Feasibility To Employ Enterococcus Faecalis Phages To Combat Poultry Infections

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***Abstract*— Phage therapy has gained increased attention as an alternative modality to antibiotics in order to control bacterial diseases and prevent the spreading of multidrug-resistant bacteria in poultry farms. In this study, Enterococcus faecalis, the most prevalent species from poultry farm to cause infections to human was targeted. Enterococcus faecalis phage was isolated from chicken fecal samples collected from poultry farms. This phage was characterized to be highly specific against Enterococcus faecalis and it could withstand high temperature up to 90°C and acidic conditions. The isolated phage which exhibits temperature and pH resistance and high specificity could be used as biocontrol agent to eliminate the existence of E. faecalis in poultry farms.**

Keywords*— *Enterococcus faecalis, biocontrol agent, poultry infections.

I. INTRODUCTION

Enterococcus faecalis is a gram positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals [1].E. faecalis can cause life-threatening infections in humans. Moreover, the antibiotic resistance exhibited at high levels by E. faecalis contributes to its pathogenicity [2]. Several reports state that enterococcal species possess the property of causing a variety of diseases in humans [3, 4]. It is known from previous studies that Enterococcus faecalis can cause endocarditis, bacteremia, urinary tract infections, meningitis and other infections in humans [5, 6, and 7]. Enterococcus faecalis and Enterococcus faecium are the most predominant species of clinical isolates accounting for more than 90% [8]. Enterococci are resistant to general antibiotics like cephalosporins and tetracycline and frequently express resistance to gentamicin at a greater rate [9, 10]. The National Nosocomial Infections Surveillance (NNIS) report showed that more than 28.5% of enterococcal isolates are resistant to vancomycin [11, 12, 13]. Recently, infections with vancomycin-resistant Enterococcus (VRE) have become a threat in nosocomial settings, with an increased incidence reported worldwide [14, 15, 3, 16, 17].

Nature of virulence and pathogenicity have been described by virulence factors encoded by genes (agg, gelE, ace, cylLLS, esp, cpd, fsrB) isolated from resistant Enterococci. The capability of forming biofilms and the production of gelatinase and hemolysin have been documented [18, 7].

E. faecalis is capable of surviving even at extreme environmental conditions. It grows in high salt concentrations, wide temperature range and tolerates a broad pH range and starves until an adequate nutritional supply is provided. Enterococcus faecalis has the ability to establish mono-infections in medicated root canals [19]. Enterococcus faecalis is often the predominant species in poultry. Forty two percent of Enterococcus faecalis from conventional farms were found to be multi-drug resistant which poses a major threat to human health [20].

Some studies reveal the dissemination of resistant Enterococcus from animals to man [21]. Multidrug resistance is common among Enterococci which makes treatment problem [9, 22]. The continuous overuse or misuse of effective antibiotics has led to the ongoing emergence of new antibiotic-resistant pathogenic bacteria [23]. The continuous vicious cycle between rapidly increasing multidrug resistance and new drug development needs intervention. Bacteriophage therapy involves the utilization of a virus as a biocontrol agent to target and destroy disease causing bacteria and is predicted to be a practicable alternative therapy because of its long history of successful use in the eastern countries [24, 25, 26, 27, 28]. Major advancements in the field includes the specificity of the interaction, the gene networks of coevolving partners, and the relative importance of the coevolving interaction in complex communities and environments [29,30,31,32].

Phages are bacterial viruses that are extremely abundant in nature and are believed to be important in controlling bacterial populations in natural systems [33, 34, 35]. The property of self-replication, which results in increased concentrations as infection persists and the narrow host range of phage, which prevents harm to beneficial and naturally occurring micro flora

gained attraction to be employed as therapeutic agents [36, 37, 38]. Sequencing genomes of bacteriophages with therapeutic potential can be sequenced which increases the quantity of genomic data to study about the taxonomy of phages for its better utilization [39, 40].

For instance, phage with lytic activity against Vibrio harveyi has been isolated and phage therapy was applied to luminous vibriosis [41]. The ability of the microorganism to acquire resistance to many antibiotics, disinfectants and dehydration assures its long-term survival in hospital settings. The application of bacteriophages is a potential tool to control bacterial infections [42, 43, 44, 45]. The FDA-approved Phase I physician-led trial was completed at a wound care centre in Lubbock, Texas using a mixture of bacteriophages targeting P. aeruginosa, S. aureus and E. coli [46]. Salmonella phage was found to be able to lyse either of the Salmonella serovars Typhi, Typhimurium, or Enteritidis [47, 48]. The aim of the present study is to isolate, purify and characterize Enterococcus faecalis phage to employ it as a biocontrol agent in poultry farms against enterococcal infections..

I. MATERIALS AND METHODS

A. *Isolation of Enterococcus faecalis*

- Sample collection: The chicken fecal samples were collected from poultry farms of Erode, Tamilnadu. The collection tubes containing samples were immediately transported to the laboratory and kept at 4°C prior to use.
- Serial dilutions of samples: 1g of fecal sample was added to 10 mL of 0.1% peptone water and serially diluted. The diluted samples were plated onto nutrient agar and incubated at 37°C for 24 h.
- Screening of bacterial isolates: Biochemical tests were performed which include Gram staining, Motility test, Catalase test, H2S production test, Citrate test, Urease test, Indole test, Gas production test and Methyl red test.

B. *Isolation of bacteriophage*

- Phage enrichment from fecal sample: Chicken excreta samples were suspended (1:10) in SM buffer and the bacteriophage was allowed to stabilize at 4°C overnight with gentle shaking. The supernatant was centrifuged at 10,000 rpm for 10 min and filtered through 0.22 µm syringe filter.
- Turbidity test: The filtered phage culture was added to the overnight grown bacterial culture and incubated at 37°C for 24 h. The bacteriophage lytic activity was observed by the change in turbidity of the broth. Confirmation of bacteriophage: To confirm the presence of phage in the crude phage lysates, the soft agar overlay method was followed. Different dilutions of bacteria and phage lysates were added and incubated at 37°C for 5 min to aid in the process of adsorption. The mixture was then added to 5.0 mL of soft agar (0.5%). The inoculated soft agar was poured onto hard agar (1.5%). The plates were then incubated at 37°C for 24 h.

C. *Concentration of phages*

The isolated plaques were precipitated using Poly Ethylene Glycol (PEG) 6000. The plaques along with the soft agar were resuspended in distilled water and centrifuged at 1000 rpm for 30 min at 4°C. To the supernatant, 10% PEG was added and dissolved at room temperature. It was kept in ice for 1 h. Centrifugation was repeated again and the pellet was resuspended in 5mL of saline (0.9% NaCl) [49].

D. *Host range analysis*

Phage host range was established by using the spot test method. The bacterial isolates were analyzed for their sensitivity to the phage lysates. The plate inoculum consisted of 5 mL of soft agar was mixed with 100 mL of the overnight bacterial culture and equal volume of CaCl2 (300 mM). The mixture was overlaid onto the surface of hard agar. 3 µL of phage lysate was spotted onto the inoculated hard agar plates and the plates were incubated at 37°C for 24 h. Bacterial sensitivity to the bacteriophage was determined by the appearance of clear zone at the spotted area. A control plate maintained using SM buffer alone, showed no zone of clearance [50].

E. *Thermal sensitivity test*

To analyze the temperature sensitivity of phages, 5 tubes containing 900 µL of sterile distilled water each were preheated to temperature, ranging from 50°C to 90˚C. Then 100 µL of phage solution (1.14×106 PFU/ mL) was added to the preheated water. It was then heated at different temperatures for 30 min and then kept at room temperature. Surviving phage titer was assayed by the double layer method [51].

F. *pH sensitivity test*

The phage (at the final concentration of 1.14×106 PFU/ mL) was incubated overnight at 25°C in phosphate buffered saline at pH ranging from 2 to 12. The phage samples were then re-adjusted to pH 7.0 and the double layer method was performed to determine the final phage titer [51].

II. RESULTS

A. *Isolation of bacteria*

Bacterial isolates were obtained from the fecal sample of chicken.

- Biochemical tests: Enterococcus faecalis was isolated based on the outcome observed for each of the biochemical tests performed as tabulated in TABLE 1 [52].

B. *Isolation of bacteriophage*

Clear plaques on a well spread bacterial lawn were observed as shown in Fig. 1. The optimized ratio of bacteria to the different dilutions of phage lysate was 1:10. The phage isolate,

showing a concentration of 1.5x104 Plaque Forming Unit, PFU/ mL was used for further studies.

- Turbidity analysis: Change in turbidity observed after 24 h shows the lytic activity of phages as shown in Fig. 2.

C. Concentration of phages

The number of plaques obtained from PEG purified phages were calculated by double layer overlay method. The PFU of phages was found to be 1.14x106 PFU/ mL after being concentrated (Fig. 3.).

D. Host range analysis

The specificity of Enterococcus faecalis phage was determined against different bacterial strains as shown in the TABLE 2.

E. Thermal sensitivity test

The initial titer of phages before temperature and pH treatment was 1.14×106 PFU/ mL. Phages were found to be reasonably stable at temperatures like 50°C, 60°C, 70°C, 80°C and 90°C with moderate reduction in titer. The thermal stability test was carried out to determine the heat resistance of Enterococcus faecalis phage. It was found that Enterococcus faecalis phage could withstand the temperature up to 90°C (Fig. 4.).

F. pH sensitivity test

Optimal pH was determined by testing the stability of phages at different pH range. Though the lytic activity was moderate at acidic and alkali environment, E. faecalis phages could withstand such extreme conditions and the activity was comparatively higher at pH 6 to pH 10 (Fig. 5.).

IV. DISCUSSION

E. faecalis has been found to be responsible for some life-threatening infections in humans, especially in nosocomial environment, where the naturally high levels of antibiotic resistance found in *E. faecalis* contribute to its pathogenicity [53, 54].

In this study, Enterococcus faecalis phage was isolated from poultry farms and further characterization of isolated phage was carried out. Enterococcus faecalis phage has narrow host range since this phage did not form zone of clearance against Staphylococcus aureus, Bacillus cereus, Bacillus pumilus, Pseudomonas. Hence phage cannot be used as a biocontrol agent against these bacterial strains. This phage is found to be highly active and specific against Enterococcus faecalis strain.

The phage activity has been found to be sensitive to the physiological state of the host, which has been affected by its growth conditions such as temperature, nutrient availability and oxygen tension [55, 56]. The virulence nature of a phage may depend on the phage–host interaction and environmental conditions such as pH and temperature [57]. Temperature may affect the ecological balance between phages and their hosts. Treating Vibrio cholerae phages with various temperatures, bacteriophage titer was found to be non-significantly different during 30-60°C [58]. Reduction of bacteriophage titer was observed from 70 to 80°C and got completely destroyed at 90°C. Thermal stability of the Enterococcus faecalis phage was assayed [51]. E. faecalis phage exhibited a decreasing activity at temperature ranging from 50°C to 90°C. Phage lytic activity was found to be comparatively high at the temperature 50°C, higher the temperature, lytic activity was found to be decreased. E. faecalis phage could withstand higher temperature at 90°C, suggesting that this phage could be used in therapy as the most prevalent biocontrol tool in poultry farms, since it is found to be thermostable.

Acidity and alkalinity of the environment are other important factors influencing phage stability. Bacteriophage AR was the most sensitive to acid and alkaline conditions and showed 100% activity only at pH 7 and pH 8 [59]. In this study, Enterococcus faecalis phage activity was comparatively low in acidic and alkaline environment. In acidic condition Enterococcus faecalis activity was low but there was no complete decline, revealing its survival efficiency at acidic and in alkaline environment. The phage shows more activity at the range of pH 6 to pH 10. Poultry processing involves high temperature and acidic treatments which becomes ineffective such that the pathogenic microorganisms escape these extreme conditions and make their survival possible. Phages that are not resistant to temperature and acidic conditions are also affected during processing. The isolated phage showing much interesting properties such as narrow host range, resistance to acidic and high temperature environments may well offer an important tool to support global efforts to reduce the burden of human foodborne disease transmission from poultry farms.

V. CONCLUSION

Enterococcus faecalis infections prevalent in poultry ecosystem create a major impact on human health. Due to the failures observed in using antibiotics and vaccines against microbial infections, phage therapy is suggested to be an alternative treatment strategy. In this study, Enterococcus faecalis phages have been isolated and characterized to analyze the efficiency in employing them as biocontrol agents. Enterococcus faecalis is not completely eliminated by different poultry processing conditions. To overcome this, Enterococcus faecalis phages which can withstand these processing conditions and also eliminate the sustaining microbe can be used. Phages used in this study were found to be resistant to both temperature and pH involved in poultry processing conditions. This phage shows narrow host range and it is highly specific to Enterococcus faecalis. The isolated phage could be subjected orally (with chicken feed) or sprayed to eliminate the existence of E. faecalis in poultry farms. Enterococcus faecalis phages could be employed as the most potential biocontrol tool towards eradicating Enterococcal infections in poultry farms.

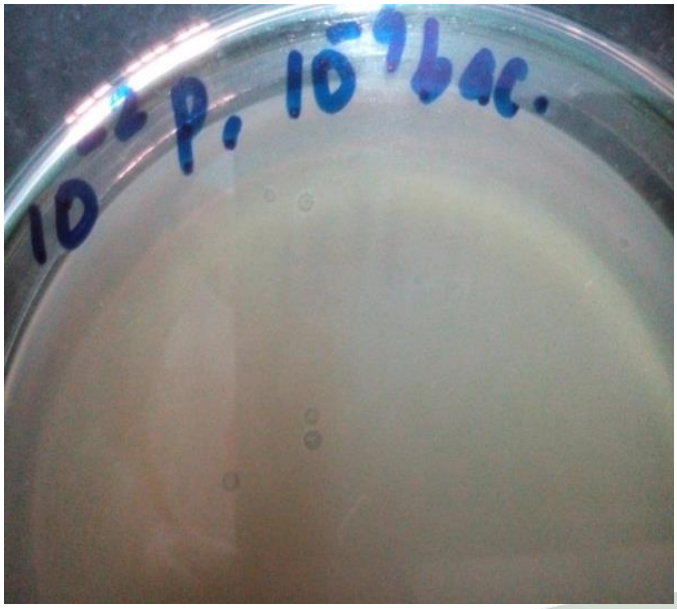

Fig. 1. Plaque assay. The appearance of clear plaques confirms the action of bacteriophage over the bacterial lawn.

Fig. 2. Turbidity analysis. Bacterial culture a) before phage inoculation b) After phage inoculation.

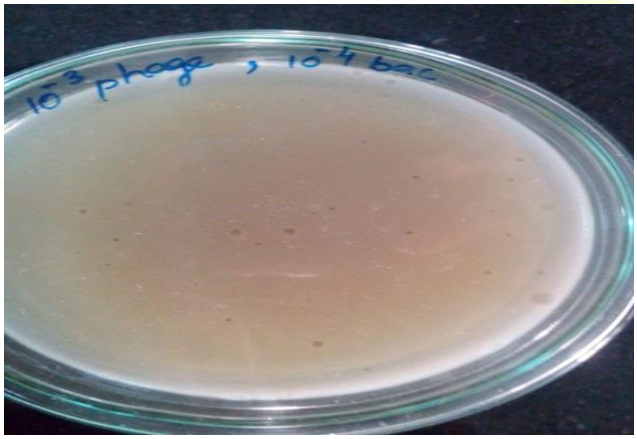

Fig. 3. Concentration of phages (1.14×106 PFU/ mL).

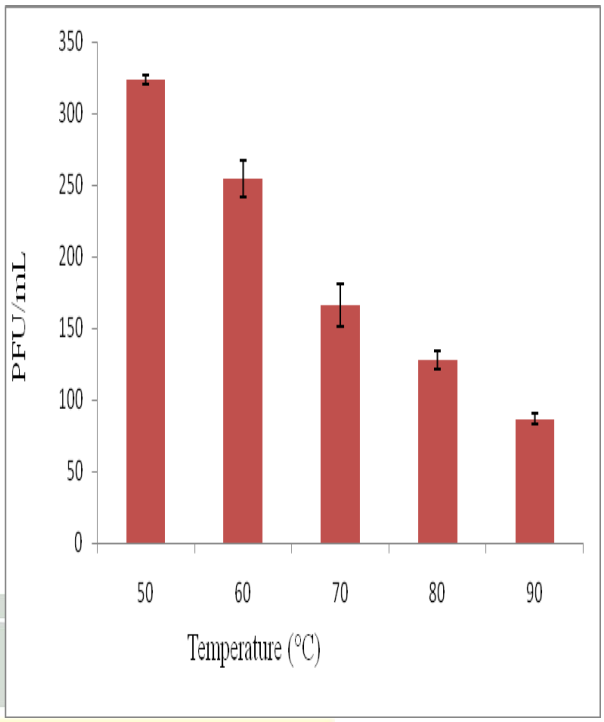

Fig. 4. Temperature sensitivity of the isolated phages

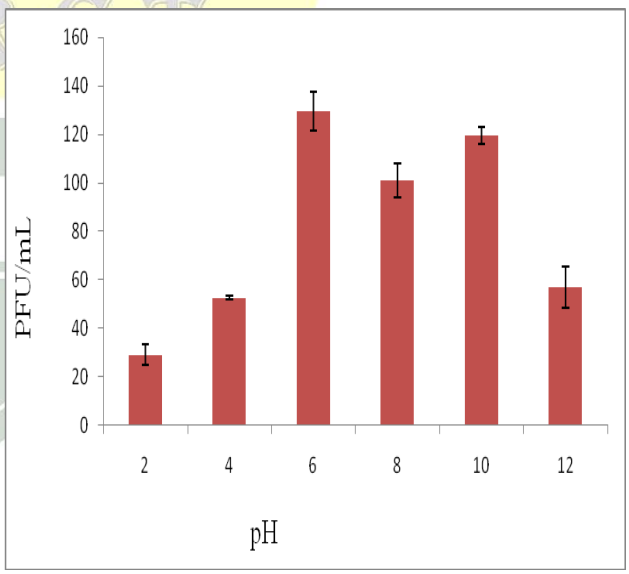

Fig. 5. pH sensitivity of the isolated phages

TABLE 1. LIST OF BIOCHEMICAL TESTS AND THEIR OUTCOMES

S.No.	Biochemical Tests	Test Outcome
1	Gram staining	Gram positive, coccus shaped bacteria
2	Motility test	non-motile
3	H_2S Production test	Negative
4	Citrate test	Negative
5	Urease test	Negative

6	Indole test	Negative
7	Gas production test	Negative
8	Catalase test	Negative
9	Methyl red test	Negative

TABLE 2. PHAGE HOST SPECIFICITY

Bacterial strains	Phage activity
Staphylococcus aureus	Negative
Bacillus cereus	Negative
Bacillus pumilus	Negative
Pseudomonas	Negative
Enterococcus faecalis	Positive

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