RESEASRCH ARTICE

Genetic Diversity and Clonal Linkages of *Clostridium perfringens* Type-A Isolates from Animal-Origin Foods and Human Diarrhoeal Cases using ERIC-PCR: Insights from One Health Perspective

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ABSTRACT

Clostridium perfringens type A is a notable pathogen transmitted through food that causes gastroenteritis and diarrhoea in humans and animals. Gaining insight into its genetic variation and spread through the food chain is vital for public health, food safety, and the management of zoonotic diseases. This study explored the genetic connections among *C. perfringens* type-A isolates obtained from meat, fish, dairy products, and human diarrhoeal stool samples using Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR), with a focus on the One health perspective. A total of 62 *C. perfringens* type-A isolates from meat and meat products (n=16), fish and fish products (n=19), milk and milk products (n=16), and human diarrhoeal stool samples (n=11) were analyzed using ERIC-PCR. Dendrograms were created using the UPGMA method based on the Dice similarity coefficients to evaluate the genetic diversity and potential transmission between sources. ERIC-PCR uncovered a complex genetic landscape with both clonally related and highly diverse strains across food and human isolate categories. Several clusters included isolates from both food products and human stool, indicating possible zoonotic and foodborne transmission. The discriminatory indices varied from 0.7583 (milk) to 0.8713 (fish), indicating different levels of genetic diversity among food items. The clustering patterns suggest shared contamination sources, cross-sectoral transmission routes, and the potential persistence of *C. perfringens* strains in food production environments, posing a significant risk to public health. This study provides molecular evidence of the genetic overlap between animal-derived food products and human diarrhoeal isolates of *C. perfringens* type-A, underscoring the importance of the One health approach for ensuring food safety. Integrated surveillance, improved hygiene practices, and stringent control measures across the human-animal-environment interface are crucial for reducing the risk of foodborne *C. perfringens* infections.

Key words: *C. perfringens*, ERIC-PCR, Genetic diversity, Food-borne pathogens, One health, Zoonoses.

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INTRODUCTION

C. perfringens is a Gram-positive, anaerobic bacterium capable of forming spores and is commonly found in the environment, such as in soil, sediments, decaying organic matter, and the gastrointestinal tracts of humans and animals (Vinod *et al*., 2014; Prescott *et al*., 2016). It ranks among the top causes of foodborne illnesses globally and is the second most prevalent bacterial cause of food-borne diarrhoea in the United States, following Salmonella (Brynestad and Granum, 2002; Scallan *et al*., 2011). The ability of the bacterium *C. perfringens* to form highly resistant spores enables it to withstand harsh environmental conditions, including high temperatures. Animal-derived foods, especially meat, fish, and dairy products, are common carriers of this pathogen (Potter, 2001; Santos *et al*., 2002). Under favourable conditions, such as temperature abuse or insufficient refrigeration, spores can germinate, leading to bacterial growth. The consumption of contaminated food leads to intestinal sporulation and enterotoxin production, causing gastrointestinal symptoms such as watery diarrhoea and abdominal cramps (McClane, 1996). Molecular genotyping has become crucial for studying foodborne pathogens because it enables precise identification of clonal relationships, source tracing, and outbreak investigations. A fundamental concept in microbial epidemiology is that isolates from related outbreaks or

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sources usually share genetic markers that distinguish them from unrelated strains. However, because similar subtypes can appear in unrelated environments, high-resolution genotyping methods are necessary to confirm epidemiological links (Anjay *et al*., 2015).

While pulsed-field gel electrophoresis (PFGE), remains the benchmark for its discriminatory ability (Alavandi *et al*., 2001), simpler methods like random amplification of polymorphic DNA (RAPD-PCR), and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) are preferred in resource-limited settings (Versalovic *et al*., 1991; Talon *et al*., 1996). ERIC-PCR provides discrimination comparable to that of RAPD-PCR (Szczuka and Kaznowski, 2004) and requires minimal DNA for routine use (Thomas *et al*., 2009). ERIC-PCR has been used to study *C. perfringens* type A strains from food and human diarrhoeal cases in the past. Studies have shown varying results regarding the genetic diversity of *C. perfringens*. Xue-qin *et al*. (2009) found low genetic variability in healthy poultry isolates using AFLP and ERIC-PCR, while Afshari *et al*. (2016) found higher diversity in poultry isolates using RAPD-PCR. This study aimed to assess the genetic diversity and epidemiological relationships of *C. perfringens* type-A isolates from meat, fish, dairy products, and human diarrhoeal samples using ERIC-PCR to improve the understanding of genetic links among isolates and aid in the development of surveillance and control strategies.

MATERIALS AND METHODS

Bacterial Isolates and Revival

The study was conducted in the Department of Veterinary Public Health and Epidemiology, Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar, India following approval by the Institutional Animal Ethical Committee. A total of 62 *C. perfringens* type-A isolates were included in this study. These isolates were collected from various sources, including meat and meat products (16 isolates), fish and fish products (19 isolates), raw and pasteurized milk and milk products (16 isolates), and human diarrhoeal stool samples (11 isolates). The isolates were revived by inoculating a loopful of culture into 2 mL of modified cooked meat medium (MCMM) broth, followed by incubation at 37°C for 24 h under anaerobic conditions.

Genomic DNA Extraction

Genomic DNA was extracted from overnight cultures using the Qiagen DNA Mini Kit, following the manufacturer's protocol with minor modifications. The cultures were centrifuged at 11180 x g for 10 min to pellet the cells, and the supernatants were discarded. The bacterial pellets were resuspended in 180 µL of ATL buffer and 20 µL of proteinase K, followed by thorough mixing via vortexing. The mixture was incubated at 56°C for 30 min in a water bath to ensure complete lysis of the cells. Subsequently, 200 µL of ATL buffer was added to the lysate, mixed thoroughly by vortexing, and 200 µL of ethanol was added to the mixture. The entire solution was transferred into a spin column provided with the kit and centrifuged at 7160 x g for 1 min. The column was then washed with 500 µL of AW1 buffer and centrifuged at 7160 x g for 1 min. Subsequently, 500 µL of AW2 buffer was added to the column and centrifuged at 21950 x g for 3 min to remove any residual contaminants from the column. DNA was eluted by adding 30 µL of AE buffer to the column, incubating it at room temperature for 2 min, and centrifuging it at 7160 x g for 1 min. To maximize the DNA yield, the elution step was repeated using an additional 30 µL AE buffer. The extracted DNA was stored at –20°C until further use. The concentration and purity of the DNA were measured using a nanometer spectrophotometer (Genova Nano, UK).

ERIC-PCR Amplification

ERIC-PCR was conducted to assess the genetic relatedness of the isolates using the primers ERIC-1 (5'-ATG AAG CTC CTG GGG ATT CAC-3') and ERIC-2 (5'-AAG TAA GTG ACT GGG GTG AGG-3'), as described by Versalovic *et al*. (1991). The PCR reaction mixture comprised 2.5 µL of 10X PCR buffer, 3 µL of a 2 mM dNTP mixture, 2.5 µL of $MgCl_2$, 3 µL of each primer (30 pmol), 0.2 µL of Taq DNA polymerase (1 unit), 3 µL of template DNA (containing approximately 20-40 ng) and rest nucleus free water to make final volume up to 25 µL. The amplification process began with an initial denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 90 s, and extension at 72°C for 1 min. A final extension step was performed at 72 °C for 7 min to complete the reaction.

Electrophoresis and Data Analysis

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide at a concentration of 0.5 µg/mL. The electrophoresis process was conducted at 80 volts for 2 h, utilizing a 1 kb DNA ladder from MBI, Fermentas, as a molecular size marker to estimate the sizes of the amplified DNA fragments. DNA bands were visualized and captured using a Gel Documentation System (Vilber, France). The banding patterns derived from ERIC-PCR were examined using PyElph 1.4 software to create dendrograms and assess the genetic similarity among the isolates using the unweighted pair group method with the arithmetic mean (UPGMA). The discriminatory power of ERIC-PCR was assessed by calculating the discriminatory index (D-value) through an online tool available at http://insilico.ehu.es/mini_ tools/discriminatory_power/.

RESULTS AND DISCUSSION

ERIC-PCR profiles of the 16 meat-derived isolates exhibited 2-7 bands, ranging from <250 to 2000 bp. Cluster analysis using UPGMA and Dice similarity coefficients grouped these into five distinct clusters (A-E) (Fig. 1), reflecting varied genetic relatedness. Cluster A was predominant, comprising seven isolates (43.75%) with closely related fingerprints (minimum distance: 19.2), suggesting clonal dissemination, possibly via shared contamination sources in the meat processing chain. Other clusters showed moderate to high divergence, such as Cluster D (single, divergent isolate CM12) and Cluster E (two closely related but distinct isolates). The discriminatory index (D) was 0.7667, indicating moderate heterogeneity in the study. These findings aligned with earlier reports of limited diversity in meat-associated isolates (Xue-Qin *et al*., 2009) with occasional clonal expansion events.

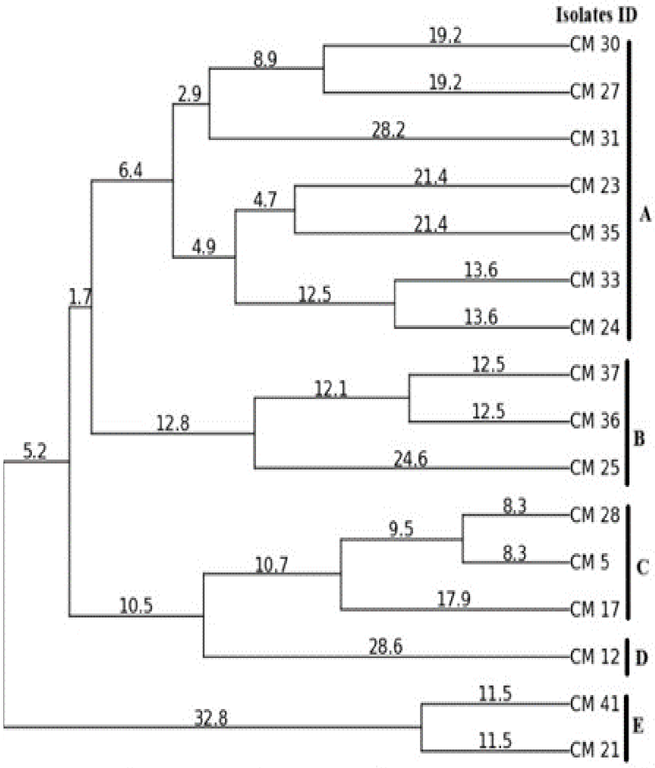

Fig. 1: Diagram showing genetic relationships among *C. perfringens* type A isolates from meat & meat products based on ERIC-PCR. The similarity was calculated from Dice coefficients by the UPGMA method.

ERIC-PCR patterns of the 19 fish-derived isolates yielded 2-8 bands (size range: <250-1500 bp). Dendrogram analysis revealed six clusters (A-F), reflecting substantial intragroup diversity (Fig. 2). Some clusters, such as Cluster A (F28 and F30) and Cluster C (F41 and F38), exhibited close genetic relationships, implying clonal origin or common environmental exposure. Other clusters, such as Clusters E and F, displayed greater internal diversity, suggesting multiple contamination events or evolutionary divergence. The high discriminatory index of 0.8713 indicates notable genetic variability, reinforcing fish as a potential reservoir of diverse *C. perfringens* strains in the aquatic environment. This expands upon previous findings that focused primarily on poultry and meat products (Deguchi *et al*., 2009).

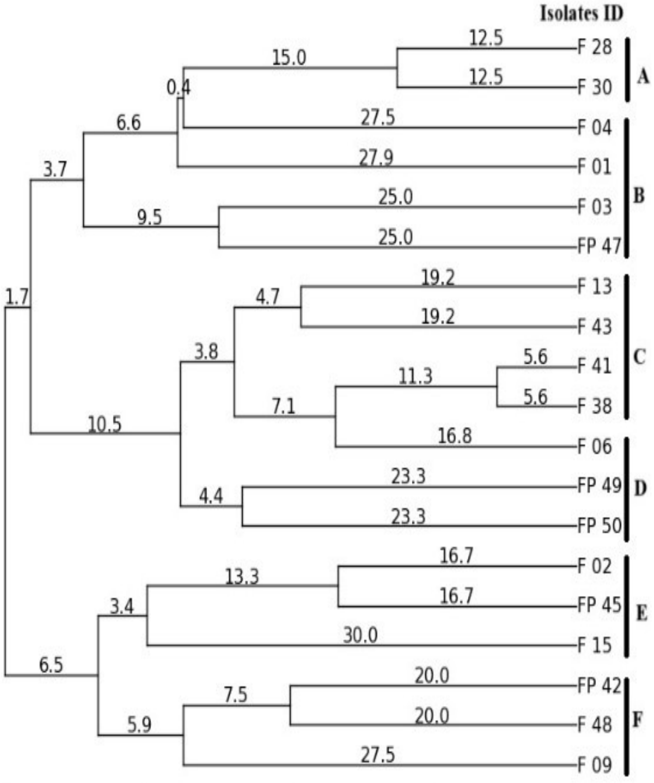

Fig. 2: Diagram showing genetic relationships among *C. perfringens* type A isolates from fish & fish products based on ERIC-PCR. The similarity was calculated from Dice coefficients by the UPGMA method.

Among the 16 isolates from milk and milk products, ERIC-PCR generated 1-7 bands (size range: <250-1000 bp). Cluster analysis (Fig. 3) grouped the isolates into five clusters (A-E) based on their similarities. Cluster A, the largest, showed clonal relationships between certain isolates, *viz.,* MP01/MP08, PM13/PM12 with genetic distance of 0.0, pointing to persistent strains or cross-contamination within dairy processing environments. Cluster E (PM04 and PM19) represented a genetically distinct lineage. The overall discriminatory index (D = 0.7583) reflected moderate to low diversity, in agreement with Madoroba *et al*. (2010), who noted clonal clustering among foodborne isolates. The inclusion of milk-based isolates in this study provides valuable insights into lesser-studied transmission pathways.

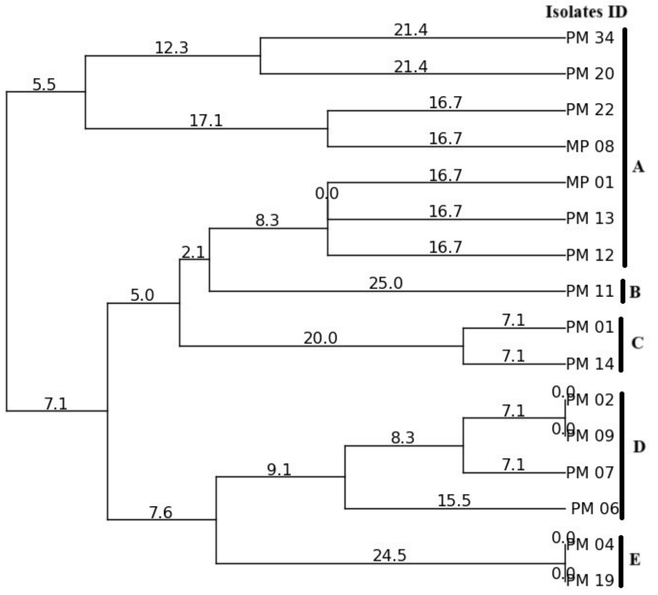

Fig. 3: Diagram showing genetic relationships among *C. perfringens* type A isolates from milk & milk products based on ERIC-PCR. The similarity was calculated from Dice coefficients by the UPGMA method.

ERIC-PCR of 11 human diarrhoeal stool isolates produced profiles with 3-7 bands (<250-750 bp), showing the highest variability among all sources. Four distinct clusters (A-D) were identified (Fig. 4), including closely related pairs (HS16/HS08; distance = 2.0) and genetically divergent strains (HS21 and HS47). The genetic distances (range: 2.0-26.9) reflect complex transmission dynamics, potentially involving diverse exposure routes, including food, water, and environmental sources of exposure. The discriminatory index (D = 0.7636) supports the high heterogeneity observed, echoing the findings of Deguchi *et al*. (2009) and Afshari *et al*. (2016) on human-associated isolates.

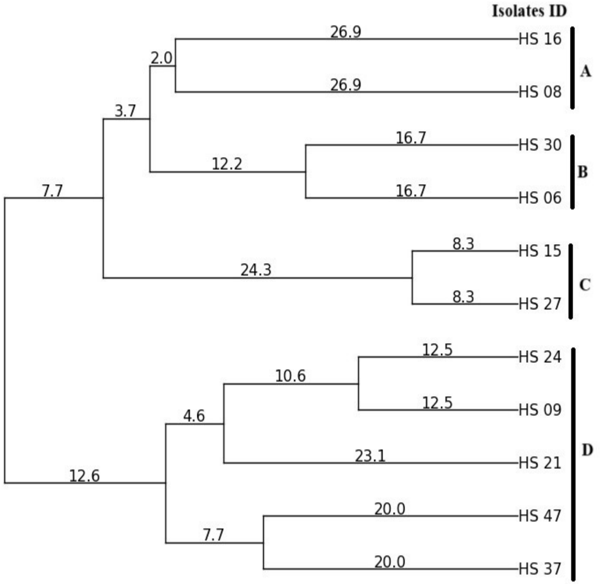

Fig. 4: Diagram showing genetic relationships among *C. perfringens* type A isolates from human stool based on ERIC-PCR. The similarity was calculated from Dice coefficients by the UPGMA method.

To explore cross-source genetic relationships, a combined dendrogram of meat and human isolates was generated (Fig. 5), resulting in eight clusters (A-H). Mixed clustering of food and human isolates in multiple clusters (Cluster B: CM21, HS24, HS09; Cluster F: CM27, HS15) underscores the potential zoonotic or foodborne transmission routes. Shared profiles within clusters imply possible contamination linkages, whereas the presence of distinct lineages reflects environmental and handling-related factors. The genetic distances (1.4-27.8) further support a combination of clonal propagation and independent introductions.

A dendrogram comparing fish products and human stool isolates (Fig. 6) revealed ten clusters (A-J), with several clusters demonstrating cross-origin similarities (eCluster A: F28, F43, HS06; Cluster C: FP47, HS16). Some isolates (F09 in Cluster F and HS37 in Cluster J) exhibited a marked genetic divergence. Overall, the genetic distances (0.0-41.1) highlighted substantial heterogeneity. The co-clustering of fish and human isolates supports the hypothesis of inter-source transmission and reinforces the significance of aquatic environments as reservoirs of *C. perfringens*.

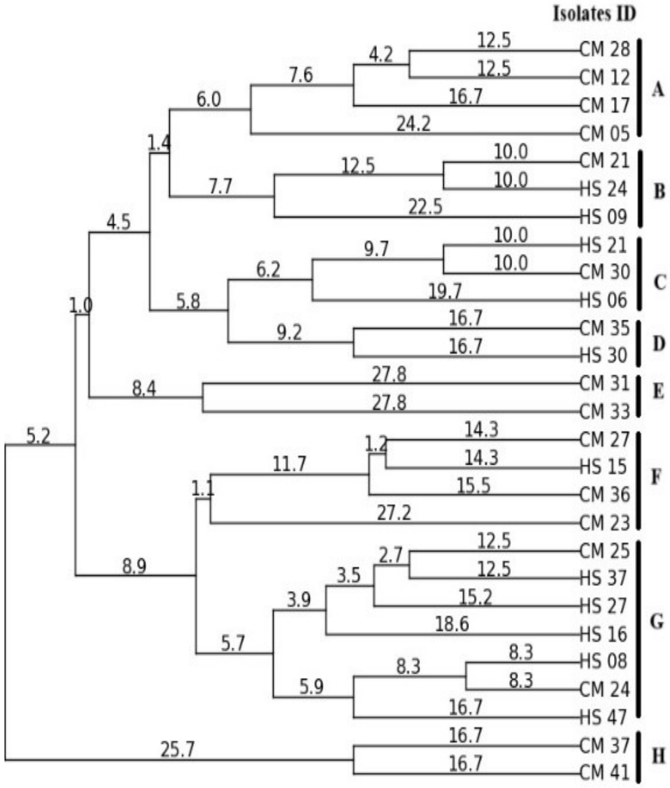

Fig. 5: Diagram showing genetic relationships among *C. perfringens* type A isolates from meat & meat products and human stool based on ERIC-PCR. The similarity was calculated from Dice coefficients by the UPGMA method.

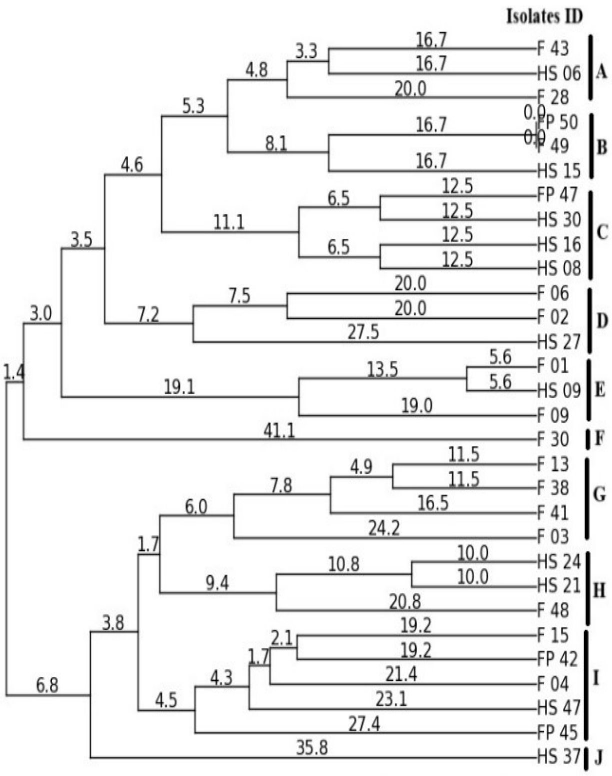

Fig. 6: Diagram showing genetic relationships among *C. perfringens* type A isolates from fish & fish products and human stool based on ERIC-PCR. The similarity was calculated from Dice coefficients by the UPGMA method.

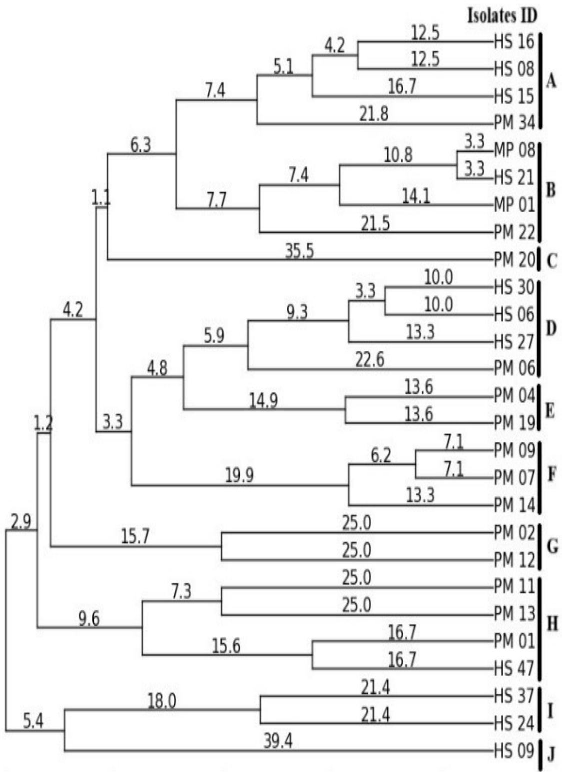

Fig. 7: Diagram showing genetic relationships among *C. perfringens* type A isolates from milk & milk products and human stool based on ERIC-PCR. The similarity was calculated from Dice coefficients by the UPGMA method.

The combined dendrogram of milk and human isolates revealed ten clusters (A-J) (Fig. 7). Several clusters contained both human and milk isolates (Cluster A: HS08, HS15, PM34; Cluster B: MP08, HS21), supporting the possibility of cross-contamination in the dairy supply chain. Highly divergent isolates (PM20 in Cluster C and HS09 in Cluster J) indicated unique contamination or evolutionary events. The range of genetic distances (0.0-39.4) reflects a complex genetic landscape with overlapping and distinct lineages.

Collectively, these findings indicate that *C. perfringens* type A isolates from diverse food sources, such as meat, fish, and dairy, share significant genetic similarities with human diarrhoeal strains. The co-clustering of food and human isolates across multiple dendrograms suggests possible zoonotic transmission and emphasizes the critical role of food as a reservoir for pathogenic *C. perfringens*. These results extend prior observations (Xue-Qin *et al*., 2009; Afshari *et al*., 2016; Deguchi *et al*., 2009; Madoroba *et al*., 2010) and offer new insights into underexplored sources, such as fish and dairy. Moderate to high discriminatory indices (0.7583-0.8713) reflect a balance between clonal propagation and genetic diversity, suggesting the need for robust molecular surveillance. Preventive strategies should focus on strict hygiene, proper food handling, and thorough cooking, especially in informal or minimally processed food systems. Additionally, potential reverse transmission (human to environment/animal) and contamination through environmental exposure cannot be ruled out, particularly in regions with poor sanitation conditions. These findings underscore the importance of integrating food safety, human health, and environmental monitoring using a One Health approach to effectively control *C. perfringens* infections.

CONCLUSION

This extensive analysis using ERIC-PCR demonstrated a strong genetic similarity between *C. perfringens* type A strains found in animal-based foods and those associated with human diarrhoea, suggesting possible pathways for foodborne and zoonotic transmission. These findings underscore the importance of maintaining rigorous food hygiene standards, implementing effective cooking methods, and establishing cross-sectoral monitoring programs in accordance with the One Health approach. Enhancing cooperation among the public health, veterinary, and food safety sectors is essential to curb the spread of *C. perfringens* and prevent food-borne diseases at the intersection of human, animal, and environmental health.

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