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Research article

Identification of phages against *Clostridium perfringens* isolated from diarrhea cases of Humans

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Abstract

Background: *Clostridium perfringens* is a gram positive, rod shaped, anaerobic and spore forming bacteria. It causes diarrhea among humans which lead towards the severe health and economic losses worldwide. It is also an important pathogen which causes the food born and gas gangrene illness in both humans and animals. Alternative therapies are required for the cure of the bacterial infections because of the increase in antimicrobial resistance in bacteria. Now a day's bacteriophages play a key role for the curing of bacterial infections that are resistant to antibiotics. **Aim of the study:** The objective of this study was to isolate *Clostridium perfringens* from the clinical cases of diarrhea from individuals and to identify bacteriophages against the diarrhea causing *Clostridium perfringens* strain. **Materials and methods:** A total of 10 feces samples were collected from the diarrhea clinical cases of different individuals from Allied Hospital Faisalabad. RCM, BHI and 5% defibrinate sheep blood agar were used for *Clostridium perfringens* isolation and the biochemical tests were performed for further confirmation of the bacteria. Bacteriophages were isolated from the diarrhea cases of humans and its lytic activity was examined against the *Clostridium perfringens* through the spot test technique and agar overlay method. **Results:** Out of these 10 clinical specimens, *Clostridium perfringens* was isolated in 6(60%) samples. It showed blackening in RCM broth, turbidity in BHI broth and beta hemolysis occurred in Blood agar. **Conclusion:** Routine periodic sampling of faeces would facilitate the selection of appropriate method. *Clostridium perfringens* were isolated and bacteriophages were identified against it.

Keywords: BHI, *Clostridium perfringens*, RCM

Introduction

Clostridium perfringens is a gram-positive, rod shaped, anaerobic spore forming bacterium which is present mostly in the sewage, soil as well as in gastro-intestinal tract of the humans and animals as an essential part of the gut macrobiotic (Labbe and Juneja., 2013).

Strains of the *Clostridium perfringens* has been divided in five different classes (A-E) because of the production of the toxins of four types known as beta, alpha, iota and epsilon toxins (Myers *et al*., 2006).

Type A of *Clostridium perfringens* strain causes gas gangrenes as well as mild diarrhea and necrotic enteritis in human (Uzal *et al*., 2014). During World War I, it has been estimated that millions of the soldiers were died because of the gas gangrene having injuries during battlefield and *Clostridium perfringens* was identified as an essential organism of the gas gangrene. Different spores and vegetative cells enter in body because of any injury in the body, as the organism cultivate very quickly into host tissues which ultimately produces different enzymes and toxins which leads toward the excessive degeneration of host tissue (Stevens *et al*., 2012). In severe cases, infection might be led to shock, systemic

toxemia, as well as death unless surgical treatments and prompt antibiotics are given. In toxigenic clostridia species, like *Clostridium difficile*, *Clostridium botulinum* and *Clostridium tetani, Clostridium perfringens* is most important specie for the genetic study, due to its ability to manipulate genetically, oxygen tolerances and high growth rate (almost 8-10 minutes generation time during optimum condition). Different enzymes and toxins have been studied and mostly their structural genes have been sequenced and cloned (McClane, 2003). Different approaches, like genetic and physical mapping, have been introduced in order to express genomic structure of the strain of different *Clostridium perfringens* (Myers *et al*., 2006).

A similarly, Type A of *Clostridium perfringens* strain which produces alpha but not produces iota, beta or epsilon toxins, are the most important cause of both histotoxic and gastrointestinal (GI) infections in domestic animals and humans (Li *et al*., 2013). Gene (*cpe*) which encoded of the 14 *Clostridium perfringens* toxins like CPE (*Clostridium perfringen*s enterotoxin) is available in less than five percent of the global *Clostridium perfringens* isolates (Briggs *et al*., 2011).

Bacterial viruses were first time reported by Fredrick William Twort in 1915 when he saw the lysis in the micrococcus bacterial cultures during the description of the transmissible smooth transformation. After the widespread use of antibiotics, bacteriophages were marketed by L'Oreal in France for the treatment of the bacterial infection. Until the 1940s, bacteriophage products were sold by the Eli Lilly Co. for the humans use. After that time bacteriophage's early clinical studies were not clearly understood in the Western Europe and United states. Bacteriophages for the treatment of bacterial infection were sold and continued in the Eastern Europe and Russian Federation (Sulakvelidze *et al*., 2001).

Bacteriophages have many forms and it may be single-stranded DNA or RNA and may be double-stranded DNA or RNA and their genome have different sizes. Examined all bacteriophages by electron microscope only 3.7% are filamentous, pleomorphic or polyhedral and the remaining 95% reported as tailed bacteriophages (Ackermann, 2003). The tailed bacteriophages belong to the Caudovirales order and on the basis of tail morphology is divided into further three families and has double-stranded linear DNA genome that's size range from 11 to 500 kb. The heads of the most bacterial viruses are icosahedral and phages having contractile tails structure belong to the Myoviridae family and those having non-contractile short tail are members of the Podoviridae family whereas phages with a non-contractile long tail structures are placed in the bacteriophage family Myoviridae (Ackermann, 2006). Isolation of bacteriophages against the *C. perfringens* can be a raising point towards the discovery of a new tool to fight necrotic enteritis and also a new implementation to control the necrotic enteritis disease causing strain of the *C. perfringens.* In general, it would help to find new strategies for controlling and treating bacterial infections and phages can be used as antimicrobials against antibiotic resistant bacteria.

Materials and Methods

This study was conducted in the Allied Hospital Faisalabad, Pakistan and the Institute of Microbiology, UAF. Faecal swab samples from patients admitted in hospitals were processed during the period from February 2019 till May 2019. The present study included 10 diarrheal patients among them 5 were male individual and remaining were female.

Sample processing

A total of 10 fecal samples were collected from hospitalized patients, suffering from diarrhea. These samples were brought to Microbiology research lab for isolation of *Clostridium perfringens.* Samples were inoculated in Robertson's cooked meat broth. RCM is selective media for growth of *Clostridium perfringens* because it contains cooked meat pieces which reduce the oxygen from media.

Samples were inoculated in Brain heart infusion broth. It is an enriched media for growth of *Clostridium perfringens*. Turbidity in inoculated BHI broth shows growth of *Clostridium perfringens.*

Blood agar is a differential media for growth of Clostridium perfringens because it differentiates *Clostridium perfringens* from other species. Only *Clostridium perfringens* give beta hemolysis on blood agar. Other species do not give beta hemolysis on blood agar.

Results

According to the results, 60% of the isolated bacteria were *C. perfringens* and remaining 40% were other bacterial species (Figure 1). *C. perfringens* was grown on different cultural media like in RCM broth it shows blackening of the media, in BHI broth its growth shows turbidity and on sheep blood agar it shows beta hemolysis (Figure 2). Gram staining was performed after the isolation of *Clostridium perfringens*. It shows purple rods under microscope.

For further confirmation, biochemical tests were performed. Catalase test was -ve with no bubble formation, TSI test was positive with yellow slant and butt, Gelatin hydrolysis test was positive with liquefaction of gelatin.

Bacteriophages were isolated by double agar overlay method and spot test technique (Figure 3). They can be preserved at 4°C in SM buffer by making stock solution. Isolated bacteriophages can be used as antimicrobials against antibiotic resistant *C. perfringens*.

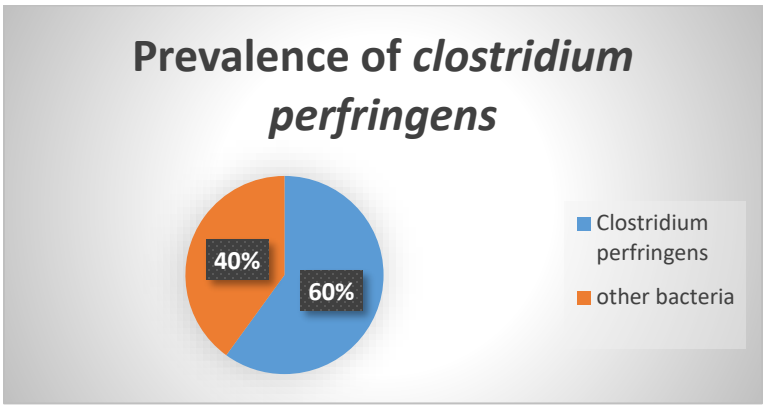

Fig. 1 showing prevalence of *C. perfringens*.

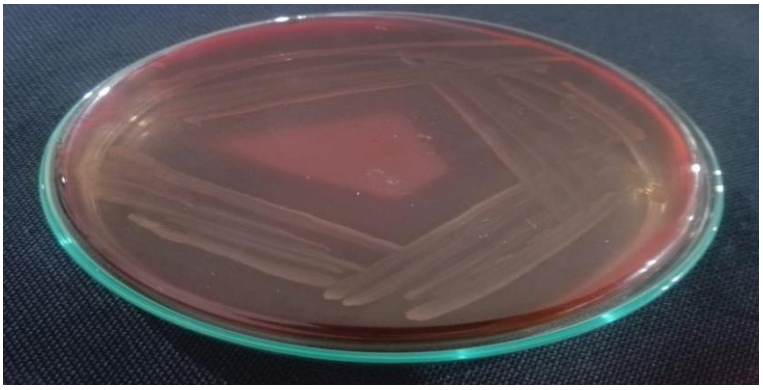

Fig. 2 showing beta hemolysis on Blood agar

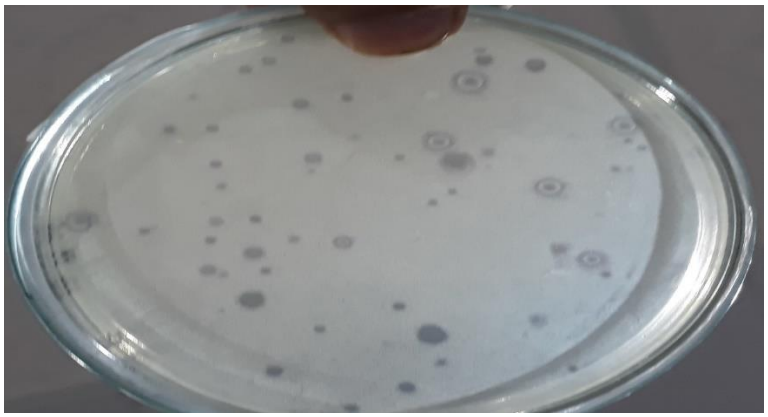

Fig.3 Bacteriophages isolation on a petri plate.

Discussion

Diarrhea is mainly caused by *C. perfringens*. To develop and discover new antimicrobial tools for treatment of diarrhea a study was conducted. The present methods and therapies used for the treatment of diarrhea are not so good. *C. perfringens* shows an ability to resist multiple drugs that makes it difficult to treat diarrhea. On the other hand, it is also seen that there are many disadvantages of the use of antibiotics. For example, the antibiotic residuals which are present in the meat are reused by the human.

So, there is a dire need to develop a new antimicrobial agent that can be used for diarrheal treatment. Formation of bacterial biofilms is causing antibiotic resistance in diarrhea is another factor (Jorge *et al*., 2015). This is experimented and evident that bacteriophages have been a good replacement of antibiotics for the treatment of diarrhea because it has an ability to fight against biofilms as well.

Multiple drug resistant *C. perfringens* has become an insistant problem whole world. This increasing level of multi drug resistant's is an economical threat because of enhancing resistance. Diarrhea is most commonly caused by *C. perfringens* and it cause health losses in humans. This increasing threat has led the scientists to think and develop a new alternative which can be used in place of antibiotics for the treatment of diarrhea. Present review shows the high prevalence of *C. perfringens* causing diarrhea in humans and the use of bacteriophage against *C. perfringens* isolated from the feces of humans suffered from diarrhea.

In this review, 10 different fecal samples were collected from Allied Hospital of District Faisalabad. These samples were screened for *C. perfringens*. These samples were also analyzed to check the presence of *C. perfringens*. In an anaerobic environment, culturing of these samples was done on different types of media. The growth of *C. perfringens* was confirmed after its culturing through gram staining, spore staining and different biochemical tests.

After isolation of *C. perfringens*, the identification of bacteriophages was done. The presence of phages was checked in both soil and water samples. These samples were collected from hospital's runoff. The isolated bacteriophages showed a positive activity against *C. perfringens*. The spot test and double layer agar method was used to determine the phage activity

against the host (Kropinski *et al*., 2009). This recent study was conducted to isolate and identify the C. perfringens from sewage. For this purpose, overlayer method was used. Two experiments were performed in this study. First experiment was conducted for identification of *C. perfringens* from infected human's feces. On the other hand, the second experiment was done to check isolated bacteriophage activity in vivo. First of all, human was exposed to *C. perfringens* and then treated with phage cocktail. In-vivo bacteriophage cocktail challenge determined phage activity by reducing the diarrhea in humans. Although this is a time consuming and laborious method. The proposed phage cocktail method appeared as an alternative of antibiotics (Ross *et al*., 2010).

In previous study, the *C. perfringens* were isolated from the stool of the young adults and older individuals by choosing some material from the conventionally cultivated every sample. Thus, isolates of bacteria represented the dominance of *C. perfringens* bacterial population in feces of both young adults and elderly people. For development of diarrhea the intestinal factor is also a key risk because of the reason that *C. perfringens* is a normal biota of human intestine. The growth of *C. perfringens* is promoted because of intake of undercooked meat. In intestinal tract of human, the high prevalence of *C. perfringens* was observed (Harmon and Kautter., 1978). For presence of *C. perfringens*, the screening of intestinal contents of human was done. It was observed that almost 60 percent of human were exposed to colonization of *C. perfringens* bacteria. These bacteria were also showing diarrheal signs with small proportions.

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