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New insight of apparently healthy animals as a potential reservoir for *Clostridium perfringens:* a public health implication

Dalia Hamza[1], Sohad M. Dorgham[2], Mahmoud Elhariri[3],
Rehab Elhelw[3], Elshaimaa Ismael[4]

[1]Department of Zoonoses, [3]Department of Microbiology, [4]Department of Veterinary Hygiene and Management,
Faculty of Veterinary Medicine, Cairo University, 12211, Cairo, Egypt
[2]Department of Microbiology and Immunology, National Research Centre, 12211, Cairo, Egypt
daliahamza@cu.edu.eg

Abstract

Introduction: *Clostridium perfringens* is commonly found in the gastrointestinal tract of animals and humans and continues to cause one of the most prevalent foodborne diseases in man. **Material and Methods:** A total of 355 samples were examined for the occurrence of *C. perfringens:* rectal swabs from cattle, sheep, and goats, fresh stool samples from diarrhoea sufferers having been in contact with these animals, irrigation water and soil samples from the husbandry sites, and pre-harvesting fresh produce from farms irrigated with the sampled water. All samples were collected from Cairo and Giza governorates, Egypt. PCR analysis was carried out with positive isolates using the α-toxin gene. Sequence analysis of the gene of *C. perfringens* isolates was performed using the neighbour-joining approach. Bootstrap analysis was executed with 1,000 resamplings. **Results:** 174 *C. perfringens* strains were isolated with a 49.01% prevalence. The highest prevalence of *C. perfringens* in apparently healthy animals was found in sheep (65.45%) followed by goats (58%), buffaloes (55%), and cattle (47.1%). Its prevalence in humans being in contact with these animals was 47.5%. The bacterium's isolation from the soil and irrigation water was achieved in 40% and 31.7% of samples, respectively, posing a risk, particularly when the water and soil contact food in the field, shown by the fresh produce isolation of 40%. A significant relationship between the prevalence of *C. perfringens* in animal and environmental samples was identified (P < 0.05). A significant relationship was identified neither between animal species and *C. perfringens* prevalence, nor between the environmental source and *C. perfringens* prevalence (P > 0.05). All isolates were positive for the α-toxin gene by PCR. The sequence analysis and the phylogenetic relationship of the α-toxin genes from different samples revealed that *C. perfringens* from faeces of apparently healthy cattle, buffaloes, sheep, and goats is a significant threat in places where it can contaminate the soil and water. In addition, the sequence of *C. perfringens* from humans suffering from diarrhoea was found in the same cluster with the sequence from cows, goats, and sheep. **Conclusion:** The role of apparently healthy animals in transmitting *C. perfringens* to humans, either through being in direct or indirect contact *via* water or soil in the cultivation of vegetables and fruits, was demonstrated.

Keywords: humans, apparently healthy animals, *Clostridium perfringens,* soil, water, public health.

Introduction

The foodborne human diseases caused by *Clostridium perfringens* remain some of the most prevalent diseases. *C. perfringens* is responsible for two forms of enteritis: type A produces diarrhoea with abdominal pain within 8–20 h after ingestion of the contaminated food (24 h clinical course), and type C is responsible for necrotising enteritis (bloody diarrhoea with abdominal pain, shock and peritonitis) (4).

This bacterium is commonly found in vegetables and crops as well as meat products (12), including cooked foods, because as these gradually cool, heat-stable spores of *C. perfringens* may germinate and proliferate (16).

C. perfringens is spore producer, is impervious to heat, chlorination, and other stress factors, and has a strong capability to survive in contrast to vegetative cells like *E. coli* and *enterococci,* which are less resistant to hostile environments (15). *C. perfringens* is

reported to display a certain acceptance of aerobic conditions in foods. This is a noteworthy in comparison with other anaerobes, that it can grow not only in canned foods but also in bulk food if reducing substances are present (4), and this indicates wide sources of infection. Meanwhile *C. perfringens* is a normal inhabitant of the intestine of most animal species and humans and also is widespread in the environment (14). The soil is a reservoir of *C. perfringens* (13), so this organism can contaminate vegetables during cultivation and subsequent harvest. Foodborne pathogens that are associated with fresh produce originate mostly from intestinal tract and faecal material of humans or animals (12). Contamination of vegetables and fruits with spores of bacteria such as *B. cereus*, *C. botulinum*, *or C. perfringens* present in the soil is common (11).

In this work, we have determined the presence of *C. perfringens* in apparently healthy animals which might be a source of contamination of fresh produce through water and soil, and a potential risk factor for consumers.

Material and Methods

Sample collection and preparation

Animal and human samples. Rectal swabs from 70 cattle, 40 buffaloes, 55 sheep, and 50 goats in Cairo and Giza governorates, Egypt, and 40 fresh stool samples from people suffering from diarrhoea and having been in contact with these animals were collected and transferred directly without delay to the laboratory.

Water samples. Water was taken in a total of 60 samples from the irrigated water around the animal facilities (1 L for each sample) to sterile glass bottles with sodium thiosulphate as a dechlorinating agent, and then transferred to the laboratory.

Soil samples. The soil was collected from different locations around the examined animals. Each of the 30 samples contained 1 g of soil, which was collected in a 15 mL sterile plastic tube.

Fresh produce. A total of 10 regular market-sized fresh products were collected from farms prior to harvesting, which were previously irrigated with the examined water. All samples were placed in sterile polyethylene bags, labelled, and placed in an ice box for further isolation and identification.

Isolation and identification of *C. perfringens*

Faecal samples. Rectal swabs were inoculated onto a tube of sterile freshly prepared cooked meat broth (Oxoid, UK), then incubated at 37°C for 24–48 h in an anaerobic jar, using anaerobic gas generating kits. A loopful from a previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 μg/mL). The plates were incubated anaerobically at 37°C for 24–48 h.

Water samples. Water samples were concentrated by filtration through 0.45 μm pore nitrocellulose filters (Sartorius, France), and then the filters were vortexed in peptone broth for recovering the bacteria. After removing the filters, the bacteria were cultivated in cooked meat broth (Oxoid, UK), then incubated at 37°C for 24–48 h in an anaerobic jar, using anaerobic gas generating kits. A loopful from a previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 μg/mL). The plates were incubated anaerobically at 37°C for 24–48 h (6).

Fresh produce. The surface of products was sanitised by dipping it in a 70% ethanol solution and then allowing it to dry. The products were dissected using a sterile scalpel into stem scar and pulp, as described by Guo *et al.* (9). Each sample was placed in a sterile stomacher bag with 9 mL of sterile 0.1% peptone water and stomached for 2 min. One millilitre of the sample was added to 9 mL of cooked meat media and completion of sample preparation ensued as previously.

Soil samples. A 10 mL aliquot of sterile differential reinforced clostridial broth medium (DRCM; Merck, Germany) was added to 1 g of each soil sample in a 15 mL sterile tube. After gentle mixing, 1 mL aliquots of DRCM suspension containing a soil sample were added to two tubes, each containing 9 mL of sterile DRCM. To identify and encourage the germination of any *C. perfringens* spores present in soil samples, one tube of each paired set was heat shocked at 75°C for 20 min before incubation at 43°C for 16 h, and the other tube was directly incubated at 43°C for 16 h to enrich for *C. perfringens* vegetative cells or self-germinating spores which were present in the sample. A loopful from the previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 μg/ml). The plates were incubated anaerobically at 37°C for 24–48 h (12).

The typical suspect colonies with the double zone of haemolysis were identified after conducting the anaerobic tolerance test by different biochemical tests such as Gram staining, phospholipase C (lecithinase) test, indole, urease production, gelatin hydrolysis, and fermentation of sugars like glucose, lactose, and mannitol (8).

DNA extraction and PCR assay. Genomic DNA of *C. perfringens* was extracted using a QIAmp Mini Kit (Qiagen, Germany). Specific oligonucleotide primers for the α-toxin gene of *C. perfringens* were used: forward GTTGATAGCGCAGGACATGTTAAG and reverse CATGTAGTCATCTGTTCCAGCATC with molecular weight 402 bp (1).

The assay was carried out in 25 μL of PCR reaction mixture (25 μL) containing 3 μL of template DNA, 12.5 μL of master mix, 0.5 μL of one of the primers (10 pmol/μL), and 8.5 μL of DNase-free water.

The PCR reaction mixtures were placed in a Biometra PCR thermal cycler (Biometra, Germany). Following initial denaturation for 5 min at 94ºC, the samples were subjected to 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min. After the last cycle, a final extension for 10 min at 72ºC was performed (7). The PCR reaction mixtures were analysed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of a 100 bp DNA ladder (Fermentas Life Science, part of Thermo Fisher Scientific, USA).

Sequence of α-toxin gene. PCR products of eight *C. perfringens* isolates obtained from different animal species, human, soil, water, and fresh produce were purified using a QIA quick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions, and sequencing was conducted using a Big Dye Terminator V3.1 sequencing kit (Applied Biosystems, USA.) by using forward and reverse primers of α-toxin genes.

GenBank submission. The sequences of the α-toxin genes have been deposited in the GenBank database under accession numbers: KJ729010, KJ729012, KJ729013, and KJ729016 from cow, sheep, goat, and buffalo, respectively, kF383124 from human, KX793135 and KX793136 from soil and water respectively, and MG252853 from fresh produce.

Sequence and phylogenetic analysis of nucleotide (nt) sequences of the α-toxin gene were compared with the sequences available in public domains using NCBI, BLAST server, and the representative sequences of α-toxin genes of *C. perfringens* from different animals and soil obtained from GenBank. Sequences were downloaded and imported into BIOEDIT version 7.0.1.4 (Tom Hall of Ibex Pharmaceuticals, USA) for multiple alignments according to their amino acid deduced using the CLUSTAL W programme of BIOEDIT. Phylogenetic analysis was performed with MEGA version 6 (Molecular Evolutionary Genetic Analysis, Kumar S, Stecher G, and Tamura K. USA), using the maximum likelihood approach. Bootstrap analysis was performed with 1,000 resamplings.

Statistical analysis. PASW Statistics, SPSS 18.0 software (IBM SPSS, USA) was used to analyse the data. The chi-squared test was used to determine whether there is a relation between the occurrence of *C. perfringens* and species of animals and to compare its presence in different types of samples. Differences were considered statistically significant if the P value was <0.05.

Results

A total of 174 *C. perfringens* strains (49.01%) were isolated from 355 examined samples. Samples totalling 120 (55.81%) were positive for *C. perfringens* from 215 rectal swabs from various animals, 19 (47.5%) samples were positive from 40 human stool samples, 31 (34.44%) samples were positive from 90 environmental samples, and 4 (40%) samples were positive from 10 fresh produce samples (Table 1). The difference between animal and environmental samples was significant as tested by the chi-squared test (P < 0.05).

Isolation of *C. perfringens* from animals. The prevalence of *C. perfringens* in cattle (33/70) was 47.10% and in buffaloes (22/40) was 55%. The prevalence of *C. perfringens* in sheep (36/55) was 65.45% and in goats (29/50) was 58%. No significant relationship among animal species and *C. perfringens* prevalence was observed (P > 0.05) (Table 1).

Isolation of *C. perfringens* from the environment surrounding the animal populations. *C. perfringens* strains were isolated from 19 out of 60 water samples used for land irrigation to give 31.7% prevalence. *C. perfringens* was isolated from 12 (40%) samples of the 30 soil samples (Table 1). No significant relationship between the environmental source and *C. perfringens* prevalence was found (P > 0.05) (Table 1).

Table 1. Prevalence (%) of *C. perfringens* in apparently healthy animals and humans, environment, and fresh produce (n = 355)

Samples	Number of samples	*C. perfringens* strains	
		Positive	%
Animals			
Cattle	70	33	47.10
Buffaloes	40	22	55.00
Sheep	55	36	65.45
Goats	50	29	58.00
Total	215	120 [a]	55.81
Human	40	19 [ab]	47.50
Environment			
Water	60	19	31.70
Soil	30	12	40.00
Total	90	31 [b]	34.44
Fresh products	10	4 [ab]	40.00
Total samples	355	174	49.01

[a, b] Different superscripts indicate significance at P < 0.05

Table 2. Prevalence (%) of *C. perfringens* in soil and water samples collected from areas around various apparently healthy animal populations (n = 90)

Samples	Number of samples	*C. perfringens* strains	
		Positive	%
Soil			
Cattle and buffaloes	10	3	30
Sheep and goats	10	4	40
Mixed species	10	5	50
Total	30	12	40
Water			
Cattle and buffaloes	20	6	30
Sheep and goats	20	6	30
Mixed species	20	7	35
Total	60	19	31.70
Total environmental samples	90	31	34.44

Sheep and goats were kept in one area, cattle and buffaloes were kept in another, while in the third area all animal species mixed freely
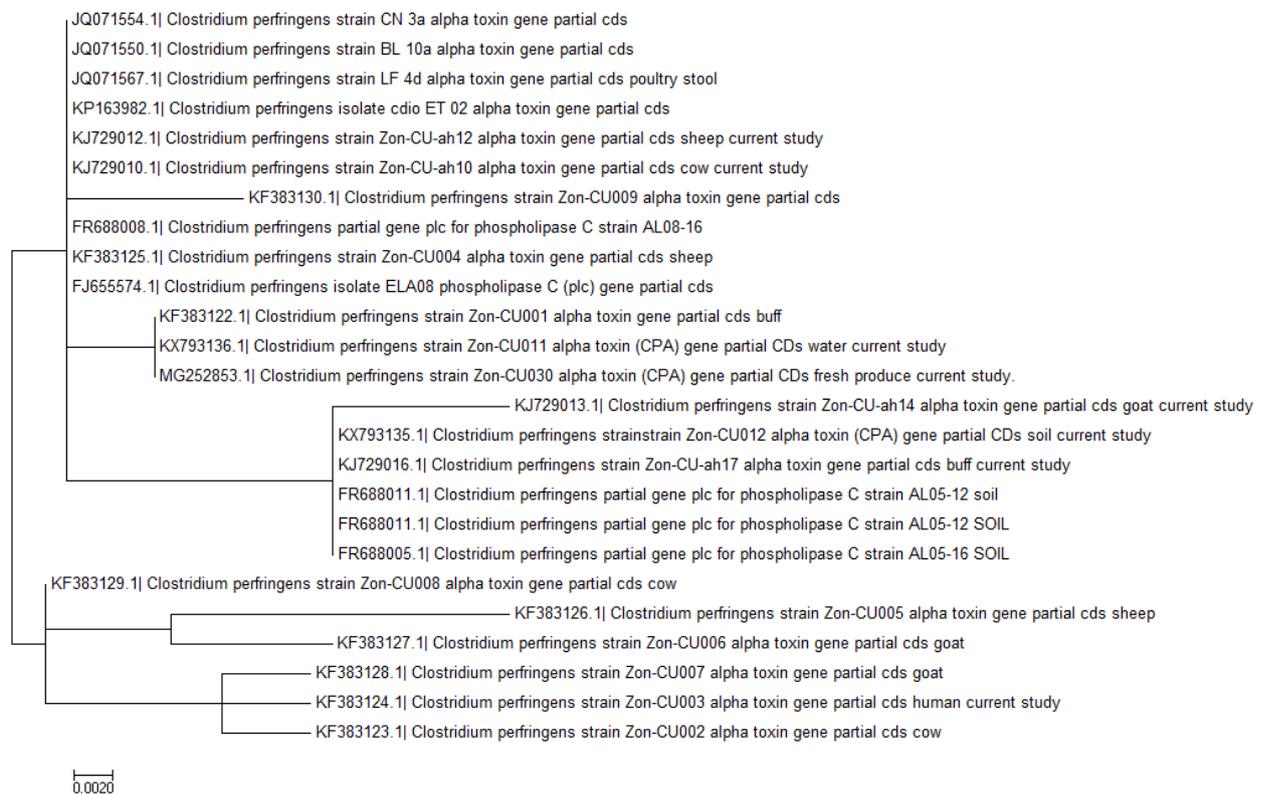
Fig. 1. Phylogenetic relationship determined using maximum likelihood method. The analysis was conducted based on the partial sequence of α-toxin gene of *C. perfringens*. The evolutionary analysis was performed with MEGA version 6

The prevalences of *C. perfringens* in soil and water by animal species raised in proximity to that soil or water are presented in Table 2. Soil-isolated *C. perfringens* strains sampled near cattle and buffaloes (3/10), sheep and goats (4/10), and mixed animal populations (5/10) had 30%, 40%, and 50% prevalences, respectively. The prevalences of positive isolation from water sources around cattle and buffaloes (6/20), sheep and goats (6/20), and mixed animal populations (7/20) were 30%, 30% and 35%, respectively.

All isolates were positive for the α-toxin gene by PCR. The sequence analysis of the eight *C. perfringens* isolates from different samples and the phylogenetic relationship revealed that the sequence of *C. perfringens* from humans suffering from diarrhoea was found in the

same cluster with sequences from cow, goat, and sheep, and in the same group as those isolated from soil, water, and fresh produce.

Discussion

As indicated by our results, 174 *C. perfringens* strains were isolated from 355 samples (faecal samples of apparently healthy cattle, buffaloes, sheep, and goats, humans, water, soil, and fresh produce) with an overall prevalence rate of 49.01%. A significant relationship between prevalence in animals and environmental samples was identified ($P < 0.05$). A significant relationship was identified neither between animal species and *C. perfringens* prevalence, nor between the environmental source and the prevalence ($P > 0.05$).

C. perfringens were isolated more frequently from sheep, followed by goats, buffaloes, and cattle (63.6%, 58%, 55%, and 47.1%, respectively). This outcome thoroughly concurs with the established certainty that *C. perfringens* is widely distributed in nature and normally found in the intestines of animals, as reported previously (2).

Moreover, the isolation of *C. perfringens* from the soil around animal husbandry sites was 40%, which could be a result of the common use of animal manures as fertilisers. Alternatively, it is possible that *C. perfringens* can grow in soil (13). In addition, the presence of *C. perfringens* in irrigation water (31.7%) could be a potential risk, particularly when the water interacts with food. Prevalence of *C. perfringens* in soil and water was not significantly related to the species of animals raised around them (Table 2), and this indicates that the spreading of the organism could happen irrespective of the animal species raised in proximity.

Our results stated that the α-toxin gene was isolated from all positive samples of *C. perfringens,* and the phylogenetic relationship of the selected sequences illustrated that the presence of *C. perfringens* in faeces of apparently healthy animals (cattle, buffaloes, sheep, and goats) represents a major hazard that could contaminate the soil and water (Fig. 1).

Since many types of life form can contaminate the surface of vegetables, microbial contamination can occur through irrigation with water contaminated with high levels of faecal bacteria and the sewage sludge of wastewater (10). *C. perfringens* can contaminate vegetables through soil or water at any stage from production to consumption as it was found at high prevalence in both water and soil.

The related foodborne diseases are caused by *C. perfringens* enterotoxin produced during sporulation (4). This enterotoxin is not produced by all known strains. Enterotoxin effects only ensue after ingestion of a large number of life forms (10^8–10^9).

The occurrence of *C. perfringens* in people that have contact with apparently healthy animals and suffer from diarrhoea was high, with prevalence of 47.5% (Table 1). The sequence analysis of *C. perfringens* isolated from humans suffering from diarrhoea was found in the same cluster with sequences from cow, goat, and sheep, and in the same group as those isolated from water, soil, and fresh produce. These results elucidate the role of animals in transmitting *C. perfringens* to humans, either through humans having direct contact with these animals or through indirect contact by using contaminated water and soil in the cultivation of vegetables and fruits. This is supported by Al-Kharousi *et al.* (3) who identified some microbial communities of fresh imported fruits and vegetables. The authors isolated *Enterobacteriaceae* with 60% prevalence from fruits and 91% from vegetables, *Enterococcus* also was isolated from 20% of fruits and 42% of vegetables. *E. coli* and *S. aureus* were also isolated from 22% and 7% of vegetables, respectively. The different microbes in fresh fruits and vegetables can passthrough the stomach to the intestines where they set up the particular relationship with the host and bring their different consequences for human health (5). Further study of isolation of *C. perfringens* from fresh produce is required.

In conclusion, the role of apparently healthy animals in transmitting *C. perfringens* to humans being in contact with those animals was illuminated by sequence analysis of *C. perfringens* isolates' α-toxin gene and was proved by the presence of the α-toxin gene sequence from human and animal isolates in the same cluster. This potentiates the enormous risk of transmission of *C. perfringens.* These animals can shed the organism in a continuous manner to the environment, including soil and water, and finally to fruit and vegetables during the cultivation and subsequent harvest or through surface contamination.

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