Anaerobe 20 (2013) 74–78

Contents lists available at SciVerse ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Pathogenesis and toxins

Glyphosate suppresses the antagonistic effect of *Enterococcus* spp. on *Clostridium botulinum*

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ARTICLE INFO

Article history:
Received 23 May 2012
Received in revised form
15 January 2013
Accepted 29 January 2013
Available online 6 February 2013

Keywords:
C. botulinum
Glyphosate
Enterococcus spp.

ABSTRACT

During the last 10–15 years, an increase of *Clostridium botulinum* associated diseases in cattle has been observed in Germany. The reason for this development is currently unknown. The normal intestinal microflora is a critical factor in preventing intestinal colonisation by *C. botulinum* as shown in the mouse model of infant botulism. Numerous bacteria in the gastro-intestinal tract (GIT) produce bacteriocines directed against *C. botulinum* and other pathogens: Lactic acid producing bacteria (LAB) such as lactobacilli, lactococci and enterococci, generate bacteriocines that are effective against *Clostridium* spp. A reduction of LAB in the GIT microbiota by ingestion of strong biocides like glyphosate could be an explanation for the observed increase in levels of *C. botulinum* associated diseases. In the present paper, we report on the toxicity of glyphosate to the most prevalent *Enterococcus* spp. in the GIT. Ingestion of this herbicide could be a significant predisposing factor that is associated with the increase in *C. botulinum* mediated diseases in cattle.

1. Introduction

Clostridium botulinum associated diseases in cattle appear in two forms, acute or chronic. The acute form of botulism occurs after the uptake of preformed botulinum neurotoxin (BoNT) in feeds causing flaccid paralysis and death by respiratory failure. Graham and Schwarze, [1] described the acute form of botulism as developing of paralysis without previous symptoms, leading to sudden death or slow recovery after complete muscle relaxation. In contrast, the chronic form of the disease is characterized by weakness, local paralysis, emaciation, muscular stiffness and recumbency of varying degrees. A neurologic manifestation may be highly aggressive behaviour resulting in damage to feeding troughs, mangers or fences. Animals may also display visual disturbances [1]. The causal bacterium, *C. botulinum*, is an ubiquitous Gram-positive, spore-forming obligate anaerobic bacterium. Strains generate neurotoxins that block the release of acetylcholine at the neuromuscular junctions. Amino acid variation within the BoNT results in seven immunologically distinct BoNT serotypes (A–G) which are further divided into subtypes [2–5]. Two other bacteria, *C. baratii* and *C. butyricum*, can also produce BoNT F and E, respectively. The factors that determine the severity of the disease and the factors that prevent intestinal colonisation by *C. botulinum* spores are incompletely characterized [6]. In the mouse model of infant botulism, the normal intestinal microflora has been shown to be a critical factor in preventing intestinal colonisation by *C. botulinum* [7]. Wang and Sugiyama [8] reported that mice developed symptoms of botulism when they were treated with metronidazole before oral application of *C. botulinum* A and B spores. Some mice harboured relatively large amounts of toxin in the large bowel without displaying any clinical signs, possibly bound to phospholipids and gangliosides [9]. This is in line with other intestinal diseases, where the normal enteric microflora has been found to protect against colonization by a variety of bacterial pathogens [6]. The microflora of the GIT of domestic animals consists of a balanced composition of facultative and obligatory anaerobic bacteria. The mature microbiota profile varies considerably along the length of the GIT and may be specific to animal species and individuals [10].

Numerous bacteria of the GIT produce bacteriocines directed against some other bacterial species including pathogens. Among others, lactic acid bacteria like lactobacilli, lactococci and enterococci may generate such bacteriocines. Specifically, *Enterococcus faecalis* (*E. faecalis*) may generate an enterocin 1146 that was shown to be very effective against *C. butyricum* and *C. perfringens* [11–14]. Glyphosate, N-(phosphonomethyl) glycine, the most widely used

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http://dx.doi.org/10.1016/j.anaerobe.2013.01.005

herbicide in the world, is the active ingredient in Roundup® and contains also adjuvants such as polyethoxylated tallowamine [15]. Glyphosate is a strong systemic metal chelator and was initially patented for that purpose in 1964 [16]. It is also a selective and potent, microbiocide. Its herbicidal action is generated by chelating manganese required in the reduction of the FMN co-factor for the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme in the shikimate pathway, inhibiting this metabolic pathway of plants and also many microorganisms [17]. The extensive use of glyphosate as a broad-spectrum herbicide in agriculture, and especially the direct application of glyphosate to Roundup Ready® soya, corn, rapeseed, cotton, sugar beets, and alfalfa fed to animals, leads to incorporation of residual glyphosate into the GIT. Moreover, glyphosate showed differences in sensitivity between microorganisms [18,19] which could disturb the normal gut bacterial community. The aim of this paper is to document the inhibitory effect of glyphosate on *Enterococcus* spp. which antagonises *C. botulinum.*

2. Materials and methods

2.1. Isolation and identification of Enterococcus strains

Enterococcal isolates were isolated from cattle, horses, and algae (*Chlorella vulgaris*, (Ökologische Produkte Altmark Co, Germany) by plating specimens on citrate- acid-tween-carbonate (CATC agar, Oxoid, Germany) and incubated aerobically at 37 °C for 48 h. Typical red colonies were sub-cultured on Caso agar (3.5% Casein-Soya, 0.3% yeast extract, 0.1% glucose, 1.5% Agar Agar). These colonies were examined for Gram reaction and cellular morphology. All Gram positive, catalase-negative cocci isolated on this medium were presumptively identified as *Enterococcus* spp. The sugar fermentation profiles of these isolates were checked for glucose, trehalose, arabinose, mannitol, salicin, raffinose, dulicit, xylose and lactose utilization. Haemolysis of these isolates was checked by culturing on blood agar medium. Species identification of isolated strains was based on their matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) profile (Table 1). Briefly, about 10 mg of cell material of the cultured strains were suspended in 300 μl of sterile water. 900 μl of absolute ethanol was added and the mixture was centrifuged at 10.000 rpm for 2 min. The supernatant was discarded and the pellet was suspended in 50 μl formic acid (70% v/v). After adding 50 μl acetonitrile (AN), the mixture was centrifuged at 10,000 rpm for 2 min. 1 μl of the clear supernatant was transferred to the MALDI target and allowed to dry followed by addition of 1 μl α-cyano-hydroxy-cinnaminic acid (Bruker Daltonik Co, Bremen, Germany) in a standard organic solvent mixture (2.5% trifluoroacetic acid, 50% AN in water). All chemicals used were of the highest quality (Merck, designated to be especially suitable for HPLC or MALDI-based techniques). Before each MALDI run, *E. coli* 1917 strain Nissle (Ardeypharm GmbH, Herdecke, Germany) was analysed to serve as the positive control and calibration standard. The MALDI–TOF MS analysis was performed using a Bruker microflex LT mass spectrometer (Bruker Daltonik CO, Bremen, Germany) and the spectra were automatically identified using the Bruker BioTyper™ 1.1 software.

2.2. C. botulinum strains

C. botulinum type A (7272), type B (7273), *C. botulinum* type C (8264) and type E (8266) were obtained from National collection of type culture (NCTC), while *C. botulinum* type D (Pasteur 1873-D) was obtained from Institute of Pasteur, Paris, France. *C. botulinum* strains were cultured anaerobically in a cooked meat medium at 37 °C for 5 d, followed by cultivation in reinforced clostridial medium (RCM, Sifin, Germany) anaerobically at 37 °C for 3 d. *C. botulinum* types A and B were heated at 80 °C for 10 min while types C, D and E were heated at 60 °C for 30 min and left aerobically at room temperature. Cultures were analysed daily for sporulation using a Gram or Rakette stain.

Table 1
Effect of different *Enterococcus* spp. on neurotoxin production in a co-culture with *C. botulinum* types A, B, D and E.

Enterococcus spp.[a]	Lab. designation	Source	Neurotoxin determination by ELISA[b]			
			BotNt A	BotNt B	BotNt D	BotNt E
E. faecium	E1	*Chlorella vulgaris*	–	–	–	–
E. fecalis	E1	*Chlorella vulgaris*	–	–	–	–
E. faecium	E3	Cattle	–	–	–	–
E. hirae	E4	Cattle	–	–	–	–
E. faecalis	E5	Horse	–	–	–	–
E. malomaoldoratus	E6	Horse	–	–	–	–
E. derrisei	E7	Horse	+/–	–	–	+
E. durans	E8	Cattle	–	–	–	–
E. faecium	E9	Cattle	–	–	–	–
E. feacalis	E10	Cattle	–	–	–	–
E. faecalis	E11	Cattle	–	–	–	–
E. faecalis	E12	Horse	+/–	–	–	–
E. hirae	E13	Horse	–	–	–	–
E. casseliflavus	E14	Cattle	+/–	–	–	–
E. coli strain (Nissle 1917)			++	++	++	++
–			++	+++	++	++

Enterococcus spp. prevent toxin production of *C. botulinum* types A, B, D and E while *E. coli* 1917 strain Nissle has no effect.
[a] Species identification performed by MALDI-TOF.
[b] ELISA results expressed as negative (–), low positive (+), positive (++) and highly positive (+++) compared with standards *C. botulinum* neurotoxins with known concentrations.

2.3. Glyphosate

- Roundup UltraMax (Monsanto, USA) which contains 450 mg/ml of glyphosate was used in this study.
- N-(Phosphonomethyle)glycine (Sigma Aldrich, Taufkirchen, Germany).

2.4. Effect of Enterococcus on C. botulinum types A, B, C, D, and E

To study the effect of *Enterococcus* spp. on *C. botulinum* strains, heat treated spores or vegetative cells were added to RCM medium at a final concentration of 10^4 cfu/ml. The inhibitory effect of different *Enterococcus* spp. was studied by addition of different bacterial dilution (10^1–10^9 cfu/ml to *C. botulinum* culture medium. The mixture was incubated anaerobically at 37 °C for 5 d. *C. botulinum* was quantified using the most probable number (MPN) estimation method using Differential Reinforced Clostridial broth (DRCM, Sifin, Germany) and the neurotoxins were tested using an ELISA.

2.5. C. botulinum neurotoxins ELISA

C. botulinum neurotoxins were tested using ELISA as described by Krüger and co-workers [20]. All ELISAs were performed in flat-bottomed ELISA plates (96 wells, high binding; Costar, Corning, New York, USA). Standard volumes were 100 μl per well and the standard incubation condition was 1 h at room temperature (1 h at RT) on a microtitre plate shaker (400 rpm). The coating buffer was 0.1 M $NaHCO_3$ and the wash solution (WS) was 0.9% NaCl with 0.1% Tween 20 (Sigma–Aldrich, Taufkirchen, Germany). After coating