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Selection of phage display peptides against *Pasteurella multocida* using suspension method of biopanning

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ABSTRACT

P. multocida contain various surface-associated antigens that could be used as a target for both therapeutics as well as diagnostics. The current study was planned to select ligands using Ph.D.-12 phage display library. This library was amplified and subjected to the alternate selection/subtraction methodology of biopanning using the suspension method in which alternate rounds of positive selection against *P. multocida* and negative selection against *Haemophilus influenzae* and *Actinobacillus lignieresii* were performed. After completing biopanning, out of 48 selected phages, 16 clonal phages were selected for indirect phage ELISA to check their binding efficiency with *P. multocida*. Out of these 16, five clonal phages bound their target with high intensity giving higher OD values at 450 nm and their binding efficiency was compared with closely related *Actinobacillus lignieresii* and *Hemophilus influenzae* using 10^7 pfu/ml at 450 nm wavelength which was found to be less against these bacteria.

Keywords: Biopanning, Indirect ELISA, *Pasteurella multocida*, Phage display peptide

Hemorrhagic septicemia (HS) is an acute, fatal and septicemic disease of cattle and buffaloes caused by *Pasteurella multocida*, an important disease in tropical regions of the world, especially in African and Asian countries (Dutta *et al*. 1990). *P. multocida* reside mostly as a commensal in the nasopharynx of animals (El-Jakee *et al.* 2016).The prevalence of HS has been well documented with predominant isolation of *P. multocida* serotypes B:2 and E:2 (El-Jakee *et al.* 2016).

P. multocida, a gram-negative and non-motile bacterium, have five capsular serogroups (A, B, D, E, and F) and 16 physical serotypes (1 to 16) (Qureshi *et al.* 2018, Dinos 2017, Verma *et al.* 2013, Chen *et al.* 2018, Chen *et al.* 2019). Not only in cattle and buffaloes, *P. multocida* is also involved in a variety of diseases in livestock (De Alwis 1996). In India, HS is of great economic importance mainly due to the high mortality in susceptible populations and has been quantified as the number one bacterial killer disease among cattle and buffaloes (Dutta *et al.* 1990). In India, about 50,000-55,000 deaths and losses exceeding ₹10 million have been recorded in bovine (Dutta *et al.* 1990).

P. multocida contain various surface virulence factors such as the capsule, lipopolysaccharides, adhesins, toxins, siderophores, sialides, and outer membrane proteins (Harper *et al.* 2006). May *et al.* (2001) reported that *P. multocida* encodes for 104 putative virulence-associated genes which make 7% of the coding density of the genome, means the major portion of *P. multocida* for their virulence capability still need exploration.

At present, we lack good diagnostic tools for the diagnosis of HS in the live animal at an early phase of the disease and also lack better therapeutics to treat HS. For development of specific and sensitive diagnostic (ready to use kit) and better therapeutics (vaccine and drugs), phage display technique can play an important role.

Phage display is generally a new strategy which can be used to select antibodies and peptide (polypeptides) on the surface of bacteriophage. Filamentous bacteriophages (f1, fd and M13) are commonly used for phage display (Bazan *et al.* 2012). Most antibodies and peptides are displayed at phage proteins pIII (Cho *et al.* 2012) and pVIII (Hess *et al.* 2012). Peptide libraries displayed on the surface of phages have multiple application viz. drug discovery, identification of ligand-binding activity, identification of biomarkers, and epitope mapping (Gallop *et al.* 1994, Lam *et al.* 1991). Use of this technique to find surface-associated antigen of pathogen play important role in diagnostics and therapeutics (Cesareni 1992). For identification of surface-associated antigens, the phage library having several phage clones and each phage clone expresses a particular type of fusion with one of the surface proteins of the bacteriophage and in this way, the desired (poly) peptide get chosen by binding to different target by biopanning (Smith 1985).

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The isolated individual clone's specificity can be analyzed by enzyme-linked immunosorbent assay (ELISA) and characterized by DNA sequencing (Hoogenboom *et al.* 1998). After that, specific phage can be isolated and confirmed using phage specific PCR. Further, peptide from a specific phage can be isolated and can be sequenced to know the amino acid sequence. Keeping the above points in mind, this study was planned to select phages that show high binding efficiency for *P. multocida* B:2 using indirect ELISA.

MATERIALS AND METHODS

Phage display peptide library (Ph.D.-12) and bacterial isolates: The stock phages were propagated in supplied *Escherchia coli* strain (ER2738) and harvested to prepare working phages as per Ph.D.-12 phage display kit protocol. Two bacterial cultures *Actinobacillus lignieresii* (MTCC 3351) and *Hemophilus influenzae* (MTCC 3826) were procured from the MTCC (Microbial type culture collection and gene bank) and cultured. *Pasteurella multocida* B:2 isolate was procured from the Department of Veterinary Microbiology, Dr G. C. Negi College of Veterinary and Animal Sciences, CSK HPKV, Palampur, India and cultured as per standard protocol (Garcia 1998). Confirmation of *Pasteurella multocida* B:2 isolate was done by PM-PCR using primers and molecular characterisation of capsular B antigen by using specific primers (Table 1).

Phage amplification: Phage broth (20 ml; 5g/l KCl, 10g/l trypton and 2% Maltose) containing tetracycline (20 mg/ml) in 250 ml flask were inoculated with 200 µl of overnight *E. coli* culture and 1 µl of phage stock, incubated overnight in a shaker incubator at 37°C, 250 rpm. After the overnight incubation, the culture was transferred to a falcon tube (Tarson) and centrifuged at 4500 g for 10 min at 4°C (Cooling centrifuge, Eppendorf). Without disturbing the cell pellet, the supernatant was decanted into a new falcon tube. The centrifugation was repeated and after the final centrifugation, upper 80% of the supernatant was pipetted into a fresh tube and 1/6 volume of PEG/NaCl solution (20% polyethylene glycol and 2.5 M sodium chloride) was added to the supernatant and left to precipitate overnight at 4°C. On the next day, PEG precipitate was centrifuged in oak ridge tubes at 12,000 g for 15 min at 4°C. The supernatant was decanted and the tube was re-centrifuged for 1 min to remove any residual supernatant using a pipette. The pellet was resuspended in 1 ml of TBS (Tris-buffered saline), then transferred to a microcentrifuge tube and centrifuged for 5 min at 4°C to pellet residual cells. The supernatant was transferred into a fresh microcentrifuge tube.

Phage titration: To perform phage titration, 1 µl of an overnight culture of *E. coli* was inoculated into the 10 ml BHI broth containing tetracycline (20 mg/ml) in a 250 ml Erlenmeyer flask and incubated at 37°C for 4-5 h in a shaker incubator (250 rpm). Meanwhile, top agar (5 g/l KCl, 10 g/l tryptone, 1% glycine, 2% maltose, 9 g/l agarose) was melted in a microwave and 1 ml of top agar was dispensed into sterile tubes (one per expected phage dilution) these tubes were kept at 50°C in a water bath to avoid the solidification. Later on, this top agar was used to overlay the *E. coli*-phage mixture on phage agar plate coated with X-gal and IPTG. Phage agarose plates (3 g/l $CaCl_2$, 5 g/l NaCl, 10 g/l tryptone, 11 g/l agarose) containing tetracycline (20 mg/ml) were then coated with 10 µl of X-gal (50 mg/ml) and 100 µl of 0.1M IPTG with L-spreader. The plates were then pre-warmed at 37°C for 30 min. Hundred fold serial dilutions of phage was prepared in TBS. *E. coli* culture (500 µl) was dispensed into 100 µl of each phage dilution. The tubes containing the *E. coli*-phage mixture were incubated for 15 min at room temperature and then mixed with top agar. Top agar containing mixture of *E. coli* and phage was poured onto the prewarmed IPTG-Xgal-agarose plate after thorough mixing. The plates were gently tilted to spread the mixture, cooled till top agar solidifies, and incubated overnight at 37°C. The plates with blue plaques (30-300) were counted and each number was multiplied by the dilution factor for that plate to get phage titer in plaque forming units (pfu) per ml.

Biopanning: In this study, suspension method of biopanning was followed as described by Lakzaei *et al*. (2019) with few modifications to eliminate the non-specific binding to Bovine Serum Albumin (BSA) present in blocking buffer and to the surface of the microtiter plate. In this study, the biopanning was done against *P. multocida*, which shares similar surface antigen with other members of *Pasteurellaceae* like *H. influenzae* and *A. lignieresii* to prevent the selection of phages that show cross-binding with these two other members of *Pasteurellaceae*, a biopanning strategy with alternate three rounds of positive and two rounds of negative selection was followed.

Positive selection was done against *Pasteurella multocida* B:2 in a U-bottom culture vial. In this, suspension of 100 µg/ ml of fresh *P. multocida* pellet was prepared in 0.1 M $NaHCO_3$ and 100 µl of this solution was transferred to the culture vial. Then, 100 µl of amplified phages of concentration 2.1×10^{13} pfu/ml were added to the 100 µl *P. multocida* solution. The suspension was incubated at room temperature with constant agitation at 150 rpm for 2 h. After 2 h of binding, the suspension was centrifuged

Table 1. Primers used for the detection and capsular typing of *P. multocida* isolates (Townsend *et al.* 1998)

Primer	Primer Sequence (5'-3')	Gene	Product size (bp)
KMT1T7	ATCCGCTATTTACCCAGTGG	KMT1	450
KMT1SP6	GCTGTAAACGAACTCGCCAC		
Cap B F	CATTTATCCAAGCTCCACC	Cap B	760
Cap B R	GCCCGAGAGTTTCAATCC		

at 4500 g for 5 min at 4°C. Supernatant with unbound phages was discarded. Pellet left at the bottom of the microcentrifuge tube contained phages bounded to bacteria. After giving washings with the TBST (Tris-buffered saline + 0.4% Tween 20) following repeated centrifugation, the next three washings were given with TBS in order to wash out non-specific bindings. Pellet was properly resuspended in 1 ml of elution buffer [0.2 M Glycine HCl (pH 2.2), 1 mg/ml BSA] and kept at 4°C for 1 h. After 1 h of elution, neutralization buffer [1M Tris-HCl (pH- 9.1)] was added @150 µl/ml of elution buffer and centrifuged at 4500 g for 5 min. The supernatant containing phages was collected in a new centrifuge tube and the pellet was discarded.

For negative selection, these eluted phages were first amplified and titered to know the concentration of amplified phages. In negative selection, the panning was done against *H. influenzae* and *A. lignieresii* using 100 µl of 10^{13} pfu/ml concentration of phages. Unlike positive selection, in negative selection, phages that would remain unbound in the supernatant were used for next positive selection. At the end of each round of biopanning, eluted phages were titered to calculate the amount of phage using equation as follow:

$$\text{Amount of phage (pfu/ml)} = \frac{\text{Number of plaques} \times \text{Dilution factor}}{\text{Volume of diluted phage solution (ml)}}$$

After final biopanning round, eluted phages were titered again. Few plaques from plate were picked up by directly stabbing blunt microtip onto plaque. Those agarose plugs containing phages were then kept for elution in 1ml of prewarmed suspension buffer (55˚C) for 2-4 h. These eluted phages were then amplified separately to the concentration of 2×10^{13} pfu/ml. Next day amplified phages were precipitated and titered to know the concentration of amplified phages to carry out the phage indirect ELISA.

Phage iELISA (10^7 pfu/ml): In this study, the indirect ELISA protocol was followed according to Valadon and Scharaff (Valadon and Scharff 1996) with few modifications. A 96-well flat-bottom microtitre plate was coated with a suspension of *P. multocida*. After coating, uncoated sites were blocked using a blocking buffer [0.1 M NaHCO3 (pH 8.6), 5 mg/ml BSA]. Thereafter, 100 µl of 10^7 pfu/ml phages were applied to the blocked plate. Two antibodies viz. anti-M13 filamentous phage antibody (1:2500) and anti-mouse IgG peroxidase-conjugated antibody (1: 10,000) were used. After adding the substrate OPD to the plate, absorbance was measured at 450 nm wavelenght after 15 min. Same, protocol was followed for both *H. influenzae* and *A. Lignieresii* as a negative control to ascertain non-specific binding.

RESULTS AND DISCUSSION

Biopanning: The concentration of phage stock provided in the phage peptide display library Ph.D.-12 was found to be 1.8×10^{11} pfu/ml. For biopanning, 2.1×10^{13} pfu/ml of amplified phages was used as an initial concentration for the first round of positive selection (PS1) against *P. multocida*. After the first positive selection round, 5.8×10^6 phages were recovered and these were amplified to carry out the first round of negative selection (NS1). At the beginning of each round of biopanning, the concentration of eluted amplified phages of PS1 was adjusted to 10^{13} pfu/ml to keep intial phage input constant.

Summary of phages recovered and amplified during all three rounds of positive and negative selection of alternate selection methodology of panning against *P. multocida* is given in Table 2.

Table 2. Phages recovered and amplified during all three rounds of positive and negative selection

Selection	Round No.	Recovered (pfu/ml)	Amplified (pfu/ml)
Positive	PS1	5.8×10^6	1.6×10^{14}
Negative	NS1	2.8×10^{11}	4.1×10^{14}
Positive	PS2	1.3×10^{11}	2.2×10^{12}
Negative	NS2	1.4×10^{12}	9.1×10^{16}
Positive	PS3	4.8×10^{12}	

Plaque selection for phage indirect-ELISA (Phage iELISA): A total of 16 individual and separate plaques were picked up from plate of final round of positive selection. The 16 phages selected were: A1, A2, A3, A4, A5, A6, A7, A8, B1, B2, B3, B4, B5, B6, B7, B8. These were tested for their specific binding to *P. multocida* using phage iELISA. The iELISA was performed at two phage concentrations of 10^7 pfu/ml and 10^{12}pfu/ml.

Phage iELISA to determine the binding efficiency of selected phage clones: Out of these 16 phages, the optical density of 6 phage clones was found comparatively higher over the rest of the 11 phages. Comparative OD values of these high-affinity phages tested using iELISA against *P. multocida*, *A. lignieresii* and *H. influenza* as shown in Fig. 1.

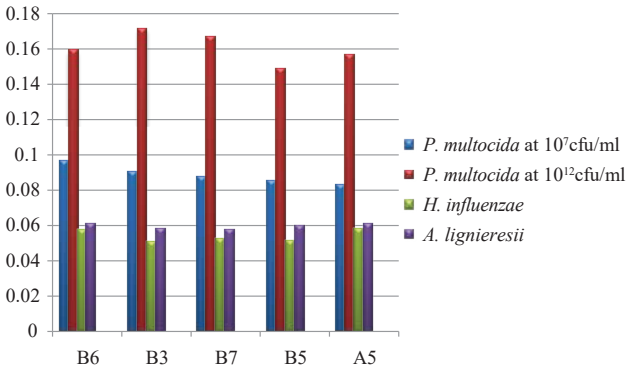

Fig. 1. Comparison in absorbance of *P. multocida, A. lignieresii* and *H. influenzae* at a phage concentration of 10^7 pfu/ml and 10^{12} pfu/ml at wavelenght 450 nm.

In the second iELISA, the concentration of phages was increased to 10^{12} pfu/ml, at this concentration higher absorbance values were recorded at 450 nm wavelength. There was a 1.7 times increase in the absorbance values in the second experiment of iELISA. This demonstrates the need for optimal concentration of different reactants

in developing a robust ELISA for optimal and consistent results. iELISA using these phages were also performed with *A. lignieresii* and *H. influenzae* and but they failed to bind their target with the same intensity as seen with *P. multocida*. The optical density readings for both the bacteria at both phage concentrations were <0.061.This increase in optical densities has been described in many other studies, in which it has been explained that from the affinity of the selection process, is largely driven by the density and accessibility of cell surface antigens (Smith 1985, de Haard *et al.* 1999).

The specificity, as well as avidity of peptides binding to its target *P. multocida*, could have been increased by incorporating some changes in the selection protocols such as increasing the stringency during washing and elution steps, altering the concentration of target, use of different buffers or changing the biopanning format. In washing buffer, instead of using 0.1% tween as per recommendation, 0.4% tween concentration was used which helped in removing unbound phages efficiently. Similarly, other modifications at various levels could be done to achieve specific binders. Generally 3 rounds of selection are sufficient but more number of selection rounds could be employed to increase the pool of more specific phages (Hyman 2019). In this study, the suspension method of selection was followed over the surface panning procedure to overcome the limitation of selecting non-specific phages to BSA in format (Panagides *et al.* 2022). However, in the suspension format of selection, non-specific binding phages to the other proteins and surfaces could be avoided.

In conclusion, the Ph.D-12 library was successfully tittered and amplified. The initial concentration of the library was found to be 1.8×10^{11} pfu/ml. With every round, there was a marked decrease in the recovered phages indicative of a rational selection. Out of 16 phages against *P. multocida* B:2, five clonal phages bounded their target with better intensity indicating that it is possible to select peptide ligands against the intended target, with high specificity and intensity.

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REFERENCES

Bazan J, Całkosiński I and Gamian A. 2012. Phage display-A powerful technique for immunotherapy: 1. Introduction and potential of therapeutic applications. *Human Vaccines and Immunotherapeutics* **8**(12): 1817-28.

Cesareni G. 1992. Peptide display on filamentous phage capsids An new powerful tool to study protein-ligand interaction. *FEBS Letters* **307**(1): 66-70.

Chen Y, Guo G, Sun E, Song J, Yang L, Zhu L, Liang W, Hua L, Peng Z, Tang X and Chen H. 2019. Isolation of a T7-Like lytic *Pasteurella* bacteriophage vB_PmuP_PHB01 and its potential use in therapy against *Pasteurella multocida* infections. *Viruses* **11**(1): 86.

Chen Y, Sun E, Song J, Yang L and Wu B. 2018. Complete genome sequence of a novel T7-like bacteriophage from a *Pasteurella multocida* capsular type A isolate. *Current Microbiology* **75**(5): 574-79.

Cho W, Fowler J D and Furst E M. 2012. Targeted binding of the M13 bacteriophage to thiamethoxam organic crystals. *Langmuir* **28**(14): 6013-20.

De Alwis M C L. 1996. Haemorrhagic septicaemia: clinical and epidemiological features of the disease. In International Workshop on Diagnosis and Control of HS, Bali, Indonesia. pp. 28-30.

de Haard H J, van Neer N, Reurs A, Hufton S E, Roovers R C, Henderikx P, de Bruïne A P, Arends J W and Hoogenboom H R. 1999. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *Journal of Biological Chemistry* **274**(26): 18218-30.

Dinos G P. 2017. The macrolide antibiotic renaissance. *British Journal Pharmacology* **174**(18): 2967-83.

Dutta J, Rathore B S, Mullick S G, Singh R and Sharma G C. 1990. Epidemiological studies on occurrence of haemorrhagic septicaemia in India. *Indian Veterinary Journal* **67**(10): 893-99.

El-Jakee J K, Ali S S, El-Shafii S A, Hessain A M, Al-Arfaj A A and Mohamed M I. 2016. Comparative studies for serodiagnosis of haemorrhagic septicaemia in cattle sera. *Saudi Journal of Biological Sciences* **23**(1): 48-53.

Gallop M A, Barrett R W, Dower W J, Fodor S P and Gordon E M. 1994. Applications of combinatorial technologies to drug discovery. *Journal of Medicinal Chemistry* **37**(9): 1233-51.

Garcia L S. 2010. *Clinical Microbiology Procedures Handbook* (Vol. 1). American Society for Microbiology Press.

Harper M, Boyce J D and Adler B. 2006. *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *FEMS Microbiology Letters* **265**(1): 1-10.

Hess G T, Cragnolini J J, Popp M W, Allen M A, Dougan S K and Spooner E. 2012. M13 bacteriophage display framework that allows sortase-mediated modification of surface-accessible phage proteins. *Bioconjugate Chemistry* **23**: 1478-87.

Hoogenboom H R, de Bruıne A P, Hufton S E, Hoet R M, Arends J W and Roovers R C. 1998. Antibody phage display technology and its applications. *Immunotechnology* **4**(1): 1-20.

Hyman P. 2019. Phages for phage therapy: isolation, characterization, and host range breadth. *Pharmaceuticals* **12**(1): 35.

Lakzaei M, Rasaee M J, Fazaeli A A and Aminian M. 2019. A comparison of three strategies for biopanning of phage-scFv library against diphtheria toxin. *Journal of Cellular Physiology* **234**(6): 9486-94.

Lam K S, Salmon S E, Hersh E M, Hruby V J, Kazmierski W M and Knapp R J. 1991. A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* **354**(6348): 82-84.

May B J, Zhang Q, Li L L, Paustian M L, Whittam T S and Kapur V. 2001. Complete genomic sequence of *Pasteurella multocida*, Pm70. *Proceedings of the National Academy of Sciences* **98**(6): 3460-65.

Panagides N, Zacchi L F, De Souza M J, Morales R A, Karnowski A, Liddament M T, Owczarek C M, Mahler S M, Panousis C, Jones M L and Fercher C. 2022. Evaluation of phage display biopanning strategies for the selection of anti-cell surface receptor antibodies. *International Journal of Molecular*

Sciences **23**(15): 8470.

Qureshi S, Saxena H M, Imam N, Kashoo Z, Sharief Banday M, Alam A, Malik M Z, Ishrat R and Bhat B. 2018. Isolation and genome analysis of a lytic *Pasteurella multocida* Bacteriophage PMP-GAD-IND. *Letters in Applied Microbiology* **67**(3): 244-53.

Smith G P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**(4705): 1315-17.

Townsend K M, Frost A J, Lee C W, Papadimitriou J M and Dawkins H J. 1998. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *Journal of Clinical Microbiology* **36**(4): 1096-1100.

Valadon P and Scharff M D. 1996. Enhancement of ELISAs for screening peptides in epitope phage display libraries. *Journal of Immunological Methods* **97**(1-2): 171-79.

Verma S, Sharma M, Katoch S, Verma L, Kumar S, Dogra V, Chahota R, Dhar P and Singh G. 2013. Profiling of virulence associated genes of *Pasteurella multocida* isolated from cattle. *Veterinary Research Communication* **37**(1): 83-89.