Occurrence of human pathogenic *Clostridium botulinum* among healthy dairy animals: an emerging public health hazard

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The current study was conducted to investigate the occurrence of human pathogenic *Clostridium botulinum* in the feces of dairy animals. Fecal samples were collected from 203 apparently healthy dairy animals (50 cattle, 50 buffaloes, 52 sheep, 51 goats). Samples were cultured to recover *C. botulinum* while human pathogenic *C. botulinum* strains were identified after screening of all *C. botulinum* isolates for the presence of genes that encode toxins type A, B, E, F. The overall prevalence of *C. botulinum* was 18.7% whereas human pathogenic *C. botulinum* strains (only type A) were isolated from six animals at the rates of 2, 2, 5.8, and 2% for cattle, buffaloes, sheep, and goats, respectively. High fecal carriage rates of *C. botulinum* among apparently healthy dairy animals especially type A alarm both veterinary and public health communities for a potential role which may be played by dairy animals in the epidemiology of such pathogen.

Keywords: Botulism, *Clostridium botulinum*, Dairy animals, Public health

Introduction

Botulism is a fatal neuroparalytic illness caused by botulinum neurotoxin (BoNT) produced by *Clostridium botulinum*. This neurotoxin is considered as the most lethal toxin known for humans at few nanogram quantities (25–50 ng).^{1,2} *C. botulinum* was firstly introduced to the scientific community in 1897 by van Ermengem who isolated such bacterium and identified it to be the cause of a severe paralytic illness outbreak that occurred in Belgium 1895; *Bacillus botulinus*. Afterward, the organism was assigned to the genus *Clostridium* and was named *Clostridium botulinum*. *C. botulinum* is a Gram positive spore-forming anaerobic bacterium which comprises four distinct phenotypic groups (I–IV), with groups I and II being pathogenic for humans. Furthermore, *C. botulinum* strains produce seven serologically different types of neurotoxins that were designated A–G, but types A, B, E, and F were the most implicated toxins in human outbreaks, while types C and D were associated with animal botulism.³ Botulism is usually associated with the ingestion of already preformed (BoNTs) in food and thereby the toxins enter the human body through gastrointestinal tract to produce the classical form of botulism which is called food-borne botulism. Nevertheless, other relatively uncommon forms of botulism were reported either when *C. botulinum*

colonized the intestinal tract of young infants and adults (infant botulism and adult intestinal colonization botulism) or when contaminated wounds become infected with such bacterium (wound botulism), germinating and producing neurotoxins.⁴ The clinical symptoms of botulism usually include double vision, dry mouth, dysphagia, dysphonia, paralysis of limb muscles which may rapidly extend to involve respiratory muscles, with subsequent respiratory failure and death.⁵ However, although food-borne botulism outbreaks seem to be relatively rare when compared with other causes of food poisoning, they show serious public health implications because of severe clinical outcomes and high treatment cost compared to other enteric infections.⁶ Food-borne botulism is usually associated with canned vegetables, meat, fish, and dairy products.⁷ Although, milk and milk products have not been frequently incriminated in food-borne botulism outbreaks, an increase in the number of outbreaks of botulism in dairy cattle was noted, alarming the public health community about the safety of milk and milk products.^{8–11} Notably, dairy products such as cheese and yogurt were previously traced as sources for several outbreaks of botulism.^{12–14} On the other side, much remains unknown about the occurrence of *C. botulinum* in the feces of apparently healthy dairy animals which may be considered as the main source of contamination of milk and milk products with *C. botulinum* spores. Therefore, the current study was carried out

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Table 1 Primers used in the multiplex PCR

Type	Primer	Sequence 5' – 3'
<i>C. botulinum</i> type A	Forward	AGC TAC GGA GGC AGC TAT GTT
	Reverse	CGT ATT TGG AAA GCT GAA AAG G
<i>C. botulinum</i> type B	Forward	CAG GAG AAG TGG AGC GAA AA
	Reverse	CTT GCG CCT TTG TTT TCT TG
<i>C. botulinum</i> type E	Forward	CCA AGA TTT TCA TCC GCC TA
	Reverse	GCT ATT GAT CCA AAA CGG TGA
<i>C. botulinum</i> type F	Forward	CGG CTT CAT TAG AGA ACG GA
	Reverse	TAA CTC CCC TAG CCC CGT AT

Table 2 Prevalence of *Clostridium botulinum* in the feces of the examined dairy animals

<i>Clostridium botulinum</i> group	Number of the examined animals	Number of positive	Percentage
Group I	203	6	3
Group II		0	0
Group III		11	5.4
Group IV		21	10.3
Total	203	38	18.7

Table 3 Distribution of human pathogenic *Clostridium botulinum* among different dairy farm animals species

Animal species	Number examined	<i>Clostridium botulinum</i> type A	
		No. of positive	%
Cattle	50	1	2
Buffalo	50	1	2
Sheep	52	3	5.8
Goats	51	1	2
Total	203	6	3

to investigate the occurrence of *C. botulinum* in the feces of apparently healthy dairy animals (cattle, buffalo, sheep, and goats) and clarify the actual role of these animals in the epidemiology of this infection, prevention, and control.

Materials and methods

Fecal samples were collected from the rectum of 203 dairy animals (50 cows, 50 buffaloes (*Bubalus bubalis*), 52 ewes, and 51 goats) using sterile gloves, inserted in sterile cups and transported directly to the laboratory in an ice box. All were lactating animals, apparently healthy and fed on pasture and concentrates (silage was not included in animal ration). Upon arrival, fecal samples were heated at 60 °C for 10 min then each was inoculated into two tubes of tryptose peptone glucose yeast extract (TPGY) broth. One tube was incubated at 37 °C, while the other was placed at 28 °C. All tubes were incubated for 5–7 days under anaerobic conditions using anaerobic jars and AnaeroGen™ gas-generating kits (Oxoid, UK). The enrichment cultures were further streaked on sheep blood agar plates and another incubation period was allowed for 3 days at duplicate temperatures, 28 and 37 °C as mentioned above under anaerobic conditions. Suspected colonies of *C. botulinum* showed narrow zones of beta hemolysis and were identified by Gram's stain, proteolytic activity in cooked meat media (Oxoid, UK), lipase test, catalase test, and other biochemical tests for confirmation.¹⁵

Molecular identification of human pathogenic *C. botulinum* isolates

All *C. botulinum* isolates were screened by multiplex PCR for identification of human pathogenic strains:

DNA extraction

DNA extraction from all *C. botulinum* isolates was performed according to the previously described protocol.¹⁶ Briefly, Bacterial cells of each isolate were harvested from 1 ml of overnight TPGY broth culture. The cells were washed with 1-ml TE buffer and suspended in 1-ml nuclease free water. The suspensions were then heated at 99 °C for 10 minutes followed by centrifugation at 10000×g for 5 min and the supernatants were used as DNA templates.

Multiplex polymerase chain reaction

Multiplex PCR was carried out using Taq PCR Master Mix kit (Qiagen, Germany) with four sets of primers specific for the genes encoding toxins types A,B,E,F (human pathogenic toxins) (Table 1).

All primers were synthesized by Invitrogen (Life Technologies, USA).¹⁶

The thermal profile of the amplification was:

Initial denaturation at 94 °C for 3 min followed by 27 cycles at 95 °C for 30 s; 60 °C for 25 s; 72 °C for 85 s then final extension at 72 °C for 3 min. PCR products then underwent an electrophoresis step using 2% agarose

Figure 1 Multiplex PCR for identification of human pathogenic *C. botulinum* isolates. Lane M: DNA ladder 100 bp; Lane 1: negative control; Lanes 2–7 positive *C. botulinum* type A showed specific bands at 782 bp.

gel to detect specific bands of 782, 205, 389, 543 bp for *C. botulinum* types A, B, E, F, respectively.¹⁶

Results

The isolation rate of *C. botulinum* from the examined fecal materials of dairy animals was 18.7% with the following distribution among different phenotypic groups, 3, 5.4, 10.3% for groups I, III, IV, respectively. Group II was not recovered from all examined animals (Table 2) and six of them yielded human pathogenic *C. botulinum* strains (one cow, one buffalo, three ewes, one goat) with the following recovery rates, 2, 2, 5.8, 2%, respectively (overall prevalence = 3%, Table 3). All human pathogenic *C. botulinum* strains were genetically classified as *C. botulinum* type A (Fig. 1).

Discussion

Botulism is a serious food-borne illness with a climbing public health impact and potential outbreaks with high fatality rates and enormous economic losses due to contaminated food.¹⁷ Packing of contaminated food with *C. botulinum* spores accompanied by improper storage conditions seems to be the cornerstone in the initiation of such outbreaks. The contamination of milk and milk products with this organism usually occurs during milking and processing of milk and so, dairy animals and dairy environment are incriminated as the major source of milk contamination.¹⁸ The results of the current study revealed a high prevalence rate of *C. botulinum* among dairy animals (18.7%), a result which is higher than that obtained by Schmid et al.¹⁹ who failed to detect *C. botulinum* types (A–F) in the feces of apparently healthy dairy cattle after examination of samples from 34 dairy cattle farms. Additionally in the present study, human pathogenic strains of *C. botulinum* were recovered from the feces of all examined dairy animal species (cattle, buffalo, sheep, and goats) with an overall prevalence of 3%. All human pathogenic strains were classified as type A.

C. botulinum type A is responsible for the production of type A (BoNT) which is the most potent botulinum toxin and commonly involved in human outbreaks.²⁰ Therefore, the recovery of *C. botulinum* type A from the feces of such

dairy animals constitutes a potential public health hazard as the milk of these animals may become easily contaminated with this pathogen.¹⁸ *C. botulinum* type A spores are highly heat resistant and can survive pasteurization temperature. They are able to germinate and release their lethal toxin during improper storage and refrigeration since the minimum required temperature for the growth of *C. botulinum* type A is 12 °C.²¹ This finding was reported in 1990s in Italy when an outbreak of botulism appeared in persons after consumption of mascarpone cream cheese. It was concluded that *C. botulinum* type A stood behind this outbreak after discontinuation of the cold chain at a retail store. Further surveillances provoked by such outbreak revealed the high occurrence of *C. botulinum* spores in 32.5% of the examined mascarpone cheese samples and 2.7% of other milk products.^{22,23} The high heat resistance of *C. botulinum* type A spores makes heat treatment of milk a critical process. These spores need a temperature of 121 °C for 3 min to be destroyed and so, even ultra heat-treated milk (UHT, 135 °C for 1–2 s) may become a vehicle for *C. botulinum* type A toxin. Any slight error or heating insufficiency may lead to survival of *C. botulinum* type A spores, germination, and toxin production, given the observation that UHT milk has an extended shelf storage period at room temperature and free from other competitive organisms.²⁴ Moreover, the shedding of *C. botulinum* type A in the feces of dairy animals gives the chance for such bacteria to contaminate the udder, teat canal and thereby leads to ascending infection of the udder tissues which may be accompanied by germination and release a potent toxin in milk; a matter which bears a significant hazard for people who consume raw unheated milk or even pasteurized milk, since (BoTN) is destroyed by heating at 85 °C for 5 min which exceeds commonly used conditions during milk pasteurization (72 °C for 15 s).^{25,26} In a recent study in Germany, *C. botulinum* types A, B, E and their toxins were detected in the udder tissues and/or milk from suspected, clinically affected, or dead cows with botulism.²⁷ Accordingly, possible contamination of milk may extend to involve milk powder which is a serious item that is largely ignored. Milk powder is prepared from pasteurized milk after drying at 75–80 °C. This temperature range is too low to eliminate *C. botulinum* type A spores.¹⁸ Although, milk

powder does not promote the germination of *C. botulinum* spores, but it is involved in the production of infant milk formulas and thereby *C. botulinum* type A spores find their way to the intestinal tracts of infants rendering them under a potential hazard from developing infant botulism, given the small number of *C. botulinum* type A spores that can produce infant botulism.²⁸ Furthermore, a case of infant botulism was traced to a contaminated infant milk formula in UK.²⁹ It is noteworthy that, the public health importance of our findings goes beyond the possible contamination of milk and other milk products, as the shedding of *C. botulinum* type A and *C. botulinum* group III in the feces of dairy animals leads to contamination of pastures used for feeding of animals or the production of silage which may lead to animal outbreaks by both botulism intoxication and/or visceral botulism which may be accompanied by shedding the toxins in milk.^{9,30,31} Interestingly, we isolated *C. botulinum* type A from the feces of grazing sheep (6%) and goats (2%), which pass long distances every day during grazing, shedding *C. botulinum* type A everywhere and increasing the environmental load with such bacteria and contaminate grasses and vegetables regarding the vast majority of botulism outbreaks in humans are usually linked to contaminated vegetables. Botulism in sheep and goats was totally overlooked however suspected outbreaks were recorded.^{32,33} On the other hand, the presence of *C. botulinum* type A in the feces of the examined animals points out to the direct hazard for veterinarians and farmers during handling of animals and rectal examination. Although developing wound botulism may be relatively rare, it is serious enough to be underlined here.³⁴

Conclusion

The findings of the current study alarm both veterinary and public health communities about the occurrence of human pathogenic *C. botulinum* in the feces of apparently healthy dairy animals which is the initial source of such pathogen in dairy environment, milk, and milk products. So, it is to be noted that continuous surveillances are needed for dairy farms to determine the prevalence of *C. botulinum* among the dairy herd while keeping strict hygienic measures during milking process. On the other hand, adequate quality control in dairy plants during heat treatment processes and also monitoring the cold chain in retails during storage of milk products should be applied in order to combat such deadly pathogen.

Conflict of interest

Nothing to declare

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