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Prevalence of Gastrointestinal *Clostridium difficile* Carriage in Australian Sheep and Lambs

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Recently, *Clostridium difficile* has been isolated from a wide variety of animals, particularly production animals, mainly cattle and pigs. Concurrently, the incidence of *C. difficile* infection (CDI) in humans has increased in the community, with some suggestions that food-borne transmission of *C. difficile* is occurring. Interestingly, sheep and lambs appear not to have been investigated for carriage/colonization with *C. difficile*. The aim of this project was to determine the prevalence of carriage of *C. difficile* in sheep and lambs in Australia by culturing fecal samples. A total of 371 sheep and lamb fecal samples were received in seven batches from three different geographic areas in eastern Australia and two in Western Australia. The overall rate of detection in sheep and lambs was low (4.0%); however, carriage/colonization in lambs (6.5%) was statistically significantly higher than that in sheep (0.6%) ($P = 0.005$). Seven distinct PCR ribotype patterns were observed, three of which were known international ribotypes (UK 056 [$n = 1$], UK 101 [$n = 6$], and UK 137 [$n = 2$]), while the remainder were unable to be matched with our available reference library. This low rate of carriage/colonization in Australian ovines suggests they are unlikely to be a major source/reservoir of human infections.

First described in 1935 as a component of neonatal intestinal microflora (1), *Clostridium difficile*, a ubiquitous, Gram-positive spore-forming anaerobic bacillus, is now widely recognized as the leading cause of health care-related infectious diarrhea in developed countries (2, 3). In the last decade, both the incidence and severity of *C. difficile* infection (CDI), and the associated economic burden to global health care systems, have increased markedly, attributed to the emergence of epidemic strains of *C. difficile* (4). Data from the United States show a doubling in the number cases of CDI between 1996 and 2003, with an estimated 500,000 cases of CDI reported in 2006 (2).

Traditionally, hospitalized and elderly individuals with exposure to antimicrobials were considered to be most at risk of developing CDI (5) due to reduced "colonization resistance" in the large intestine (5). However, there have been an increasing number of reports in recent years of persons outside hospital environments acquiring CDI (6, 7). Community-acquired CDI (CA-CDI) represents a further burden to health care systems worldwide, with an estimated 40% of CDI cases now originating from the community (6, 7).

One possible source of *C. difficile* in the community is animals. *C. difficile* ribotype 078 (RT078) is the predominant strain of *C. difficile* in production animals worldwide and in some retail meat products in North America and Europe (8–12). RT078 is currently the third most common human isolate in European hospitals (13) and an emerging cause of CDI in the United States (6). Strains of RT078 infecting both animals and humans were genetically related by multilocus variable-number tandem-repeat (MLVA) typing (14), suggesting both a common source and a potential zoonosis (15).

To date, few studies have looked at *C. difficile* in sheep and lambs and the potential risk to public health. Pigs and cattle in Australia are colonized with *C. difficile* strains different from those reported in the Northern Hemisphere (16, 17), so we hypothesized that sheep and lambs would also be different. Therefore, we undertook a study of the prevalence of *C. difficile* in Australian sheep and lambs and characterized strains isolated by genotyping and PCR ribotyping.

MATERIALS AND METHODS

Study samples. Three hundred seventy-one sheep and lamb fecal samples were collected and analyzed. Initially, 300 samples of feces (approximately 50 g) from sheep (defined as ≥1 year of age) and lambs (defined as <1 year of age) were received in four batches from abattoirs in South Australia (SA), Victoria (VIC), and New South Wales (NSW) during the period of May 2011 to June 2011. Batches comprised animals from multiple farms (farm-specific data not available). Samples were taken within the processing area of the slaughter line after hosing of the whole carcass was complete. Feces were collected aseptically directly from the large intestine. A further 71 samples of feces from lambs were collected in three batches from two farms in different geographic areas in Western Australia (WA) in July ($n = 55$) and December ($n = 16$) 2012. Sampling was performed by collection of feces from the ground soon after defecation. All samples were transported to The University of Western Australia, stored at 4°C, and processed within 24 h.

Culture and identification of *C. difficile*. Isolation of *C. difficile* was based on previously described methods (18). Feces were cultured in an enrichment broth containing gentamicin, cefoxitin, and cycloserine (19) and, to enhance spore germination, directly on cycloserine cefoxitin fructose agar containing sodium taurocholate. After 48 h of incubation, broths were treated with alcohol for spore selection. Presumptive identification of *C. difficile* was based on their characteristic chartreuse fluorescence under long-wave UV light (~360 nm), odor (horse dung smell), and colony morphology (yellow, ground glass appearance). The identity of undetermined isolates was confirmed by the presence of L-proline aminopeptidase activity (Remel Inc., Lenexa, KS), Gram stain (18), and amplification of a segment of the 16S rRNA gene (20).

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TABLE 1 Prevalence of *C. difficile* from feces in Australian sheep and lambs

State	No. of specimens	Source	Prevalence in sheep (no. [%])	Prevalence in lambs (no. [%])
SA	50	Abattoir	1/27 (3.7)	3/23 (13.0)
NSW	100	Abattoir	0/47 (0.0)	1/53 (1.8)
SA	50	Abattoir	0/24 (0.0)	0/26 (0.0)
VIC	100	Abattoir	0/58 (0.0)	2/42 (4.2)
WA	24	Farm	NT[a]	1/24 (4.2)
WA	31	Farm	NT	0/31 (0.0)
WA	16	Farm	NT	7/16 (0.0)
Total[b]	371		1/156 (0.6)	14/215 (6.5)

[a] NT, not tested.
[b] Prevalence for total numbers of sheep and lamb were significant ($P = 0.005$ by Fisher's exact test).

PCR ribotyping and toxin profiling. Template DNA was prepared by resuspension of cells in 5% (wt/vol) Chelex-100 (Sigma-Aldrich, Castle Hill, NSW, Australia). All isolates included in this study were screened by PCR for the presence of toxin A (*tcdA*), toxin B (*tcdB*) (21), and binary toxin (*cdtA* and *cdtB*) genes (22) and to identify changes in the repeating region of *tcdA* (23). PCR ribotyping was performed as previously described (24), and reaction products were concentrated using the Qiagen MinElute PCR purification kit (Ambion Inc., Austin, TX). PCR products were run on the QIAxcel capillary electrophoresis platform (Ambion Inc., Austin, TX). BioNumerics software, package v.6.5 (Applied Maths, Saint-Martens-Latem, Belgium), was used for dendrogram and cluster analysis of PCR ribotyping band patterns (Dice coefficient). Isolates that could not be identified with the available reference library were designated with internal nomenclature.

Statistical analysis. Fisher's exact test was used, where appropriate, to compare the prevalence of *C. difficile* among the sampled sheep and lamb populations.

RESULTS

***C. difficile* prevalence in sheep and lambs.** The prevalence of *C. difficile* in sheep and lambs is presented in Table 1. Overall, *C. difficile* was isolated from 1 (0.6%) of 156 fecal specimens from sheep and 14 (6.5%) of 215 fecal specimens from lambs. Recovery of *C. difficile* from lambs at slaughter (4.2%) and on farms (11.2%) was not significantly different ($P = 0.08$). The overall rate of detection was 4.0%, and the observed age-related differences in prevalence were statistically significant ($P = 0.005$).

Toxin gene profile of *C. difficile* isolates. Toxin gene profiles for all isolates are shown in Fig. 1. Of the 15 isolates of *C. difficile* recovered from sheep and lambs, 93.3% (14/15) were positive for *tcdA* and *tcdB* (A^+ B^+) but negative for binary toxin genes (CDT^-); the remaining isolate was A^- B^+ CDT^+.

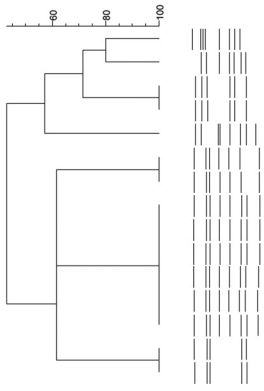
PCR ribotyping of *C. difficile* isolates. Seven distinct PCR RTs were identified (Fig. 1). Nine isolates (60%) were assigned one of three internationally recognized RTs (UK 056 [$n = 1$], UK 101 [$n = 6$], and UK 137 [$n = 2$]). The remaining six isolates (including the single isolate from a sheep) gave four distinct banding patterns but were unable to be assigned PCR ribotypes based on our reference library at the time. These isolates were designated with internal nomenclature (QX029 [$n = 1$], QX049 [$n = 2$], QX098 [$n = 2$], and QX137 [$n = 1$]) (Fig. 1).

DISCUSSION

This study represents the largest sampling of ovines worldwide for *C. difficile* to date. A few smaller studies in Europe and the United States have shown various levels (0 to 18.2%) of *C. difficile* in the feces of sheep and lambs (25–28). Most of these studies did not specify the age of the animal at the time of sampling, and only one study characterized the isolates that were found (RT015 and RT097) (26). In addition, one previous study has looked for *C. difficile* in retail sheep meat and found none, although the sample size ($n = 7$) was very small (29).

In this study, *C. difficile* prevalence was low in both lambs and sheep (overall rate of detection of 4.0%). Of the seven RTs identified, none was epidemic RT027 or RT078; however, most would be considered pathogenic, having been isolated from human cases of CDI in Australia previously (unpublished data). The finding of RT056 is interesting, as it has been found previously in poultry (26) and cattle (17) and is associated with a complicated disease outcome in cases of nosocomial CDI (13). The low proportion of binary toxin-producing isolates (6.7%) was surprising and differs from studies of other livestock (11, 30), where a high proportion of isolates have been binary toxin producers. This result is indicative of the lack of predominance of RT078 or other toxinotype V strains associated with animals in the Northern Hemisphere (31).

We found a significantly higher rate of *C. difficile* detection in



Isolate No.	Age group	Origin	PCR Ribotype	Toxin gene profile	Collection Year
AI 0466	Lamb < 12 months	WA	QX 029	A+B+CDT-	2012
AI 0192	Sheep > 12 months	SA	QX 137	A-B+CDT+	2011
AI 0193	Lamb < 12 months	SA	UK 137	A+B+CDT-	2011
AI 0194	Lamb < 12 months	SA	UK 137	A+B+CDT-	2011
AI 0195	Lamb < 12 months	SA	UK 056	A+B+CDT-	2011
AI 0196	Lamb < 12 months	NSW	QX 098	A+B+CDT-	2011
AI 0304	Lamb < 12 months	WA	QX 098	A+B+CDT-	2012
AI 0463	Lamb < 12 months	WA	UK 101	A+B+CDT-	2012
AI 0465	Lamb < 12 months	WA	UK 101	A+B+CDT-	2012
AI 0460	Lamb < 12 months	WA	UK 101	A+B+CDT-	2012
AI 0461	Lamb < 12 months	WA	UK 101	A+B+CDT-	2012
AI 0462	Lamb < 12 months	WA	UK 101	A+B+CDT-	2012
AI 0464	Lamb < 12 months	WA	UK 101	A+B+CDT-	2012
AI 0202	Lamb < 12 months	SA	QX 049	A+B+CDT-	2011
AI 0203	Lamb < 12 months	SA	QX 049	A+B+CDT-	2011

FIG 1 Summary of *C. difficile* PCR ribotypes, toxin gene profiles, and isolate demographics from Australian sheep and lambs. PCR ribotyping pattern analysis included a neighbor-joining tree, Dice coefficient (optimization, 1.00%; tolerance, 0.5%). UK, internationally recognized PCR ribotype; QX, unidentified ribotype pattern, internal nomenclature.

lambs than in sheep (4.2 and 0.6%, respectively). The observed decrease in the rate of *C. difficile* detection with increasing age is interesting and has been reported for pigs and cattle as well (10, 17). The intestinal microflora of neonatal animals is often underdeveloped, and the gut is rapidly colonized after birth. If the environment into which the animal is being born is contaminated with *C. difficile*, it is very likely that colonization with *C. difficile* will occur in these younger animals (32). As the age of the animal increases, there is an increase in the gut microbiota responsible for colonization resistance and the numbers of *C. difficile* reduce.

The reasons for the differences in *C. difficile* prevalence between lambs and other neonatal animals, such as pigs and calves, are unknown and require further investigation. The age of the animal at sampling could be a contributing factor. In the present study, both sheep and lambs were investigated. Given that a lamb may be aged up to 12 months (defined by incisor development), it is possible that neonatal lambs, like other neonates, will have a higher rate of carriage/colonization. Other factors, such as diet, husbandry practices, and herd density, also may explain the low level of carriage in sheep and lambs.

C. difficile is associated with disease in neonatal piglets (33). In Australia, there is evidence of the widespread use of antimicrobials on swine herds (34). It is possible that the prolonged and injudicial use of antimicrobials contributes to the colonization and onset of disease in neonatal piglets, a situation analogous to that of humans and nosocomial infections. Little is known of the use of antimicrobials in the Australian sheep industry. The low prevalence reported in this study could be indicative of low exposure of animals to antimicrobials. Given the size of Australia's sheep industry, it is imperative that exposure of neonatal lambs to antimicrobials remains low.

In conclusion, the low rate of carriage reported in this study, combined with the lack of RTs known to significantly contribute to CDI in the community, suggests Australian ovine are unlikely to be a major source/reservoir of human infections.

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