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Prevalence of *Clostridium perfringens* and Detection of Its Toxins in Meat Products in Selected Areas of West Kazakhstan

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Abstract: Objectives. The current study aimed to investigate the prevalence of *Clostridium perfringens* in meat products at meat fairs in four cities of West Kazakhstan from April to October 2021. **Methods**. In total, 240 samples were collected and subsequently examined for the presence of *Clostridium perfringens* and its associated toxins using a standard culture method and multiplex PCR assay. **Results**. In the 240 samples, 67 (30%) tested positive for *Clostridium perfringens*. All isolates were classified as biotype A with the ability to produce α toxin. The prevalence of *Clostridium perfringens* was found in almost all types of meat products tested. Beef samples 20/40 (50%) were found the most contaminated with a pathogen, followed by minced lamb 16/40 (40%), ground beef 11/40 (27.5%), lamb 9/40 (22.5%), beef intestines 7/40 (17.5%) and lamb intestines 4/40 (10%). **Conclusions**. The outcomes of our study demonstrated the high contamination rate of *Clostridium perfringens* in local meat products. This study is also the first survey on *Clostridium perfringens* prevalence in meats in Kazakhstan. The findings in this report will enhance knowledge of epidemiology and help develop coordinated actions to prevent and control possible food poisoning outbreaks.

Keywords: *C. perfringens*; toxin; beef and lamb meat; meat fair; Kazakhstan; foodborne diseases

1. Introduction

Clostridium perfringens, a spore-forming anaerobic Gram-positive bacterium, is a foodborne pathogen that induces food poisoning and enteric infections in humans and animals [1,2]. Previously, *Clostridium perfringens* was classified into five toxic strains (A-E) based on the synthesis of four significant toxins; alpha (α), beta (β), epsilon (ε), iota (ι) [3,4]. In 2018, two more biotypes were identified. They were subsequently introduced as a type F which produces α-toxin and enterotoxin (CPE), and type G, which produces α-toxin and necrotic enteritis toxin (NetB) [5].

Clostridium perfringens-associated food poisoning cases are reported in developed countries, while they are rarely investigated in developing countries [6]. In humans,

Clostridium perfringens biotypes can be found in the gastrointestinal tract and genital organs and, under certain circumstances, may serve as a causative agent of food poisoning, gas gangrene, and multiple organ failure [1,7–9].

According to the Center for Disease Control and Prevention, *Clostridium perfringens* has become the second most prevalent pathogen associated with foodborne disease in the USA, causing 1 million cases every year [10]. In Kazakhstan, food poisoning caused by foodborne pathogens, including *Clostridium perfringens,* is poorly reported; however, detection of *Clostridium perfringens* type A and its associated α toxin in Kazakh honey samples has been published recently [11]. It is suggested that food poisoning develops mostly following consumption of meat products contaminated with *Clostridium perfringens* at a level of 10^6 CFU/g [12].

Beef and lamb meat products are a major source of dietary protein among the Kazakhstani population. To the best of our knowledge, there were no studies conducted to determine the prevalence of *Clostridium perfringens* in meat products in street markets of West Kazakhstan. Additionally, the biotypes of *Clostridium perfringens* and the presence of a specific *cpe* gene in the isolates were analyzed.

2. Materials and Methods

2.1. Study Area and Sample Collection

The research was carried out in the West Kazakhstan region (51°55′–43°54′ N, 45°73′–61°82′ E) between April and October 2021. According to the national census, the oblast has a population of approximately 2.5 million in an area of 736,129 km^2. The region consists of four major oblasts and one administrative center. Three provinces (Atyrau, Aktobe, Aksay) and an administrative center (Oral) were selected for sample collection. These areas were selected as they are the main meat-producing regions in West Kazakhstan.

Overall, samples of 240 beef and lamb products (beef and lamb intestines, ground beef, minced lamb) were taken from fair markets in the area studied. Ten samples of each meat type were collected from each region. The temperature of samples taken varied between 13 and 19 °C. All samples collected were stored at 4 °C in a 50-mL sterile plastic tube and immediately transported to the laboratory. Microbiological and PCR examination of samples were run within 6 h following delivery.

2.2. Microbiological Examination

Examination of the samples for the presence of *Clostridium perfringens* was conducted according to the international protocol based on ISO 7937 (ISO, 2004) [13] guidelines. In brief, each specimen (25 g) was transferred into a sterile blender jar containing 225 mL peptone dilution fluid (1:10 dilution) and subsequently homogenized for 1 min at 1500× *g*. Next, tenfold serial dilutions from 10^{-1} to 10^{-6} were made using the 1:10 dilution mentioned above. Then, 0.1 mL of each dilution was introduced into the center of solidified TSC agar supplemented with egg yolk emulsion (Neogen Perfringens Agar Base, Thermo Fisher Scientific, Billerica, MA, USA). To establish anaerobic settings, plates were then overlaid with 10 mL of TSC agar without egg yolk emulsion and allowed to incubate at 37 °C for 24 h.

Identification of presumptive *Clostridium perfringens* colonies was based on their characteristic morphology. Accordingly, plates containing 20–200 black colonies were considered positive after incubation in an egg yolk medium with an opaque white zone surrounding the colony due to lecithinase activity. Black colonies were counted, and a calculation of *Clostridium* cells per gram of specimen was made using the Quebec colony counter (Reichert, Darkfield Quebec, Thermo Fisher Scientific, Billerica, MA, USA). The experiment was run in triplicate, and plates were sent for genomic confirmation.

2.3. DNA Manipulation and PCR Amplification

For DNA extraction, a Bacterial Genomic Miniprep Kit (Sigma-Aldrich Inc, Rockville, MD, USA) was used according to the manufacturer's instructions.

Following *Clostridium perfringens* reference strains were utilized as a positive controls of toxin typing: *C. perfringens* type A, National Collection of Type Culture (NCTC) 528 (cpa); type C, NCTC 3180 (cpb), NCTC 4989 (cpb, cpb2); type D, NCTC 8346 (etx) and type E, NCTC 8084 (iap, cpe). For toxin genotyping of isolates, multiplex PCR (mPCR) was performed according to the protocol described by Baums et al. [14] with modification [15]. This technique allows the detection of *cpa*, *cpb*, *cpb2*, *cpe*, *etx*, and *iap* genes of *Clostridium perfringens* biotypes in a single reaction. The conditions for DNA amplification in a Thermal Cycler (Eppendorf Mastercycler) were as follows: 95 °C for 2 min 30 s, 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 20 s (35 cycles), and 72 °C for 2 min. The mPCR products obtained were administered in 1% agarose gel electrophoresis in the presence of *Ethidium bromide*, and the results were visualized using Bio-Imaging Systems (MiniBIS Pro, Jerusalem, Israel).

3. Results

Detection of Clostridium perfringens

The identification of *Clostridium perfringens* in samples of beef and lamb products taken from meat fairs is demonstrated in Table 1. Out of 240 samples examined, 67 (28%) tested positive for the presence of *Clostridium perfringens* strains. All strains isolated belonged to the *Clostridium perfringens* biotype A with the ability to produce the α gene. *Clostridium perfringens* isolates were found in almost every meat type sampled. Moreover, the meat fairs of each province were contaminated with this pathogen. The highest prevalence of *Clostridium perfringens* isolates was found at the meat fair of the Oral administrative unit accounting for 25/40 positive samples, followed by Atyrau 16/40, Aksay 12/40, and Aktobe 7/40 districts (Table 2). The anaerobic count of *Clostridium perfringens* ranged from 1.5×10^2 to 6×10^4 per gram. The highest bacterial load was observed in minced lamb, whereas the lowest was observed in the beef intestine (Table 3).

Table 1. Detection of *Clostridium perfringens* isolates and associated genes in examined meat products (n = 240) from meat fairs (n = 4) in four areas in west Kazakhstan.

Sample	Number of Samples Tested	Number of Samples Tested Positive/%	*C. perfringens* Toxin Type	Toxin Gene					
				cpa	*cpb*	*cpb2*	*etx*	*iap*	*cpe*
Beef	40	20/50	A	20	-	-	-	-	-
Lamb	40	9/22.5	A	9	-	-	-	-	-
Beef intestines	40	7/17.5	A	7	-	-	-	-	-
Lamb intestines	40	4/10	A	4	-	-	-	-	-
Ground beef	40	11/27.5	A	11	-	-	-	-	-
Minced lamb	40	16/40	A	16	-	-	-	-	-
Total	240	67/27.9	-	67	-	-	-	-	-

Table 2. Prevalence of *C. perfringens* isolates in meat samples (n = 240) taken from meat fairs (n = 4) in West Kazakhstan.

Districts	Number of *Clostridium perfringens* Isolates (Meat Type)	Total in Districts (%)
Atyrau	6 (beef), 1 (beef intestines), 2 (lamb intestines), 4 (ground beef), 3 (minced lamb)	16 (40%)
Aktobe	4 (beef), 1 (lamb), 2 (ground beef), 1 (minced lamb)	7 (17.5%)
Aksay	2 (beef), 2 (lamb), 3 (beef intestines), 2 (lamb intestines), 2 (ground beef), 1 (minced lamb)	12 (30%)
Oral (administrative centre)	8 (beef), 5 (lamb), 4 (beef intestines), 3 (ground beef), 5 (minced lamb)	25 (62.5%)

Table 3. The total number of vegetative *Clostridium perfringens* cells in meat products (n = 240) collected from meat fairs (n = 4) in West Kazakhstan.

Sample	*Clostridium perfringens* CFU/g, Mean
Beef	2×10^4
Lamb	4×10^2
Beef intestines	2×10^2
Lamb intestines	4.2×10^3
Ground beef	3×10^3
Minced lamb	6×10^4

4. Discussion

Clostridium perfringens is a ubiquitous anaerobic spore-forming bacteria that cause various diseases in humans and animals. It ranks as the most important foodborne pathogen causing food poisoning worldwide [10,16]. In the current study, about 30% of meat fair products tested were positive for the presence of *Clostridium* perfringens, similar to those reported by Miwa et al. [17] and Wen and McClane [18].

In developed countries, foodborne illnesses caused by Clostridium perfringens biotype-F (CPE-producing strain) are responsible for human food poisoning. Moreover, *Clostridium perfringens* foodborne outbreaks among children have been reported in Northern Greece [19].

In Kazakhstan, the presence of *Clostridium perfringens* in raw meat products has never been reported. However, our study demonstrated a high prevalence of *Clostridium perfringens* biotype-A in meat fairs in the most populous cities in West Kazakhstan. Smallholder farmers from remote rural areas are the main participants at meat fairs. Meat fairs are usually organized in the open air during the spring and autumn months. Personal communications revealed that individual farmers travel a long distance (about 500 km) before attending meat fairs. Moreover, meat products, in most cases, are transported without proper packaging and without a refrigeration system. It should be taken into account that slaughtering is often performed on the farm site in the evening before the meat fair. Carcasses and meat cuts are kept at the slaughtering site overnight and are transported to the fair at dawn. Furthermore, the circumstances are aggravated because meat counters in fairs are not equipped with refrigerators. Accordingly, farmers are forced to store their products under unfavorable conditions for an additional 5 h, from 7.00 a.m. to 12.00 p.m. On the fair site, meat cuts remain on meat counters unwrapped or uncovered, and meat delicacy (intestines, beef tongue) are left in the plastic cabinet at the ambient temperature for several hours, which increases the temperature of the meat. These factors have a positive effect on *Clostridium perfringens* proliferation. It is reported that vegetative cells of this foodborne pathogen are capable of fast and dynamic growth at 20 °C to 53 °C, whereas spores can survive up to 95 °C for 1 h [20,21]. Additionally, the violation of hygienic practices during slaughtering, processing, and handling can affect bacterial loads [22].

Previous studies indicated that meat and meat products are common sources of *Clostridium perfringens* infection [23,24]. Although the heterogeneous nature of *Clostridium perfringens* possesses high diversity of biotypes (A, B, C, D, E, F, and G) and could be explained by their recombination, in-vivo and in-vitro horizontal gene transfer, and evolutionary dynamism [5,25] our study demonstrated low heterogeneity of the isolates tested, belonging to biotype A only.

In the Oral district, the prevalence of *Clostridium perfringens* biotype A carrying the characteristic cpa gene was higher than in other districts investigated. Among meat types tested, *Clostridium perfringens* biotype A prevailed in samples taken from beef and minced lamb. No food-poisoning-associated biotype F isolates were found; additionally, the presence of *cpb*, *cpb2*, *etx*, *iap*, and *cpe* genes was negative in all isolates tested.

Our results agree with Cooper et al. [26] and [27,28] where the prevalence rate of Type A among *Clostridium perfringens* strains isolated from meat products varied 86–100%. In addition, Smedley et al. [29] reported that less than 5% of all *Clostridium perfringens*

strains are capable of producing enterotoxin. In humans, gastroenteritis associated with CP infection is usually produced by enterotoxigenic type F strains of this microorganism. Since no type F strains were found in our study, but other types of CP were found in many meat samples, this suggests that the samples analyzed were not involved in human disease. Our results suggest, however, poor hygiene and food management. Based on this, *Clostridium perfringens* biotype A is not supposed to pose a big risk for enteric disease (encodes only alpha-toxin).

However, the presence of *Clostridium perfringens* in these popularly-consumed meat products illustrates their potential to serve as an etiologic agent of gas gangrene and sepsis in humans. Therefore it is suggested that Kazakhstani sanitary authorities should take strict control measures on the microbiological quality of meat products being sold in fairs.

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