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1 **Alternative Sampling to Establish the *Escherichia coli* O157 Status on Beef Cattle Farms**

2

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9 **Key words:** beef cattle, *E. coli* O157, farm, alternative samples

10

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24

25 **Abstract**

26 Prevalence of *E. coli* O157 in cattle at the farm level is mostly determined by taking
27 individually rectal samples. From the animal welfare point of view the collection of such
28 samples on the farm is not advisable. The present study evaluated alternative sample types to
29 assess the *E. coli* O157 status of cattle farms. Twelve closed cattle farms were visited twice
30 with a time interval of six to eight months. Rectal and hide surface samples (the nose, the
31 neck, the shoulder, the flank, and the round) were collected from beef cattle within the period
32 of five months before slaughter and from their environment (overshoes from the pen bedding,
33 swabs from the pen barrier, feed and water). Statistical analysis revealed that from all samples
34 taken only the “overshoe method” may be a good sampling technique to substitute the
35 collection of individual fecal samples to establish the *E. coli* O157 status of a farm and even a
36 pen. Characterization of the isolates, using Pulsed Field Gel Electrophoresis, revealed that on
37 each positive farm only one genotype was presented, even after a period of more than six
38 months.

39 1. Introduction

40 Since *E. coli* O157 has been associated with bloody diarrhoea and severe complications such
41 as haemorrhagic colitis and haemolytic-uremic syndrome in humans in 1982, it has emerged
42 as an important foodborne pathogen. (Griffin and Tauxe, 1991). Cattle are considered to be
43 the major reservoir of *E. coli* O157 (Ørskov et al., 1987; Chapman et al., 1993) and beef
44 becomes contaminated by fecal contamination or by cross contamination during slaughter
45 (Chapman et al., 1993; Armstrong et al., 1996). Most studies on natural infected cattle at farm
46 level are based on individually taken fecal samples (Cizek et al., 1999; Keen and Elder, 2002;
47 Schouten et al., 2005). This is not only time consuming, but stress caused by this sampling
48 method can affect the animal welfare. Furthermore, as beef cattle are often housed in groups,
49 research on pathogens may be focused on the managed groups of cattle rather than on
50 individuals (Hancock et al., 1997). Epidemiological studies showed that *E. coli* O157 is
51 frequently present in the environment of cattle such as water (Hancock et al., 1998; Shere et
52 al., 1998; Sargeant et al., 2003), feed (Shere et al., 1998; Doane et al., 2007) and bedding
53 material (Conedera et al., 2001; LeJeune and Kauffmann, 2005). Alternatively, sampling of
54 the animal hide surface has been performed at the time of slaughter (Elder et al., 2000;
55 Tutenel et al., 2003), but sporadically at the farm level (Elder et al., 2000; Stephens et al.,
56 2007).

57 In the present study the potential of different sampling types was evaluated as a substitution
58 for the elaborative individually fecal sample collection in order to detect the *E. coli* O157
59 status of beef farms. Furthermore, the possible persistence of *E. coli* O157 strains on closed
60 beef cattle farms was studied to provide a more complete understanding of *E. coli* O157
61 epidemiology on such farms.

62

63

64 **2. Material and Methods**

65 **Farms**

66 Twelve Belgian cattle farms, situated in Flanders and rearing animals belonging to the
67 Belgian Double-Muscled White-Blue cattle breed, were included in this study carried out
68 between December 2004 and March 2006. All farms were closed beef cattle farms and each
69 farm was sampled twice with a time interval of six to eight months. Only animals at the age
70 within five months before slaughter (18 to 24 months old) and their environment were
71 sampled. None of the sampled animals showed clinical symptoms at the moment of sampling.

72

73 **Sampling Methods**

74 A diverse set of sample types was chosen, including feces, samples from various hide surface
75 areas of the animals, feed, water, and pen environment. In total, 208 animals dispersed over
76 62 pens were sampled. The number of animals per pen varied from one to seven.

77 Fecal samples were rectally taken using rectal gloves. Hide surface samples were taken from
78 the nose mirror and on the left side of each animal from the neck, the shoulder, the flank, and
79 the round with a sterile cotton swab moistened with 0.1 % peptone water. Except for the nose,
80 a hide area of 400 to 600 cm² was sampled. The metal barriers from each pen were sampled
81 on different places using the swab method described above, while sampling the pen bedding
82 was carried out by walking around in an “8”-shaped track in the pen using one pair of
83 disposable, liquid absorbing overshoes (Kolmi, Saint Bathélémy d’Anjou, France). Water
84 samples were collected from the paddle waterers of each pen in clean 50 ml tubes. Finally,
85 feed samples from each pen were collected with a clean glove by taking three grab
86 subsamples and pooling them to one sample. All samples were transported to the laboratory
87 under cooled conditions and processed within three hours.

88

89 **Laboratory Methods**

90 Twenty-five gram of feces, feed and water were homogenized with a stomacher blender for 1
91 min at normal speed in 225 ml sterile modified trypton soya broth (Oxoid, Basingstoke,
92 United Kingdom) supplemented with 20 mg/l novobiocin (Sigma, Aldrich, St-Louis, MO,
93 USA)(mTSBn). After incubation in a warm water bath at 42°C for 6 h, immunomagnetic
94 separation was carried out on 1 ml using 20 µl Dynabeads (Dyna, Oslo, Norway) according
95 to the manufacturer's recommendations. The washed beads were spread onto sorbitol-
96 MacConkey agar (Oxoid) supplemented with cefixime (0.05 mg/l) and potassium tellurite (2.5
97 mg/l)(Dyna, Oslo, Norway)(CT-SMAC) and incubated for 24 h at 42°C. To all individual
98 swabs and overshoes, respectively 40 ml and 250 ml mTSBn was added. The samples were
99 further processed as described before.

100 Following incubation of the selective CT-SMAC medium, per plate up to two non-sorbitol
101 fermenting colonies with typical morphology were serologically tested with the O157 antigen
102 latex agglutination assay (Oxoid). Colonies positive for agglutination were biochemically
103 tested using API 20E tests (Biomérieux, Marcy l'Etoile, France).

104 One confirmed colony per sample was examined for the presence of the *rfb* (O-antigen-
105 encoding) and *fliC* (H-antigen-encoding) genes (Osek, 2003). Isolates positive for the *rfb* gene
106 were further tested for the presence of virulence genes by the multiplex PCR according to
107 Fagan et al. (1999) using the primers for *vt1*, *eaeA* and *HlyA* described by Fagan et al. (1999)
108 and for *vt2* described by Paton and Paton (1998).

109 Pulsed field gel electrophoresis (PFGE) was performed as recommended by Pulsenet (2007).

110

111 **Statistical analysis**

112 The data obtained by different sampling methods were analysed by the Fisher exact
113 probability test at a confidence level of $P > 0.05$.

114 **3. Results**

115 A total of 1496 samples was collected on the 12 farms and *E. coli* O157 was detected in
116 samples collected on six farms. The organism was present at one sampling time on two farms
117 (B and D) and at both sampling times on four other farms (H, I, J and K). The prevalence of
118 *E. coli* O157 in the fecal content on positive farms is shown in Table 1. During both sampling
119 times, the proportion of cattle infected on these farms varied between 0 and 85 %. With the
120 exception of one pen, all pens where at least one excreting animal was present, the overshoe
121 pairs were positive for *E. coli* O157 (Table 2). Moreover, six overhoe samples on two farms
122 (B and K) were found to be positive for *E. coli* O157 whereas the organism could not be
123 isolated from the fecal material of the sampled animals present in these pens. The number of
124 positive pens on farms based on the presence of animals shedding *E. coli* O157 in a pen and
125 positive overshoes collected from the same pen was not significant different ($P>0.05$).

126 From all hide surface samples taken, only 49 samples originating from 20 animals were
127 positive for *E. coli* O157. In water, feed and on metal *E. coli* O157 could only be detected
128 respectively three, five and four times (Table 2). Contaminated water was found on one farm
129 at only one sampling day and occurred simultaneously with the detection of at least one
130 animal shedding the organism in the pen. The presence of *E. coli* O157 in the feed or metal
131 barrier was always associated with contaminated bedding material, but not with positive
132 animals in the pen.

133 All isolates from farm B, H, I, J, and K carried the *eaeA* and the *HlyA* gene and at least one of
134 the *vt* genes. On farm D, isolates possessed only the *eaeA* and the *HlyA* gene. Further
135 characterization of the isolates by PFGE revealed six XbaI patterns (Fig. 1). On each positive
136 farm one genotype was identified.

137

138

139 **4. Discussion**

140 Cattle in the fattening period were chosen as this may reflect more accurately the risk of
141 animals shedding the pathogen at the time of slaughter. Different sample types were collected
142 to evaluate the potential of such samples to determine the on-farm *E. coli* O157 status.
143 Among the types of these samples, rectal samples and overshoes allowed the correct
144 determination of the farm status. At the pen level only one pair of overshoes was negative
145 while *E. coli* O157 could be isolated from the rectal content. Such false-negative results may
146 be reduced by the application of more than one pair of overshoes since Heyndrickx et al.
147 (2002) have proven that the *Salmonella* status of broiler flocks could be determined more
148 accurately when more than two pairs of overshoes were used for the sampling of the broiler
149 houses. Remarkably, samples from the pen floor were six times positive for *E. coli* O157
150 whereas the pathogen was not detected in the feces from the animals staying in the pen. These
151 results can be explained by the intermittent excretion of *E. coli* O157 by cattle (Faith et al.,
152 1996; Robinson et al., 2004). Moreover, *E. coli* O157 has the capacity to survive and even to
153 replicate in different bedding material in the presence of urine (Conedera et al., 2001; LeJeune
154 and Kauffmann, 2005; Davis et al., 2005). Pen bedding samples collected by overshoes can
155 replace individual fecal samples to assess the presence of *E. coli* O157 at the pen level.

156 The finding that only a limited number of animals yielded positive hide swabs was not
157 consisted with data reported by Keen and Elder (2002) for feedlot cattle. The presence of a
158 small number of animals in a relative large pen may led to less hide contact between animals
159 and consequently less cross contamination. The hide of all sampled animals was visibly clean
160 indicating that not only dirty hides are contaminated with *E. coli* O157.

161 Characterization of the isolates showed that on each farm, even over a time interval of more
162 than six months, an unique genotype was present. Faith et al. (1996) showed that different
163 PFGE types can be simultaneously present on a cattle farm. Different studies reported the

164 long term survival of a predominant strain on cattle farms (LeJeune et al., 2004; Shere et al.,
165 2002; Liebana et al., 2005; Schouten et al., 2005). The fact that only one genotype was found
166 on each farm may be due to the type of the sampled cattle farms. All farms were of the closed
167 type, and consequently have no or very limited contact with other cattle farms, reducing the
168 opportunity for the entrance of other *E. coli* O157 genotypes.

169 In conclusion, the *E. coli* O157 status of beef cattle farms could be determined most
170 efficiently by the “overshoe method” whereas the hide surface samples, pen swabs, feed and
171 water can not be applied to substitute individually collected rectal samples. In addition, the
172 study demonstrated that only one genotype was present on each closed farm and could be
173 isolated for a significant period of time.

174

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178

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- 262

263 **Table 1. Prevalence of *E. coli* O157 on positive farms based on individually collected**
264 **rectal material from beef cattle.**

Farm	First visit	Second visit
B	0/20 (0 %)	5/20 (25 %)
D	0/7 (0 %)	7/14 (50 %)
H	7/14 (50 %)	3/7 (43 %)
I	11/13 (85 %)	1/11 (9 %)
J	1/6 (17 %)	1/3 (33 %)
K	2/11 (18 %)	2/12 (17 %)

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274 **Table 2. Detection of *E. coli* O157 in environmental and animal-related samples collected on positive farms.**

Farm	Visit	No. of pens	No. of animals present	Rectal material ²	Overshoe pen ³	Metal pen barrier ³	Feed ³	Water ³	Hide samples					Total ²
									Nose ³	Neck ³	Shoulder ³	Flank ³	Round ³	
B ¹	2	4	5	0/5	0/1	0/1	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5
			5	0/5	0/1	0/1	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5
			5	0/5	1/1	0/1	0/1	0/1	0/5	0/5	0/5	0/5	0/5	0/5
			5	5/5	1/1	0/1	1/1	0/1	2/5	0/5	2/5	0/5	1/5	3/5
D ¹	2	4	4	1/4	1/1	0/1	0/1	0/1	0/4	0/4	0/4	0/4	0/4	0/4
			4	1/4	1/1	0/1	1/1	0/1	0/4	0/4	0/4	0/4	0/4	0/4
			3	3/3	1/1	0/1	0/1	0/1	0/3	0/3	0/3	0/3	0/3	0/3
			3	2/3	0/1	0/1	0/1	0/1	0/3	0/3	0/3	0/3	0/3	0/3

H	1	3	7	$5/7$	$1/1$	$0/1$	$0/1$	$0/1$	$4/7$	$1/7$	$3/7$	$3/7$	$4/7$	$7/7$
			6	$1/6$	$1/1$	$0/1$	$0/1$	$0/1$	$0/6$	$0/6$	$0/6$	$0/6$	$0/6$	$0/6$
			1	$1/1$	$1/1$	$0/1$	$0/1$	$0/1$	$0/1$	$0/1$	$0/1$	$0/1$	$0/1$	$0/1$
	2	2	4	$2/3$	$1/1$	$0/1$	$0/1$	$0/1$	$0/3$	$0/3$	$0/3$	$0/3$	$0/3$	$0/3$
			4	$1/4$	$1/1$	$1/1$	$0/1$	$0/1$	$0/4$	$0/4$	$0/4$	$0/4$	$0/4$	$0/4$
	I	1	3	6	$5/6$	$1/1$	$0/1$	$0/1$	$1/1$	$0/6$	$0/6$	$0/6$	$0/6$	$0/6$
5				$4/5$	$1/1$	$0/1$	$1/1$	$1/1$	$3/5$	$2/5$	$4/5$	$4/5$	$4/5$	$4/5$
2				$2/2$	$1/1$	$0/1$	$0/1$	$1/1$	$0/2$	$0/2$	$0/2$	$0/2$	$0/2$	$0/2$
2		2	6	$1/5$	$1/1$	$0/1$	$0/1$	$0/1$	$0/5$	$0/5$	$0/5$	$0/5$	$0/5$	$0/5$
			6	$0/6$	$0/1$	$0/1$	$0/1$	$0/1$	$0/6$	$0/6$	$0/6$	$0/6$	$0/6$	$0/6$
J		1	1	6	$1/6$	$1/1$	$0/1$	$0/1$	$0/1$	$0/6$	$0/6$	$0/6$	$0/6$	$0/6$

	2	1	3	1/3	1/1	0/1	0/1	0/1	0/3	0/3	0/3	0/3	0/3	0/3	
K	1	8	3	0/2	0/1	0/1	0/1	0/1	0/2	0/2	0/2	0/2	0/2	0/2	0/2
			3	0/2	0/1	0/1	0/1	0/1	0/2	0/2	0/2	0/2	0/2	0/2	0/2
			2	0/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	1/1	1/1	0/1	1/1
			1	0/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
			2	2/2	1/1	1/1	0/1	0/1	0/2	0/2	0/2	0/2	0/2	0/2	0/2
			1	0/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
			1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
			2	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
2	5	3	0/3	0/1	0/1	0/1	0/1	0/3	0/3	0/3	0/3	0/3	0/3	0/3	
		3	0/3	1/1	0/1	1/1	0/1	0/3	1/3	1/3	1/3	1/3	1/3	2/3	
		2	0/2	1/1	0/1	0/1	0/1	1/2	0/2	1/2	0/2	1/2	1/2	1/2	
		3	1/2	1/1	0/1	0/1	0/1	0/2	0/2	1/2	0/2	1/2	1/2	1/2	
		2	1/2	1/1	0/1	0/1	0/1	1/2	0/2	0/2	0/2	0/2	0/2	1/2	

Total	33	118	40/111	24/33	4/33	5/33	3/33	11/111	5/111	11/111	9/111	13/111	20/111
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275 ¹ = All samples were negative at the first visit; ² = number of positive animals/number of animals tested; ³ = number of positive samples/total

276 number of samples

277 **Fig. 1. Dendrogram constructed by UPGMA showing the PFGE types.** One genotype of
278 each farm is presented as all isolates from one farm revealed the same PFGE type. The letters
279 represent the positive farms.

