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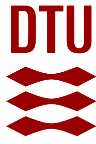

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1 Isolation and molecular characterization of *Clostridium perfringens* from healthy Merino
2 lambs in Patagonia Region, Argentina

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13 **Keywords**: *Clostridium perfringens*; lambs; enterotoxaemia; PCR; PFGE.

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20 **Abstract:**

21 The presence and molecular characterization of *Clostridium perfringens* in healthy Merino
22 lambs over a six-month period was investigated in this study. Overall, a high prevalence of
23 *C. perfringens* was detected, even in day-old lambs. Even though the majority of the
24 isolates were characterized as being of type A, types C and D were also isolated.
25 Furthermore, a high genetic diversity was observed by PFGE among the type A isolates.

26

27 *Clostridium perfringens* is an anaerobic, Gram-positive bacterium that can be found in the
28 in the gastrointestinal tract of healthy humans and animals [1,2]. Under certain
29 circumstances, *C. perfringens* is able to cause severe diseases by the production of a variety
30 of toxins [3]. Depending on the ability to produce four of these toxins (alpha, beta, epsilon,
31 iota), *C. perfringens* strains are classified into five types (A, B, C, D, E) [4]. All *C.*
32 *perfringens* types harbor the alpha toxin-encoding gene (*cpa*) and in the case of type A
33 strains only the *cpa* gene is carried. *C. perfringens* types B and C also harbor the beta toxin
34 gene (*cpb*) and additionally types B and D strains carry the epsilon toxin gene (*etx*),
35 whereas type E strains carry the iota toxin gene (*iA*). Besides these classifying toxins, all
36 types of *C. perfringens* can produce other toxins involved in the pathogenic behavior of the
37 bacterium; such as the enterotoxin (*cpe*), the beta2 toxin (*cpb2*) and the NetB toxin (*netB*)
38 [5–7].

39 In sheep, all *C. perfringens* types can produce gastrointestinal diseases [8,9]. Among the
40 different *C. perfringens* types, type A is the most common type isolated from the intestine
41 of healthy domestic animals and in the environment. However, it is also able to cause

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42 gastrointestinal disease in lambs known as yellow lamb dysentery [10–12]. *C. perfringens*
43 type B is responsible of dysentery and type C of necrotic enteritis [8]. Both diseases occur
44 mainly in newborn lambs during the first days of life and beta toxin is responsible for the
45 characteristic lesions. Enterotoxaemia is caused by type D strains that produce the epsilon
46 toxin and is one of the most common clostridial diseases in sheep [8].

47 Diseases caused by *C. perfringens* are triggered by different predisposing factors, such as
48 stress situations or sudden changes in diet, that allow proliferation of the bacterium and
49 production of toxins [8,13].

50 Prevalence studies in healthy lambs and lambs with clostridial gastrointestinal diseases
51 have reported the presence of *C. perfringens* types A, B, C and D [10,11,14]. However,
52 data from longitudinal studies is not available. In order to better understand the behavior of
53 this complex pathogen in lambs, we analyzed the presence and molecular characterization
54 of *C. perfringens* isolates collected from healthy lambs during a six-month period.

55 The study was conducted at INTA's Experimental Farm (S41°1'55" W70°35'24") located at
56 Patagonia, Argentina; from October 2014, when the lambing season started, to May 2015.
57 Fifteen healthy female Merino lambs belonging to the same flock, born within six days
58 from 31 October to 5 November 2014, were used in this study (Table 1). Samples were
59 collected during six months starting from the day the lambs were born. The age of the
60 animals at each sampling time is found in Table 1.

61 Samples were obtained from lambs using a sterile swab that was inserted 3 to 4 cm into the
62 rectum and gently rotated and rubbed against the inner wall of the rectum. Fecal swabs

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63 were then transported to the laboratory in sterile screw-capped tubes containing 5 ml of
64 glycerol 50 % v/v in saline solution at room temperature and were processed the same day.

65 Fecal swabs were inoculated into Tarozzi broth medium and incubated in anaerobic jars
66 (Oxoid, United Kindom) in an atmosphere with H_2 80% and CO_2 20% at 37ºC for 24 h [1].
67 Subsequently, blood agar (BA) plates (Britania, Argentina) were spread plated with 0.1 ml
68 of the cultivated Tarozzi broth medium and incubated anaerobically. After incubation,
69 colonies compatible with *C. perfringens* (medium-sized, bright, round, with a characteristic
70 double-zone haemolysis) were subcultured in thioglycolate medium (Britania, Argentina).
71 Gram staining and biochemical tests (production of catalase, lecithinase, reverse CAMP
72 and aerotolerance) were carried out to identify the isolates [15].

73 Some *C. perfringens* isolates were further analyzed by Matrix-assisted laser
74 desorption/ionization time of flight mass spectrometry (MALDI TOF). All the isolates
75 analyzed by MALDI TOF were demonstrated to be *C. perfringens* with a high score value
76 confirming the correct identification by classical bacteriological means (data not shown).

77 DNA was extracted from Tarozzi broth medium after 24 h of cultivation with fecal swabs
78 and from pure thioglycolate cultures with 150 µl of 5% Chelex resin (Bio-Rad, USA)
79 following manufacturer´s instructions.

80 Multiplex PCR targeting the *cpa*, *cpb, etx*, *iA*, *cpe* and *cpb2* genes was performed using
81 primers previously described [16,17]. Reactions contained a final concentration of 0.4 µM
82 of *cpa* primers, 0.36 µM of *cpb* primers, 0.5 µM of *etx*, *iA* and *cpb2* primers, 0.6 µM *cpe*
83 primers, 10 mM of dNTPs, 2 mM $MgCl_2$, 2 × PCR buffer Taq Pegasus (PB-L, Argentina),
84 1.25 units of DNA Taq Pegasus, 1 µl of template and water to 25 µl. The thermal cycling

85 was carried out with 25 cycles of denaturation at 95ºC for 1 min, annealing at 53ºC for 1
86 min, and extension at 72ºC for 1 min, finally an additional extension period of 10 min at 72
87 °C was done. PCR products were subject to electrophoresis in 2.2 % agarose gel for 40 min
88 at 100 V and stained with Gel Red (Biotium, USA). PCR product visualization and
89 documentation were performed under 254 nm UV light.

90 Nineteen *C. perfringens* isolates were analyzed by Pulsed-Field Gel Electrophoresis
91 (PFGE) as previously described [18]. Briefly, colonies from BA were picked and
92 suspended in Brain Heart Infusion broth (Becton Dickinson, USA). Then, the cultures were
93 centrifuged and washed with ice-cold PIV buffer before being embedded in agarose (Bio-
94 Rad, USA). Agarose blocks were lysed and then digested with 20 U *Sma*I (Invitrogen,
95 USA) for 3 h at 30ºC. The restricted fragments were separated in a 1% agarose gel in 0.5 ×
96 TBE buffer by using a CHEF-DR III system (Bio-Rad). Following the electrophoresis, the
97 gel was stained in aqueous ethidium bromide 2 μg/ml followed by a distained step in water
98 and photographed under 254 nm UV light. The reference strain used for the analysis was
99 *Salmonella* serotype Braenderup H9812 and was digested with *Xba*I (Invitrogen, USA) for
100 3 h at 37ºC [19].

101 PFGE gel photos were imported to BioNumerics version 7.1 (Applied Maths, Belgium) as
102 JPG files and bands were assigned to each lane. The similarity among the isolates was
103 calculated using the Dice similarity coefficient with branch matching of 2 % tolerance, and
104 the cluster analysis was based on the unweighted pair-group method with arithmetic
105 averages (UPGMA).

106 The results of the present study demonstrate a high prevalence of *C. perfringens* in healthy
107 Merino lambs from Patagonia, Argentina. *C. perfringens* was isolated at all sampling times,

108 at least from one animal throughout a six-month period (Table 1). Moreover, the bacterium
109 was isolated from lambs as young as one day old (Table 1).

110 A higher prevalence of *C. perfringens* was detected in younger animals, reaching a 100%
111 prevalence in lambs less than a month old. However, the prevalence started decreasing as
112 they grew older (> 1 month old) (Table 1). Indeed, when the lambs were two month old, the
113 prevalence of *C. perfringens* was 67% and later started decreasing and was observed to be
114 between 7-36% for the rest of the period (Table 1).

115 Based on the multiplex PCR, *C. perfringens* Type A was commonly isolated from the fecal
116 samples (Table 1) [11]. However, *C. perfringens* type C and type D (*cpe*+, *cpb2*+) were
117 also detected (Table 1). Both types were isolated at an age when lambs are especially
118 susceptible to those *C. perfringens* types [8,14]. Indeed, *C. perfringens* type C was isolated
119 from newborns and *C. perfringens* type D (*cpe*+, *cpb2*+) was isolated from lambs after
120 weaning, when the animals were 4 month old and were moved to a new paddock. This
121 sudden change in diet is one of the predisposing factors for enterotoxaemia caused by *C.*
122 *perfringens* type D [13]. Both *C. perfringens* type C and type D were only isolated at one
123 time point each and only from one animal and could not be isolated again in the following
124 sampling time.

125 The genetic diversity of 19 *C. perfringens* isolates was analyzed by PFGE. PFGE analysis
126 with restriction enzyme *Sma*I resulted in 10 different PFGE patterns (Fig. 1). All the
127 isolates analyzed produced a fragment of approx.1,100 bp, as previously reported [20] (Fig.
128 1). Five *C. perfringens* isolates collected from eleven different animals at the same time had
129 the same PFGE pattern (Fig. 1). In contrast, different PFGE patterns were detected in

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130 isolates from the same animal at different time points (Fig. 1). Indistinguishable PFGE
131 patterns were only detected in two isolates from the same animal when weekly and not
132 monthly samples were analyzed. The isolation of several different genetic clones of *C.*
133 *perfringens* over time in the same animal could be due to the characterization of only one
134 colony at each time point. However, when different isolates from the same animal within
135 the same time point were analyzed, the same PFGE pattern was detected in all of them,
136 suggesting a succession of strains in the same animal. On the basis of these results it is
137 suggested that time may be an important factor for the isolation of clustered or even
138 identical bacterial strains. Also, the presence of different clones isolated from the same
139 animal at different times suggests that *C. perfringens* could pass passively through the gut
140 because of the ingestion of spores from the environment, as previously suggested for
141 *Clostridium difficile*, instead of being a permanent inhabitant of gut flora [21]. This issue
142 remains to be further analyzed to provide new insights into the mechanism of pathogenicity
143 and physiology of *C. perfringens*. However, care has to be taken when interpreting these
144 results because of the small number of *C. perfringens* isolates.

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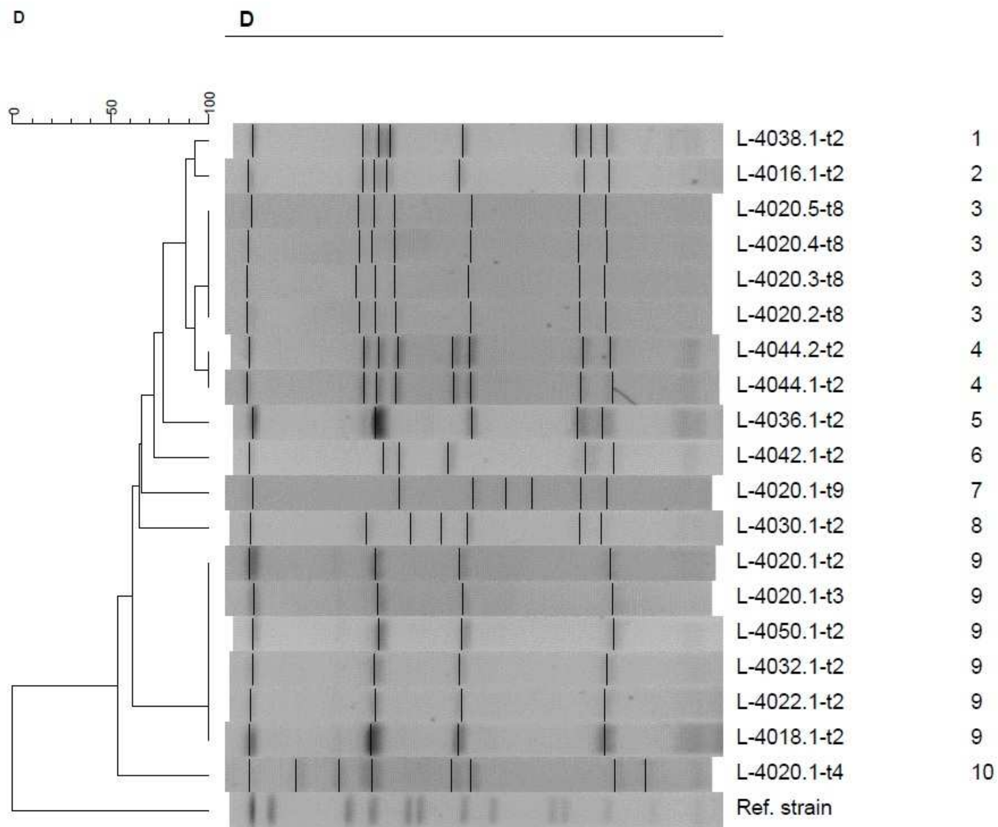

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235 **Figure 1. Dendrogram of PFGE types of *C. perfringens* isolates from healthy Merino**
236 **lambs in Patagonia, Argentina.** L is lamb, followed by the animal number. The different
237 lambs samples collected at different time points (t2, t3, t4, t8, and t9) are shown in the left
238 column, while the assigned PFGE patterns are shown in the right column. Ref. strain is the
239 *Salmonella* serotype Braenderup H9812 reference strain.

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Table 1. Detection of *C. perfringens* by culture from fecal swabs from healthy lambs over time.

Sample N°	1	2	3	4	5	6	7	8	9
Age of lambs (days)°	1	4	10	30	60	90	120	150	180
Lamb N°									
4016	-	+	+	+	+	-	-	-	-
4018	+	+	+	+	+	-	+	-	-
4020	-	+	+	+	+	+	-	+	+
4022	-	+	+	+	+	-	-	-	-
4026	-	+	+	+	-	-	-	-	+
4030	+	+	-	-	-	-	+	-	+
4032	-	+	+	+	+	-	+ **	-	+
4036	+	+	+	+	-	-			
4038	-	+	+	+	-	-	-	-	-
4040	+	+	+	+	-	+	+	-	+
4042	-	+	+	+	+	-	+	-	-
4044	-	+ *	+	+	+	+	-	-	-
4046	-	+	+	+	+	+	-	-	-
4048		+	+	+	+	-	-	-	-
4050		+	+	+	+	-	-	-	-
% of lambs with *C. perfringens*	31	100	93	93	67	27	36	7	36

°average age is shown
+ *C. perfringens* type A
+ **C. perfringens* type A and C
+ ***C. perfringens* type D (*cpe*, *cpb*2)
- negative isolation
(grey cell) sample not available

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