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Research Article

ISOLATION OF BACTERIOPHAGE FROM WASTE WATER AGAINST MULTIDRUG RESISTANT *E. COLI* FROM MASTITIC BUFFALO MILK

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ABSTRACT

Bovine mastitis is the most economically vicious disease for milk producers because of reduced milk production, treatment, labour cost, etc. It is the inflammatory disease of mammary glands in milch animals, caused by more than 200 infectious and non-infectious agents. But due to the emergence of multiple drug resistant pathogens, the traditional antibiotic treatment is failing and alternate methods of treatment like phage therapy are being sought out. In the present investigation, *Escherichia coli* was isolated from a milk sample of buffalo suffering from mastitis. The antimicrobial susceptibility test of the isolated *E. coli* revealed the bacterial isolate sensitive to only two antibiotics out of 15 tested. The waste water from Buffalo Farm, LUVAS, Hisar was processed for bacteriophage isolation by standard enrichment protocol using isolated *E. coli* as host and spot test was conducted to observe bacterial lysis. Plaque assay was carried out to isolate bacteriophage using double agar overlay technique. The phage was enriched and its titer was determined to be 7x108PFU/ml. Further characterization of the isolated phage is required to find its suitability in phage therapy against other mastitic pathogens.

Keywords: Bacteriophage, Mastitis, Multidrug resistant *E. coli*

Bovine mastitis, caused by various infectious or non-infectious agents, is an inflammatory disease of the mammary glands in milch animals. It causes substantial loss to the dairy farmers (Sharma *et al.*, 2012). In India, the annual economic losses on account of udder infection were estimated to be Rs. 7165.51 crores (Bansal and Gupta, 2009). The overall prevalence of mastitis in India ranged from 25.63 to 97.61% (mean prevalence ~44.67%) (Sudhan and Sharma, 2010), while in Haryana, it was 54.49% in both cows and buffaloes together based on samples submitted to our central laboratory (Annual Report, CCL, 2016).

The predominant causal organisms of mastitis are cell-walled bacteria, although mycoplasma, yeast and algae have also been reported to cause mastitis (Pankaj *et al.*, 2012). Indiscriminate use of antibiotics for the treatment of mastitis has led to the development of multiple drug resistant pathogens. This crisis of antibiotic resistance can be overcome by the use of a nearly forgotten strategy such as bacteriophage (phage) therapy (Mapes *et al.*, 2016). Lytic phages are the viruses ubiquitous in all environments, including the water, soil, and air, which kill bacteria. Ganaie *et al.* (2018) described the isolation and characterization of lytic phages having a potential to be used in therapy against *Staphylococcus aureus* causing mastitis. The present investigation was carried out to isolate a lytic phage for activity against multidrug resistant *E. coli* which was isolated from mastitic buffalo milk.

MATERIALS AND METHODS

Isolation of bacteria from mastitic milk samples: Milk samples (Lab ID 3183) of four quarters of a buffalo suffering from mastitis for past 20 days was received at College Central Lab (CCL), LUVAS, Hisar. A 10 µl of milk sample from respective quarters was placed near the wall of blood agar (BA) plate and then streaked onto the agar using a sterile inoculation loop. The plates were incubated at 37 °C overnight in a BOD incubator for 24 h. A single colony was picked from the Petriplate and transferred to Brain Heart Infusion broth followed by incubation at 37 °C overnight in shaker incubator at 200 rpm to obtain pure culture.

Characterization of isolated bacterial culture: The bacterial isolate was characterized by Gram's staining and its ability to grow on selective media like MLA and eosin methylene blue (EMB) agar. The presence of *E. coli* was verified by 'Hi *E. coli* Identification Kit (HiMedia)' using biochemical tests as per the manufacturer's instructions. The bacterial culture was also verified using automated VITEK®2 Compact system (bioMerieux, France).

Antibiogram: The antimicrobial susceptibility test of the isolated bacteria was performed using the disc diffusion technique (Watts *et al.*, 2008). The antibiotic discs used were: Enrofloxacin, Penicillin G, Streptomycin, Amoxycillin, Levofloxacin, Moxifloxacin, Ceftriaxone, Chloramphenicol, Oxytetracycline, Ampicillin, Gentamicin, Neomycin, Amikacin, Cloxacillin and Cefoperazone. The zone of inhibition formed around the disc was checked and measured with the help of a scale and the results interpreted as per the literature provided with the discs.

Phage collection and isolation: Water/soil sample for bacteriophage isolation was collected from four different places in Hisar *viz*. waste water sample from Buffalo Farm, LUVAS and sewage water sample from sewage treatment

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plant; soil sample from Veterinary Clinics, LUVAS and Buffalo Farm, LUVAS. The samples were processed for phage isolation and filtrates subjected to Spot test (Carlson, 2005). The phage filtrate showing positive Spot test was then used for phage isolation by double agar overlay technique (Adams, 1959). The phage filtrate was diluted 10-fold in SM buffer (HiMedia) from 10^{-1} to 10^{-7} dilutions in different tubes followed by addition of 100 µl of overnight grown isolated bacterial culture, 30 µl of 0.1 M calcium chloride and 0.1 M magnesium chloride, each and 3 ml of molten top agar. The tubes were mixed gently maintaining a constant temperature and poured onto LB agar plates labeled 1 to 7. The plates were incubated at 37 °C overnight in a BOD incubator and checked for the presence of plaques which were picked using a sterile micro pipette tip (1000 µl) and transferred in SM buffer at 4 °C overnight for leaching. The picked plaques were used for phage isolation using phage enrichment method (Bonilla *et al.*, 2016). The plaque forming units (PFU) were calculated (Jothikumar *et al.*, 2000) by counting the numbers of plaques present in maximum dilution of phage preparation and using the following formula:

PFU count= Number of plaques counted × 1/Dilution factor × 1/ Volume of phage taken

RESULTS AND DISCUSSION

The present study was an effort to isolate a bacteriophage from indigenous environment against mastitis causing *E. coli*.

Colonies for bacterial isolation: BA plates containing milk samples of four quarters of buffalo suffering from mastitis were observed for the appearance of colonies after 24 h of incubation at 37 °C. Greyish white colonies observed on BA plate indicated the presence of *Escherichia* spp.

Characterization of isolated bacterial culture: The microscopic view of Gram's staining of culture exhibiting pink coloured, rod shaped organism which indicated the Gram negative nature of organism. The presence of pink coloured colonies on MLA and metallic green sheen on EMB agar indicated that the organism was *E. coli*. The isolated bacterial culture was further subjected to biochemical tests using 'Hi *E. coli* Identification Kit (KB010, HiMedia) and the results were interpreted as per the standards given in the Result Interpretation Chart which confirmed the presence of *E. coli*. The bacterial culture was verified to be *E. coli* using automated VITEK® 2 Compact system.

Antibiogram: The isolated mastitis causing *E. coli* was found to be resistant to enrofloxacin, penicillin G, streptomycin, amoxycillin, levofloxacin, moxifloxacin, ceftriaxone, oxytetracycline, ampicillin, neomycin, amikacin, cloxacillin, cefoparazone and sensitive to chloramphenicol and gentamicin antibiotics. The antibiotic resistance of the isolated *E. coli* to several antibiotic groups may be attributed to indiscriminate use of antibiotics in the field for treatment of mastitis and its improper use. Therefore, a nearly forgotten line of treatment i.e. phage therapy may re-emerge as a rescuer to this accelerating crisis of antibiotic resistance.

Phage collection and isolation: The filtrate obtained from waste water sample of Buffalo Farm, LUVAS gave positive spot test against mastitis causing *E. coli*. The sample exhibited clear zone of lysis on *E. coli* bacterial lawn onto LB agar plate (Fig. 1). There was no zone of lysis observed in rest of the three soil and water samples. The waste water filtrate from Buffalo Farm was then used for phage isolation using double agar overlay technique. The plaques were observed in LB Petriplates using different dilutions (10^{-1} to 10^{-7}) of phage filtrate (Fig. 2). After double agar overlay technique, the LB Petri plate (10^{-7} dilution) showing single isolated plaque was selected and five plaques with clear morphology were picked. The process was repeated to get purified phages. The number of plaques in phage filtrate with dilution 10^{-6} was found to be 70. So, the phage titre was calculated to be 70 x10^{6} x10 = 7x10^{8}PFU/ml.

Phage therapy can be used as an alternative to

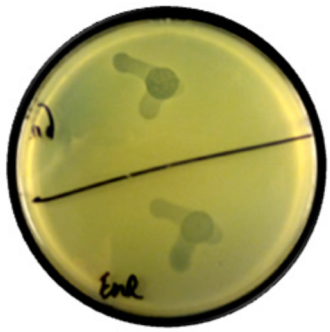

Fig. 1. Clear zone of lysis exhibited by phage filtrate against isolated *Escherichia coli* on Luria Bertani agar plate in Spot test

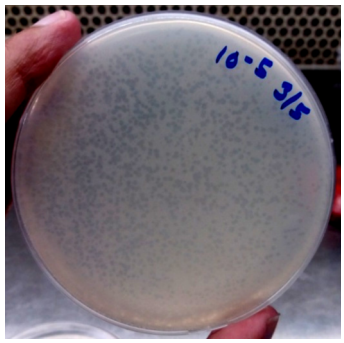

Fig. 2. Plaques in different dilutions of phage filtrate against mastitis causing *Escherichia coli* after double agar overlay technique

antibiotics and to control a variety of mastitis-causing pathogens mainly *S. aureus* and *E. coli* (Gill *et al.*, 2006; Garcia *et al.*, 2009; Ribeiro *et al.*, 2018). In the present investigation, a lytic bacteriophage against mastitis causing multidrug resistant *E. coli* bacteria has been isolated from waste water of Buffalo Farm, LUVAS. This is the first report of phage isolation against mastitis causing *E. coli* from Haryana. Further characterization of the isolated phage is required to find its suitability in phage therapy against mastitic pathogens. The applications of such lytic phages could be an asset for bio-controlling of pathogenic agents in medical and veterinary sciences.

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