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1 ORIGINAL ARTICLE –revised01.11

2 Tonsils – place of botulinum toxin production. Results of routine laboratory diagnosis in
3 farm animals

4

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14

15

16 **Abstract**

17 Since a case of a veterinarian was reported, who was likely to be infected/intoxicated by

18 botulinum during the handling of a diseases animal, tonsils in animals were tested for

19 botulinum neurotoxin and bacterial forms of neurotoxic *Clostridium botulinum* during routine

20 botulism laboratory examinations including standard samples (intestinal tract and liver) from

21 48 cattle, 11 horses, and 14 goats. Ten out of 60 samples from tonsils contained free

22 botulinum toxin, and 12 out of 59 were positive for live toxin producing bacteria. In 32 out of

23 162 intestinal samples toxin was detected. Toxin producing bacteria were found in 37

24 samples. Eight of 56 liver samples contained free toxin, and 15 out of 43 toxigenic bacteria.

25 Samples from 10 slaughter pigs were all negative, whereas from slaughter cattle tonsils had a
26 high incidence of toxin (7 of 10) or toxigenic bacteria (2 of 8). The results are discussed in the
27 context of effects on animal health and botulism as zoonosis.

28

29 *Key words:* Clostridium botulinum, botulism, neurotoxin, neuroimmunology, tonsils

30

31 **1. Introduction**

32 *1.1 Clostridium (C.) botulinum/botulism*

33 Due to its etiology botulism is considered a botulinotoxicosis. The different botulinum toxins
34 of *C. botulinum* neurotoxins (BoNT) and non-neurotoxins, here referred to as “toxin” may
35 interfere not only with the neuro-muscular junctions (classical botulism with muscular
36 paralysis), but also with different physiological circuits in the body. Recently a clinical picture
37 different from classical botulism was described in cattle (“Visceral botulism”). In this disease
38 complex, toxin formation is seen mainly in colon and caecum (Schwagerick and Böhnel,
39 2001; Böhnel et al., 2001b; Böhnel and Gessler, 2005).

40 The environment is the natural reservoir of the *C. botulinum* spores. Vegetative toxigenic
41 bacteria (VTB) of *C. botulinum* produce toxin outside the body either in food or feed, or
42 inside the body. Factors which trigger toxinogenesis remain mostly obscure. Hence the
43 disease is either an intoxication or an infection (toxicoinfection). It has to be underlined that
44 under natural conditions in a food-borne intoxication live bacteria are taken up as well. This
45 may lead to a consecutive intestinal infection if the initial amount of toxin was not directly
46 lethal, as it was predicted by C. F. Meyer already in 1928, and first reported in horses
47 (Rooney and Prickett, 1967), and in infants (Midura and Arnon, 1976). In the last decade the
48 number of bovine botulism cases is increasing in Germany (Böhnel and Gessler, 2003).
49 Toxicoinfections are becoming more important in animal production, in France they
50 outnumbered already intoxications (AFSSA, 2002).

51 Food of animal origin may be contaminated by faeces or soil carrying botulinum spores and
52 thus creating a health risk for the consumer. However during medical examination of affected
53 patients any health risk is generally denied (Smith and Sugiyama, 1988; CDC, 1998).

54

55

56 *1.2 Tonsils*

57 There are three groups which differ by situation and anatomic properties: oro-pharyngeal,
58 naso-pharyngeal, and lingual tonsils. The paired palatine tonsils (“tonsils”) are located at the
59 centre of this strategic region, the oropharynx. They are covered by a squamous epithelium,
60 and contain deep tubular crypts extending through the full thickness of the organ, forming
61 branched crypts and anastomoses (Nave et al., 2001). Bovine tonsils have a well developed
62 capsule (Velinova et al., 2001). They comprise 1-3 funnel shaped entries, and are located in
63 the muscular and connective tissue layers. In horses they are more prominent and of flat shape
64 with shallow crevices. In small ruminants, crypts can have a large lumen and are frequently
65 branched (Coquyt et al., 2005). The tonsillar crypts mainly contain degenerated cells and
66 cellular debris (Nave et al., 2001). Pigs have anatomically no pharyngeal tonsils; however the
67 lingual tonsils are prominent and taken as “tonsils”.

68

69 Hence, the hypothesis was put forward that tonsils may be carriers of botulinum toxins or
70 VTB. Further examinations should elucidate this question.

71

72

73 **2. Material and Methods**

74 *2.1 Material*

75 Our laboratory is doing routine botulism diagnosis. Samples were taken as soon as possible
76 after death, frozen immediately at -18 °C, and dispatched frozen. At the laboratory they were

77 kept at -18 °C until testing. From 2002 until January 2007, samples including parts of tonsils
78 were received from 38 cattle, 11 horses, and 10 goats.

79

80 Additional samples were taken at abattoirs during slaughter from 10 cattle and 10 pigs. The
81 slaughter cattle originated from two different unknown farms. The origin of the pigs is
82 unknown as well. The animals had passed meat inspection without any restriction.

83

84 *2.2 Method*

85 The internationally accepted mouse bio-assay was used to detect BoNT (buffer elution) or
86 VTB (anaerobic culture in bacterial medium) (CDC, 1988; Böhnelt and Gessler, 2003). About
87 20 g of a sample were soaked in 20 ml phosphate buffered saline (pH 6.2) over night at 6 °C.
88 The supernatant was then used for direct inoculation into 2 mice (0.5 ml i.p.). About 5 g of the
89 sample were transferred to 20 ml reinforced clostridium medium (pH 6.8) and incubated (4
90 d/37 °C under standardized anaerobic conditions (90 % N₂, 10 % CO₂, 10 % H₂; Anoxomat[®],
91 MART, Bocholt). The culture was centrifuged (4000 g/30 min, 6°C) and the supernatant was
92 injected into two mice (0.5 ml i.p.). Toxins and bacteria were typed by toxin neutralisation
93 assays using specific antibodies. As there are no international standard antitoxins available,
94 we used those of different sources (OBP, Onderstepoort, ZA; ID-DLO, Lelystad, NL;
95 metabiologics, Madison (Wisc.), USA; own production). Two toxin groups A+B+E and C+D
96 were formed to save laboratory animals, time, and costs. Tests for particular toxin types were
97 not done. Normally, a positive toxin neutralisation test was considered being
98 significant, hence parallel bacterial culture tests were discontinued. In some cases the
99 specimens were so small that toxin elution was not possible. Additional help for the
100 differentiation between intoxication and infection in cattle was an ELISA for serum
101 antibodies against different BoNT types (Böhnelt and Gessler, 2003) (not reported here). The
102 final diagnosis was made by the farm veterinarians considering clinics, pathology,

103 bacteriology, epidemiology, and differential diagnosis, reported as muscular (mus) or visceral
104 (vis) forms in Table 1).

105

106

107 **3. Results**

108 Samples of 93 tonsils were tested (Tables 1 and 2). Seventeen were positive, 73 negative for
109 the direct toxin elution, 14 samples were positive and 77 negative in the indirect VTB culture
110 test. Additionally, 427 tests were done from samples of intestinal tract and liver. In some
111 cases neutralisation was only possible when using a combination of five antitoxins
112 (A+B+E+C+D). Fourteen samples, which were positive for toxins or VTB, were not
113 neutralized, because typing results had already been obtained in other organs of the same
114 animal. For some samples both, toxin and bacterial tests were done. Tables 3 and 4 show the
115 results comparing tonsils and intestinal tract plus liver.

116

117 The results of bovine samples in our routine work are split according to the final clinical
118 diagnosis of muscular or visceral botulism.

119 The acute cases showed a high proportion of toxic samples in the tonsils and rumen/small
120 intestines. 6 out of 19 liver samples revealed BoNT.

121 There were no toxins detected in the tonsils in visceral forms, although 5 of 10 samples
122 harboured VTB. Liver samples were negative for BoNT, one contained VTB.

123 Those animals in which botulism finally was excluded as cause of death did contain neither
124 BoNT nor VTB with one exception, containing VTB (Table 1) in the rumen/small intestine.

125

126 In horses with (muscular) botulism, two tonsils contained BoNT. In two samples VTB were
127 found. Those animals without clinical manifestations of botulism showed neither BoNT nor
128 VTB.. In four cases typing was not done (Table 1).

129

130 The caprine samples of animals without clinical botulism were negative both for BoNT and
131 VTB. Those animals presenting (muscular) botulism had neither BoNT nor VTB in the tonsils
132 but in the intestine and liver samples (Table 1).

133

134

135 In slaughter cattle, 7 of 10 tonsils, and 1 of 10 large intestines contained BoNT. VTB were
136 detected in 2 samples each of tonsils, small intestines, and large intestines (Table 2).

137 The samples of slaughtered pigs were all negative for BoNT and VTB (Table 2).

138

139

140 **4. Discussion**

141 To our knowledge there are no reports of *C. botulinum* infections of tonsils or the presence of
142 botulinum toxin therein. This report shows for the first time the proof of botulinum toxin and
143 vegetative toxinogenic bacteria of *C. botulinum* in samples of tonsils of bovine, equine, and
144 caprine origin. Presuming that *C. botulinum* is wide spread in the environment it seems
145 logical that at least *C. botulinum* bacteria should be found in the tonsils. The obtained samples
146 did not allow to distinguish between the localisation of BoNT or *C. botulinum* on or within
147 the tissue of the tonsils. There might be anaerobic microenvironments within the tonsillar
148 crypts (Kataura and Harabuchi, 1995) leading to colonization of the tonsils by VTB, and
149 subsequent BoNT formation. However the findings in animals with visceral botulism confirm
150 the reports that toxinogenesis is found only in the hind parts of the intestinal tract (Böhnel et
151 al., 2001b).

152

153 In preceding reports it was shown that infections by *C. botulinum* in farm animals are by far
154 more numerous than estimated (Böhnel and Gessler, 2003), but there is no explanation for the

155 results in slaughter cattle. The tonsils are capable of a variety of complex immunological
156 functions in man. Microorganisms may there be phagozytosed by M cells, which may cause
157 afterwards different types of secondary diseases (Kataura and Harabuchi, 1995).

158

159 The colonisation of the tonsils by *C. botulinum* may lead to an intoxication when BoNT is
160 resorbed locally, or in the intestines after swallowing. BoNT may penetrate directly into the
161 lymphatic system. In the tonsils microorganisms may be phagozytosed by M cells, which may
162 cause afterwards different types of secondary diseases (Kataura and Harabuchi, 1995). In a
163 review, different possible ways for a passive penetration of the intestinal wall by BoNT were
164 shown (Böhnel and Gessler, 2005). Most recent findings propose that a 14-mer complex of
165 BoNT type D and adherent non-toxic components may be attached to intestinal membranes,
166 the non-toxic units serving as a “delivery vehicle” for the BoNT (Hasegawa et al., 2007). The
167 tonsils are capable of a variety of complex immunological functions in man. Simpson (1982)
168 did not find any receptors for BoNT type C in the intestines. There is one report of respiratory
169 intoxication by BoNT type A in man (Holzer 1962). Evidence for direct absorption of BoNT
170 from the buccal region is inconclusive (Lamanna et al., 1967). Recently, Park and Simpson
171 (2003) showed that there were binding and transcytosis determinants in rat alveolar epithelial
172 cells and in human pulmonary adenocarcinoma cells.

173

174 It would certainly be worthwhile to investigate the structure of the toxic complexes in or on
175 the epithelium of the affected tonsils.

176

177 As a cause of vocal fold paralysis BoNT is not considered (Richardson and Bastian, 2004) or
178 of common movement disorders affecting the larynx in man (Merati et al., 2005). Voice
179 problems, however, are a common feature in botulism (Smith and Sugiyama, 1988).

180

181 Direct action of BoNT on parts of the autonomous nervous system may cause visceral
182 botulism in cattle (Böhnel et al., 2001b) and grass sickness in horses (Hunter and Miller,
183 1999).

184

185 In man, high IgA titers of unknown genetic stimulus in the tonsils are suspected of causing
186 IgA nephropathy (Kataura and Harabuchi, 1995). SIDS infants had statistically significant
187 higher concentrations of IgG and IgA-immunocytes in tonsils compared to controls
188 (Stoltenberg et al., 1995).

189 Tonsils can serve as expression sites of locally induced antibody responses (Quidink-Järbrink
190 et al., 1995). Continuous uptake of a highly toxic BoNT by M cells in the tonsils may lead to
191 overproduction of antibodies. This may be the explanation of high antibody titres in animals
192 with visceral botulism (unpublished data). However, the question is not solved whether these
193 antibodies have protective properties (Brandtzaeg, 1999, 2007). Such immunological
194 responses may have at least a hitherto completely unconsidered effect on neuroimmunological
195 pathways (Besedovsky and del Ray, 1996), influencing diseases of the enteric nervous system
196 (Goyal et al., 1996). The connections between the various immune components are not yet
197 fully understood (Medzhitov, 2007).

198

199

200 **5. Conclusion**

201 Data of the study presented indicate that interaction of tonsils and *C. botulinum* may play an
202 important role in human and animal pathology and warrant concerted investigation. There are
203 many anaerobic bacteria species recovered from tonsillitis and abscesses in man. *C. botulinum*
204 was not looked for (Brook 2005). Bailey and Love (1991) obtained several hundred anaerobe
205 isolates from the pharyngeal tonsillar surface of healthy and diseased horses. No *C. botulinum*
206 was found. Gerber (2001), reporting on a case of tonsillitis in a child caused by *C.*

207 *perfringens*, recommended to sample the oropharynx with an unexpected infant death to find
208 infectious diseases that have become systemic. As BoNT do not provoke visible lesions in
209 affected organs, post mortem health inspection of bovine tonsils, as proposed by Duncanson
210 and Slater (1986), would not help to find carriers of BoNT.

211

212 The present results indicate that hygienic risks during clinical investigations of an animal with
213 botulism or during meat inspection may be substantially underestimated. In the case of toxin
214 bearing tonsils, saliva may contain botulinum toxin as well. This might have been the case of
215 veterinarians having inspected the muscle tonus of the tongues of cows or a horse with
216 suspected botulism. Several days later they fell sick with typical clinical symptoms of
217 muscular weakness, difficulties in swallowing, or general weakness and digestive disorders.
218 Botulism had been confirmed in both cases, caused by the same *C. botulinum* types as in the
219 investigated animals (B. Neufeld, pers. comm.; J. Blatt, pers. comm).

220

221 The results presented in this work encourage further research which may help to find BoNT as
222 cause of diseases or disease complexes which are to date of unknown origin, although the
223 term “botulism” prevents apparently an unbiased approach to botulinum toxicoses. In 2003,
224 botulism was listed as a zoonosis by the EU. Investigations in clinical sick animals, especially
225 the classical testing of tongue tonus by pulling the tip of the tongue should be done with
226 appropriate precautions to avoid danger for the investigator.

227

228

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- 306

307 **Tables/captions**

308

309 Table 1

310 Results of toxin neutralisation tests and typing of botulinum toxin originating from elution
311 (proof of direct toxin) or culture (proof of toxinogenic bacteria) of diagnostic samples

312

313

314 Table 2

315 Results of toxin neutralisation tests and typing of botulinum toxin originating from elution
316 (proof of direct toxin) or culture (proof of toxinogenic bacteria) of slaughter samples

317

318

319 Table 3

320 Summarized results of botulinum toxin neutralisation tests. Comparison between samples of
321 tonsils and upper and lower intestinal tract including liver of diagnostic samples

322

323

324 Table 4

325 Summarized results of botulinum toxin neutralisation tests. Comparison between samples of
326 tonsils and upper and lower intestinal tract including liver of slaughter samples

327
328

329 **Legend tables 1-4**

330 n = number of animals tested; di = final clinical diagnosis/meat inspection classification; mus =
331 muscular (acute) botulism/intoxication; vis = visceral (chronic) botulism/toxicoinfection; +ve =
332 clinical botulism approved, -ve = clinical botulism disapproved, pass = meat inspection passed
333 T = tonsils; SSI = stomach/rumen, small intestine; LIR = large intestine, rectum/faeces; L = liver
334 botulinum toxin neutralisation: BoNT = number of tests with toxin elution, VTB = number of tests
335 with bacterial cultures
336 i = toxin types A+B+E; ii = toxin types C+D; iii = toxin types A+B+E+C+D; iv = proof of toxic
337 substance, no specific neutralisation done, but positive neutralisation in other samples; v = negative
338 Bold figures are summarized results
339 Tables 3 +4
340 + = number of positive tests; - = number of negative tests

341

342 Table 1

n	di	T					SSI					LIR					L																				
		BoNT					VTB					BoNT					VTB					BoNT					VTB										
		i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	
bovine = 48																																					
28	mus	2	5	1	19	1	2	1	1	21	1	11	4	2	16	4	4	2	1	13	1	1	2	13	2	2	3	1	8	3	2	1	13	8	2	9	
10	vis				10	1	3	1	5					15	2				13				12	3	4	1	7				7		1	6			
38	+ve	2	5	1	29	2	5	2	1	26	1	11	4	2	31	6	4	2	1	26	1	1	2	25	5	6	4	1	15	3	2	1	20	8	1	2	15
		8			29	10			26	18				31	13				26	4			25	16			15	6			20	11			15		
10	-ve				10				10					13				1	12				8				8				9				9		
		0			10	0			10	0				13	1				12	0			8	0			8	0			9	0			9		
equine = 11																																					
5	+ve		1		3		2		3		1	2	3		2		4		1	1	1		1		2		1		4			5					
			2		3		2		3		3		3		2		4		2		1		1		2		1		4		0	5					
6	-ve				6				6				9				9				11				11				5			5					
		0			6	0			6	0			9	0			9	0			11	0			11	0			5	0		5					
caprine = 14																																					
3	+ve				8				8	3			12	1	1		13	1			6	1	1		5	1			6	1	3	3					
		0			8	0			8	3			12	2			13	1			6	2			5	1			6	4		3					
6	-ve				4				6				8				12				4				8				4			6					
		0			4	0			6	0			8	0			12	0			4	0			8	0			4	0		6					

343

344 Table 2

345

n	di	T					SSI					LIR					L															
		BoNT					VTB					BoNT					VTB					BoNT					VTB					
		i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	i	ii	iii	iv	v	
bovine = 10																																
10	pass	1	5	1	3	1	1	8	20	1	1	18	1	9	1	1	8															
		7			3	2		8	0	20	2	18	1	9	2	8																
porcine = 10																																
10	pass				10			10	10			10																				
		0			10	0		10	0	10	0	10																				

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Table 3

n	di	T				SSI+LIR+L			
		BoNT		VTB		BoNT		VTB	
		+	-	+	-	+	-	+	-
bovine = 48									
38	+ve	8	29	10	26	28	76	40	56
10	-ve	0	10	0	10	0	30	1	29
equine = 11									
5	+ve	2	3	2	3	6	8	3	11
6	-ve	0	6	0	6	0	25	0	25
caprine = 14									
8	+ve	0	8	0	8	5	24	8	21
6	-ve	0	4	0	6	0	16	0	26

Table 4

n	di	T				SSI+LIR+L			
		BoNT		VTB		BoNT		VTB	
		+	-	+	-	+	-	+	-
bovine = 10									
10	pass	7	3	2	8	1	29	4	26
porcine = 10									
10	pass	0	10	0	10	0	10	0	10

Legend table 4 see table 3