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Antimicrobial susceptibility of anaerobic bacteria

Molecular characterization and antimicrobial resistance profile of *Clostridium perfringens* type A isolates from humans, animals, fish and their environment

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ABSTRACT

The study was aimed to characterize, and determine antibiogram of *C. perfringens* type A isolated from the feces of human and animal diarrhoeal cases, as well as healthy animals, meat of pigs and goats, gills and intestine of fish and samples from fish pond. A total of 460 samples, including human diarrhoeal cases (n = 130); diarrhoeal cases of pig (n = 52) and goat (n = 50); fecal samples from healthy pig (n = 50) and goat (n = 50); meat samples *viz*. pork meat (n = 52); goat meat (n = 50) and fish including their environmental sources (n = 26) were used for isolation and identification of *C. perfringens* type A. All the biochemically confirmed isolates were positive for species-specific 16S rRNA and *cpa* genes by PCR assays. Toxinotyping of *C. perfringens* type A isolates showed that overall prevalence of *C. perfringens* type A with only cpa^+ gene was 43.2%; with cpa^+ and $cpb2^+$ genes was 45.4%; with cpa^+ and cpe^+ genes was 4.9%; however, with cpa^+, $cpb2^+$ *and* cpe^+ genes was 6.6%. Antimicrobial susceptibility testing revealed that 83.7% of isolates were resistant to three or more antibiotics.

1. Introduction

Clostridium perfringens is one of the important pathogens responsible for intestinal infections as well as histotoxic diseases in humans and animals [1,2]. In developed countries, *C. perfringens* type A food poisoning is a commonly reported food-borne illness [3]. *C. perfringens* enterotoxin (CPE) producing type A is the second most common cause of foodborne illnesses in the United States (US), with nearly 1 million cases per year, resulting in an economic loss of $382 million [4,5].

Based on the production of four major toxins i.e., alpha (α), beta (β), epsilon (ε) and iota (ι), *C. perfringens* is divided into five major toxinotypes/biotypes (A-E) [1,6]. In addition to major toxins, it also produces enterotoxin (CPE) and β2 toxins (CPB2), which are responsible for food poisoning in humans and gastroenteritis in animals [7]. Classic CPE-mediated *C. perfringens* food poisoning occurs when contaminated meat, fish and/or poultry products are undercooked and/or held at improper temperature (12 °C-60 °C), allowing for spore germination and growth of CPE-producing *C. perfringens* type A in foods [5,8].

Despite wide prevalence of *C. perfringens* type A in the environment, only a small proportion (1–5%) of *C. perfringens* type A strains isolated from humans and animals carry the CPE gene (*cpe*) [8]. Isolates originating from humans with gastrointestinal diseases carry most commonly CPE toxin and in some cases CPB2 toxin [7]. These CPE-positive organisms sporulate in the intestinal tract, producing enterotoxin (CPE), which is responsible for the

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diarrhoeal symptom of the disease [8]. Enterotoxigenic *C. perfringens* type A is also associated with antibiotic-associated diarrhoea (AAD) (approximately 5–15% of all cases of AAD) and sporadic diarrhoea [9,10]. These non-foodborne gastrointestinal tract illnesses tend to be more severe and long lasting than most *C. perfringens* type A food poisoning cases [9,11]. Foodborne isolates of *C. perfringens* most frequently harbour a chromosomal *cpe*, whereas, isolates from patients with AAD and sporadic diarrhoeal cases, and animal diarrhoeal cases usually have a plasmid-borne *cpe* gene [1]. In developing countries including India, there is lack of epidemiological studies that could highlight the magnitude of occurrence of the pathogenic *C. perfringens* type A and the characterization of its virulence genes profile from humans, animals, fish and their environmental sources. The emerging problem of antimicrobial resistance between pathogenic and commensal bacteria is also of concern [12,13]. There are a few reports on antibiogram of *C. perfringens* isolates recovered from different sources from India [14–17], but there is scarcity of data on antibiotic resistant patterns of *C. perfringens* isolates recovered from humans, animals, fish including their environmental samples. Therefore, the present study was aimed to isolate, characterize and determine antimicrobial susceptibility patterns of *C. perfringens* type A from feces of human and animal diarrhoeal cases, as well as healthy animals, meat of pigs and goats, gills and intestine of fish and samples from fish pond.

2. Material and methods

2.1. Sample collection and area of study

A total of 460 samples, comprising feces of human (n = 130) and animal diarrhoeal cases [pig (n = 52), goat (n = 50)], as well as healthy animals [pig (n = 50), goat (n = 50)], meat of pigs (n = 52) and goats (n = 50), gills and intestine of fish and samples from fish pond (n = 26) were collected (Supplementary Table 1). The human diarrhoeal samples were collected from the National Institute of Cholera and Enteric Diseases (NICED), Kolkata in screw cap polypropylene tubes containing Cary-Blair transport medium. Animal and environmental samples were collected from pig and goat farms and butchers herds in and around the area of human samples source using sterile screw cap tubes, whereas pork meat, goat meat and fish samples were collected in sterile plastic sachet. Samples were immediately transported to the laboratory in ice pack containers and processed within 24 h.

2.2. Reference strains

Reference strains of *C. perfringens* (NCTC 8237) were procured from Biological Standardization Division, IVRI, Izatnagar, India.

2.3. Isolation of C. perfringens

Robertson cooked meat (RCM) medium was used as an enrichment broth for isolation of *C. perfringens*. Fecal samples, homogenized meat and fish samples, slurry and water samples were inoculated into Robertson cooked meat medium (1:10 ratio) and heated at 75 °C for 20 min, followed by incubation at 37 °C for 24–48 h under anaerobic condition in McIntosh anaerobic jar using gas pack (HiMedia, Mumbai, India). For isolation, 100 μl of enriched inoculum were added into sterile petri plates, followed by pouring of sterile melted sulfite polymyxin sulfadiazine (SPS) (HiMedia, Mumbai, India) agar over the inoculum with mixing by rotating the plate. Plates were incubated at 37 °C for 24 h in anaerobic conditions using gas pack [18]. The characteristic black colour colonies (3–5 colonies for each sample) on SPS agar were subjected to biochemical tests for identification [19]. Isolates positive for lecithinase, gelatin liquefaction, nitrate reduction and lactose fermentation and negative for indole were presumptively identified as *C. perfringens*. The presumptive *C. perfringens* isolates were subjected to PCR assay for the detection of species-specific 16S rRNA [20].

2.4. Detection of virulence genes

The *C. perfringens* isolates were subjected for *cpa, cpb, εtx* and *ιtx* [21], *cpb2* and *cpe* genes [22,23]. The primers were from M/S Xcelris Labs Limited, Ahmadabad, Gujarat (India).

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by agar dilution method as per the guidelines provided by Clinical Laboratory Standards Institute (CLSI) using *Staphylococcus aureus* ATCC 29213 as the reference strain [24]. The antimicrobials included were ampicillin (AMP), co-trimoxazole (COT), ceftazidime (CAZ), norfloxacin (NX), ceftriaxone (CTR), ciprofloxacin (CIP), tetracycline (TE) and amoxicillin/clavulanic acid (AMC) (HiMedia, Mumbai, India).

2.6. Statistical analysis

The antimicrobial resistance data of the recovered isolates from human, animal, meat, fish and their environmental sources were statistically analyzed by Chi-squared test using SPSS software, 22.0 version.

3. Results

3.1. Isolation and identification of C. perfringens

A total of 57 (43.8%) isolates from human diarrhoeal cases, 34 (65.4%) isolates from pig diarrhoeal cases, 30 (60.0%) isolates each from goat diarrhoeal cases and healthy pigs, 18 (36.0%) isolates from healthy goats, 7 (13.5%) isolates from pork meat, 3 (6.0%) isolates from goat meat and 6 (23.1%) isolates from fish, including their environmental sources were positive for *C. perfringens*. All recovered isolates were found to be positive by PCR assays for species specific 16S rRNA and later on characterized for virulence genes *viz., cpa, cpb2, cpe, cpb, εtx* and *ιtx*. The isolates showing positivity in species specific 16S rRNA and major toxin *cpa* PCR assays were confirmed as *C. perfringens* type A; whereas, those were showing positivity for *cpb, εtx* and *ιtx* PCR assays were excluded from *C. perfringens* type A, since *C. perfringens* type A is devoid of *cpb, εtx* and *ιtx* toxin genes. By molecular detection, one isolate each from diarrhoeal cases of pig and goat were found to be positive for *C. perfringens* type C (*cpa* and *cpb* genes) and type B (*cpa, cpb and εtx* genes), respectively. While none of the isolates were found to be positive for *C. perfringens* type D and E.

3.2. Toxinotyping of recovered C. perfringens isolates

Results of PCR assays used for toxinotyping of *C. perfringens* type A showed that overall occurrence of *C. perfringens* with only cpa^+ gene was 43.2%; with cpa^+ & $cpb2^+$ genes was 45.4%; with cpa^+ & cpe^+ genes was 4.9%; however, with cpa^+, $cpb2^+$ & cpe^+ genes was 6.6% (Table 1).

Out of 57 *C. perfringens* type A isolates recovered from human diarrhoeal cases, 28 (49.1%) were found to have only cpa^+ gene, 20 (35.1%) were positive for both cpa^+ & $cpb2^+$, 3 (5.3%) were positive for cpa^+ & cpe^+ and 6 (10.5%) were positive for all three cpa^+, $cpb2^+$