the development of an effective, single antigen-based preparation is not possible in near future. To determine the protective efficacy of the lysate vaccine formulations, direct challenge tests and passive mouse protection tests were performed. In bacteriological examination of blood smear we found *P. multocida* in the peripheral blood of all unvaccinated rabbits. Isolation and identification in blood agar shown few growths of *P. multocida* with special mold odor provided evidence of bacteremia in killed animals. In blood smears from affected animals, the organisms appear as short bacilli in Leishman staining. Both *P. multocida* type B strain P52 and A: 1 showed 460 bp products in PM-PCR.

Type A had an amplification of 1044bp while type B had 760bp using multiplex PCR (Fig 1 and 2). The results of the passive mouse protection test of phage lysate vaccine against A: 1 and B: 2 were depicted in the table. All the passively immunized mice showed 75% and 50% protection against A: 1 and B: 2 respectively of 42 days rabbit sera (Table 1). Experiments showed that s/c vaccinated phage lysate of *P. multocida* A serogroup protect rabbits against challenge with high dose wild type B: 2 strain and can act as an effective killed vaccine against virulent B: 2 strain. In the direct challenge study, an unvaccinated rabbit was died due to septicemic conditions, while in 70% of protection was found in vaccinated group (Table 2).

Immunoblotting of the *Pasteurella multocida* antigen was done with pooled sera from 21 and 35 days PI of the rabbit. Total numbers of 4 bands were observed in the region between 130 kDa to 25 kDa in the protein of the phage lysate. The molecular weight of those 4 bands was 25 kDa, 35 kDa, 55 kDa and 70 kDa respectively (Fig 3 and 4).

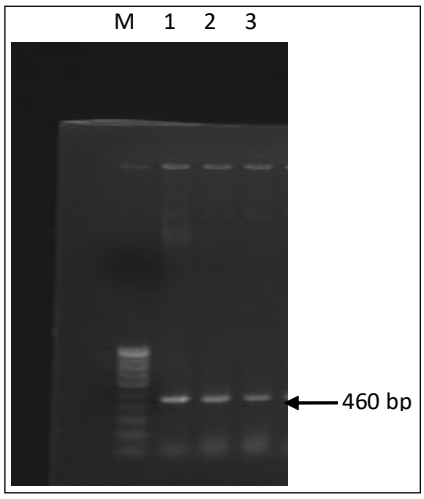

Fig 1: PM-PCR-460 bp.

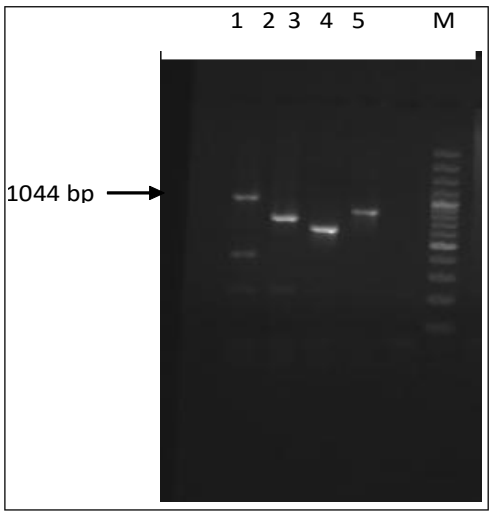

Fig 2: Multiplex-PCR- Lane 1-1044 (A), 4-760 (B).

Current vaccines are required to administer parentally, booster dose and are less efficacious (Proceeding of FAO/ APHCA workshop on HS 1991). Earlier studies showed that vaccination of buffaloes and poultry with killed bacteria generally results in serotype-specific immunity (Carpenter *et al.,* 1991; Qiang *et al.,* 2020; Shukla *et al.,* 2021) whereas, heterologous protection is conferred only with the attenuated strain (Elena Garrido *et al.,* 2008; Waffa *et al.,* 2014; Myint. 2000). Experiments using anti-Pasteurella sera suggested the significance of humoral immunity in murine pasteurellosis. Passive transfer of sera containing anti-lipopolysaccharides, outer membrane proteins and lipid antibodies could protect mice against direct challenge with virulent *P. multocida* (A: 1 and P52). Vaccines based on purified extracts or recombinant antigens will not lend themselves to large-scale production in countries where the disease exists. Though, these techniques support large-scale production in disease outbreak conditions. We found heterologous protection using phage inactivates cells with an only single administration. These results were supported

Table 1: Assessment of protective immune response of rabbits immunized with phage lysate by PMPT.

Groups	Challenge infection	
	A:1 No. mice challenged/ No. survived (% protection)	B:2 (P52) No. mice challenged/ No. survived (% protection)
Lysates	6/5 (75%)	6/3 (50%)
Control	6/0 (0%)	6/0 (0%)

Table 2: Assessment of protective immune response of rabbits by direct challenge study.

Groups	No. rabbits challenged/ No. survived (% protection)
Lysate vaccine	4/3 (70%)
Control	2/0 (0%)

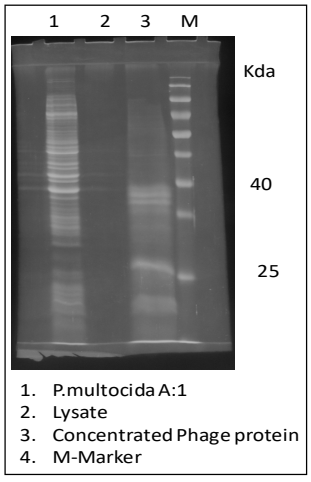

Fig 3: SDS PAGE analysis of lysate, *P. multocida* P_{52} and A:1 cell membrane protein.

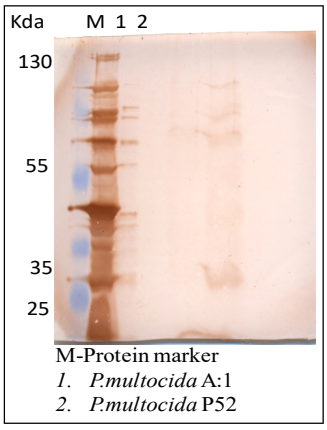

Fig 4: Western Blot analysis of rabbit pooled sera revealed 25 kDa, 35 kDa, 55k Da and 70 kDa proteins.

by the finding of Wang and Glisson (1994). Western blot and PMPT of pooled sera indicate that antibody (IgG) to identifiable protein in phage lysate were associated with protection. Esber *et al.* (1985) revealed that staphylococcal phage lysate elicit a significant increase in anti-staphylococcal IgG1, IgG2a and IgG2b level in experimented animals. Many attempts have been tried to explore specific immunogenic proteins of *P. multocida* isolates from HS and FC.

No single component of *P. multocida* is solely responsible for protective immunity. Bacterial cell wall fractions, LPS, proteins and polysaccharide contribute towards solid protective immunity. It can be opined from the present investigation that 27 kDa, 35 kDa, 55 kDa and 70 kDa proteins detected in phage lysates may be associated with a protective response against virulent challenge but, their specific role remains to be elucidated. By reviewing the current status and options for developing better immunizing preparations for Pasteurellosis, phage-lysate appears to be a methodology with great potential. Such preparations are expected not only to circumvent most limitations of live vaccines but also to have an additional advantage. Being inactivated preparations, their field use at the time of outbreak or during young age will be possible without any danger of death. Nevertheless, our results suggest that the use of phage inactivated vaccine is a good candidate for a new vaccine for Pasteurellosis and phage lysed cells of *P. multocida* provide umbrella-like protection against different serogroups of the organism. Hence, further investigations are needed for exploring the potential use of lower dose of phage lysate vaccine without booster vaccination regimen.

CONCLUSION

The results of the present study indicated that the phage lysate induced a strong antibody in the immunized rabbits. The passive transfer of antibodies to mice protects the animals against a challenge, indicating that antibodies alone were capable of giving cross-protection against different serovar of *P. multocida* infection. The phage-lysate methodology appears to be a better, technologically feasible and viable option as compared to other inactivation methods. Further immunogenicity trials in bovines may be conducted to develop suitable dose formulation that may be capable of inducing clinical protection in an endemic region.

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