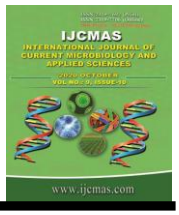
Int.J.Curr.Microbiol.App.Sci (2020) *9*(10): 732-737

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 10 (2020)**
Journal homepage: http://www.ijcmas.com



Original Research Article https://doi.org/10.20546/ijcmas.2020.910.088

Safety and Cross Protective Efficacy of Bacteriophage Lysate against *Pasteurella multocida* Infection in Mice

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Keywords

Bacteriophage, Lytic, *P. multocida* type A, Isolation, Therapeutic application

Article Info

Accepted:
07 September 2020
Available Online:
10 October 2020

A B S T R A C T

In this study established the methodology to isolate highly virulent bacteriophage and assessed the ability of this phage lysate to induce protection against wild-type challenge in mice and the natural host chickens. Subcutaneous administration of phage lysate to mice stimulated significant protection against *Pasteurella multocida* P_{52} and A1 serotypes. But, significantly phage lysate was able to induce protection through subcutaneous route against challenge with wild type *Pasteurella multocida* A1 serotype in chicken but not through intranasal administration of phage lysates

Introduction

Haemorrhagic septicaemia (HS), the fatal septic-shock of cattle and buffaloes, and Fowl Cholera (FC) of poultry are the two 'Pasteurelloses' against which safe and effective bacterins are currently available commercially, and used.

Conventional inactivated bacterins against HS and FC lack the ability to induce an across-serotype or serogroup response. HS vaccines containing killed whole cells of P_{52} (B: 2), or any of the recognized vaccine strain, along with an adjuvant, fail to induce a cross-protective response against serotypes of A (Samanta and Rawat 2005). Hence this study was made to determine bacteriophages, and phage lysate products have been identified as suitable alternative vaccine.

Also exploits cost protective solid immunity to control the different serotypes of *Pasteurella multocida* infection in poultry and livestock.

Materials and Methods

Bacteriophage isolation

To isolate lytic bacteriophages, 1 ml of log phase *P. multocida* culture in NZCYM broth (DIfco), 10 ml NZCYM broth and 5 ml farm yard slurry/pond water were combined and incubated at 37^{o}C overnight in shaking incubator at 100 rpm. Next day, culture was centrifuged at 5000 rpm to remove cell debris and clear supernatant was filtered (0.22µ millipore) to obtain bacterial free filtrate (BFF). BFF was analyzed for the occurrence of lytic phage, if any, by soft agar overlay method (Adams, 1959)). In brief, four sterile test tubes each filled with 5 ml soft NZCYM agar (0.6%) were set in a water bath at 47^{o}C to prevent agar from solidifying. First, second and third tubes were fed with one, three and six drops of BFF, whereas fourth tube was left unfed and served as control. Thereafter, each tube was added with 0.3 ml early log phase pure culture. After a brief vortex, content of each tube was poured onto four separate petri plates each containing a layer of hardened bottom brain heart infusion agar (BHI) 2.5%. The top soft agar was spread evenly on hard agar surface, allowed to stand at room temperature for 15 min, incubated at 37^{o}C for 18-24 hrs and inspected for Phage plaques. Phages were purified by streaking in individual plaque on BHI agar plates overlaid with NZCYM soft agar containing early log phase culture of respective indicator strains. Plates were incubated at 37^{o}C for 18 h and inspected for clear zones around streak line. Phages were harvested with SM buffer (50 mM Tris-HCL (pH 7.5) 100 mM Nacl, 10 mM $MgSo_{4,}$ 2% gelatin). Gross agar shreds were removed by slow speed centrifugation and supernatant containing purified phages were filtered through (0.22µ). Purified phages were confirmed by plaque assay using the soft agar overlay method [Adams (1959)]. Briefly, 100 appropriately diluted phage and 300 early log phase culture of indicator organism were added to 5 ml molten NZCYM soft agar (0.6%), mix well poured on to BHI agar plates, incubated at 37^{o}C for 18 h examined for plaques.

Production and storage of homogenous phage stocks

Bulk stocks of phages were prepared by conventional liquid culture methods described by Eisentark (1967) 1 litre of sterilized NZCYM broth in a flat bottom flask (3 litre capacity Hafkins flask) was separately inoculated with 50µl of 18 hour pure broth culture of *P. multocida* serotype A1 and incubated at 37^{0} C for 2 hours. Stock suspension of phage was inoculated to attain 1:100 final phage bacterial ratio Rawat and Verma (2007). The phage bacteria mixtures were incubated at 37^{o} C with vigorous intermittent shaking until complete lysis (approximately 6 hours was observed). The bacterial lysates were filtered through 0.22µ membrane filter and collected aseptically in sterile bottles. The stocks were stored at 4^{0} C for 1 month to eliminate residual lytic activity attributed to phage induced enzymes. The sterility of phage stock was tested by standard methods (Indian Pharmacophoea, 1996). Using appropriate quantity soyabean casin digest medium and fluid thioglycolate medium.

Determination of titration of phage of stock suspension

Serial 100 fold dilution of phage stocks was prepared in sterile SM buffer. 100µl of each dilution beginning from 10^{2} onwards to 10^{12} was mixed with 300 µl of 18 hour incubated pure NZCYM broth culture of *P. multocida* A1 in sterilized tubes. The phage bacteria mixture was allowed to stand at 37^{0}C for 20 minutes. The content of each tube was then added to 3 ml of sterile molten soft agar at

47^0C mixed thoroughly by vortexing and immediately poured onto plates containing hardened BHI agar. The plates were incubated at 37^0C for 18 hours and plaques were counted after incubation.

Safety test

Safety test of purified phage must conducted in mice. Four separate group of 5 mice each injected with phage (10^8 PFU/mouse) through i/m, s/c, i/p and i/v routes, were kept at constant monitoring under ideal condition for 20 days.

In vivo prophylactic efficacy in mice

Invivo prophylactic efficacy of phage against host bacterium (*P. multocida* serotype P_{52} and A1) was assessed in experimental mice. Healthy adult mice of either sex weighing not less than 18-20 grams where subdivided into 3 groups (G1-G3) of 3 mice each marked with positive control (G1) 1µl of phage lysate (10^8 PFU/ml) administered mice (G2) and 0.5 ml of phage lysate administered mice is G3. After 4-5 hours administration of phage lysate all the groups (G1-G3) where s/c challenged with 0.2 ml of experimentally grown 10^6 dilution of *P. multocida* serotype P_{52} culture containing 5 x 10^8 CFU following the lethal dose recommended by other investigation.

The safe experimental trial has been conducted against *P. multocida* serotype A1 as well. But the challenge dose of *P. multocida* A1 culture containing 5 x 10^9 CFU (10^7 dilution) were recommended.

In-vivo prophylactic efficacy in mice after 21 days administration of phage lysates and *in-vivo* prophylactic efficacy of phage lysate against host bacterium was assessed in experimental mice after 21 days administration of phage lysate. Healthy adult mice of either sex weighing not less than 18-20 grams where subdivided into 3 groups (G1-G3) of 3 mice each marked with positive control (G1) 1 µl of phage lysate (10^8 PFU/ml) administered mice (G2) and 0.5 ml of phage lysate administered mice is G3. After 21^{st} day of administration of phage lysate all the groups were s/c challenged with 0.2 ml experimentally grown 10 dilution containing 5 x 10^8 CFU *P. multocida* P_{52}. The same experiments were conducted against the challenging organism of *P. multocida* A1. For challenging with A1 organism the recommended mouse lethal dose 10^7 dilution containing 5 x 10^9 CFU/ml. All the mice were observed for any mortality or morbidity until for 5^{th} days from the challege of the mice.

In-vivo prophylactic efficacy in birds challenged with *Pasteurella multocida* A1 serotypes

In-vivo prophylactic efficacy of phage lysate against host bacterium (*P. multocida* A1 serotypes) was assessed apparently healthy birds (7-8 weeks) were subdivided into 3 groups G1 – G3 of 3 birds each. Marked G1 positive control, marked G2 phage lysates were administered through intra nasal and marked G3 phage lysate were administered through subcutaneous route. The dose rate of phage lysate is 100µl (1:2 dilution). All the birds were challenged with 1ml of 10^7 dilution of experimentally grown $5x10^9$ c.f.u/ml bacteria. Following the lethal dose recommended by other investigators, the birds were observed for any morbidity and mortality until 72 hr after post challenge.

Results and Discussion

Lytic phages: Four lytic phages against the *P. multocida* A1 serotypes were isolated. Phages were christened as PM-IVRI-1, PM-IVRI-2 and PM-IVRI-3 respectively. But only one lytic phage against *P.multocida* P_{52} isolates were isolated.

Safety test: All the groups of the mice which inoculated with phages were alive and active after 20 days of inoculation.

In vivo prophylactic efficacy

Mice were showing protection when it is challenged with 0.2ml of experimentally grown 10^6 dilution of *P. multocida* P_{52} culture.

In vivo-prophylactic efficacy in mice after 21 days administration of phage lysates

Mice were inoculated with single dose of upto 10^8 PFU phage lysates and 20 days apart the mice were challenged with highly virulent homologus as well as heterologus virulent bacteria. Both control mice were died after 24 hrs after challenge with lethal dose of A1 seotype. In contrast, mice immunized with phage lysate were showing 100% protected when immunized with the 1.0µl as well as 0.5 µl of phage lysate.

In vivo prophylactic efficacy in natural host by challenging with *P.multocida* A1 serotype

Birds were injected with upto 10^8 PFU phage lysates and 6 hrs and 21 days apart the bird were challenged with highly virulent wild type *P. multocida* A1 serotype lethal dose.

In S/C inoculation one bird has survived after challenged with homologus strain of *P. multocida*. In contrast, intranasaly inoculated as well as control birds were being died after 24 hrs challenge.

Table:1 Protection acquired in mice by s/c immunization with phage lysates of P. multocida against P52

Dose of phage lysate	Challenge dose P52	No of mice Challenged	Survived mice after challenge 24h	48h	72h	86h	120h	144h
1.0µl	0.2ml	3	2	1	1	1	1	1
0.5µl	0-2ml	3	3	3	2	1	1	1
Control	0.2ml	2	Sick	died	-	-	-	-

Table.2 Protection acquired in mice by s/c immunization with phage lysates of P. multocida against A1 serotype

Dose of phage lysate	Challenge dose P52	No of mice Challenged	Survived mice after challenge 24h	48h	72h	86h	120h	144h
1.0µl	0.2ml	3	3	3	3	3	3	3
0.5µl	0-2ml	3	3	3	3	3	3	3
Control	0.2ml	2	Sick	died	-	-	-	-

Table.3 Protection conferred in birds by s/c Immunization with phage lysates of *P. multocida* against wild *P. multocida* A1 serotype

Group	No of birds	Challenge of birds after 6hr Post Innoculation	Survived birds after challenge 24 hr	48 hr	72 hr	96 hr	120 hr	144hrs
S/c	3	1.0 ml (10^{-7} Dilution)	3	1	1	1	1	1
I/N	3	1.0 ml (10^{-7} Dilution)	2	-	-	-	-	-
Control	4	1.0ml (10^{-7} Dilution)	All Died		-	-	-	-

Table.4 Protection conferred in birds after 21 days immunization with phage lysates of *P. multocida* against wild *P.multocida* A1 serotype

Group	No of birds	Challege of birds after 21 day Post Inoculation	Survived birds after challenge 24 hr	48 hr	72 hr	96 hr	120 hr	144hrs
S/c	2	1.0 ml (10^{-7} Dilution)	2	2	2	2	2	2
I/N	3	1.0 ml (10^{-7} Dilution)	2	1	1	1	1	1
Control	4	1.0ml (10^{-7} Dilution)	All Died		-	-	-	-

Phage lysate were giving 100% protection when lysate were inoculated through S/C. In contrast, Intranasal inoculated birds were showing the 0% protection after 21 days immunization.

The high abundance of the *P. multocida* in livestock and poultry indicates its pathogenic importance. Minimizing its community spread and its economical loss has the primary task of modern medicine. In addition, inefficiency of most commonly used vaccines (Alum precipitated vaccine and Agar washed vaccines) advocate their ability to survive and keep them vicious cycle on in vaccine rich environment. Isolation of lytic phages against indicator *P. multocida* strain from farm yard slurry/pond illustrates the human/animals excreta is a rich repertoire of anti *P. mltocida* phages. Broad lytic efficacy of this phage to *P.mltocida* indicates it as safe antibacterial agent and thus therapeutic application of this phage would not hamber the growth of host normal microflora.

Phage lysates were recommended to safety suggest that inoculation of phage lysate at 10 fold higher doses (10^8 p.f.u/ml) to that of prophylactic dose 10^7 was safe and thus phage lysate may be used for animal safety and followed by clinical prophylactic trials.

In-vivo protection efficacy of phage lysate in mice reveals its prophylactic significance to alleviate clinical symptoms induced by *P. multocida* challenge infection. Immunizing trial in mice involved phage lysed bacteria giving better protection as that live bacteria. Likewise, phage lysed salmonella have demonstrated to induce better protection than heat killed/formalin killed strain (Muotiala *et al*, 1989). The component of phage lysate are presented to the host in their natural antigenic state, which may altered by heat, formalin or other treatments used to kill bacteria. In addition, live antigenic states induced better cell mediated immunity than killed vaccines and live bacteria, but not killed bacteria, induce macrophage to produce high level of IL-12 in vivo, which can induced naive T-cell to produce IFN ¥ (Th1 response), activating the bactericidal activity of macrophages and CD 8 cytotoxic T cells, both component of cell mediated immunity (Matsui *et al*, 1992 and Cheers 1996). The identity and function of cross-protective antigen in *P. multocida* are still not well characterized, but several studies have indicated that cross protective factor are produced exclusively. In this study, phage lysate giving protection to *the P. multocida* infection in mice model as well as natural host. In this study, phage lysate were giving protection to *the P. multocida* infection in mice model as well as natural host. The importance of the phage lysed bacterial components has been giving protection/ resistance were suggested by various studies (Sulakvelidze *et al.,* (2001), Pasterneck, 2009). However the role of phage lysate in acquired immunity has been controversial, but that is authenticated in the salmonella infection. Through the use of phage lysates, our results demonstrate for the first time that solid immunity to *P. mltocida* B2 and A1 can

be stimulated by phage lysed bacterial components.

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How to cite this article:

Durairajan, R., Harshit Verma, Awadwsh Prajapati, Mohammmed Abbas, Mayank Rawat and Rishender Verma. 2020. Safety and Cross Protective Efficacy of Bacteriophage Lysate against *Pasteurella multocida* Infection in Mice. *Int.J.Curr.Microbiol.App.Sci*. 9(10): 732-737.
doi: https://doi.org/10.20546/ijcmas.2020.910.088