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A molecular characterization of *Clostridium difficile* isolates from humans, animals and their environments

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SUMMARY

It is generally accepted that most patients with *Clostridium difficile*-associated diarrhoea acquire the organism from the environment. Recently we demonstrated that household pets may constitute a significant reservoir of *C. difficile* through gastrointestinal carriage in up to 39% of cats and dogs. These findings suggested that direct transmission from household pets, or contamination of the environment by them, may be a factor in the pathogenesis of *C. difficile*-associated diarrhoea. To investigate this possibility, we examined isolates of *C. difficile* from humans, pets and the environment by restriction enzyme analysis (REA) and restriction fragment length polymorphism (RFLP) typing using enhanced chemiluminescence. Both REA and RFLP typing methods used *Hin*d III digests of chromosomal DNA. A total of 116 isolates of *C. difficile* from pets (26), veterinary clinic environmental sites (33), humans (37) and hospital environmental sites (20) was examined. REA was far more discriminatory than RFLP typing and for all isolates there were 34 REA types versus 6 RFLP types. There was good correlation between the REA types found in isolates from pets and from the veterinary clinic environment, and between isolates from humans and from those found in the hospital environment. There was, however, no correlation between REA type of *C. difficile* found in pets and isolates of human origin. We conclude that there may still be a risk of humans acquiring *C. difficile* from domestic pets as these findings may be the result of geographical variation.

INTRODUCTION

Although initially considered to be non-pathogenic, in the late 1970s *Clostridium difficile* was recognized as the aetiological agent of most cases of pseudomembranous colitis [1] and a major cause of antibiotic-associated diarrhoea in humans [2]. Apart from humans *C. difficile* has been isolated from a variety of other animals, both domestic and wild, including camels, cattle, horses, donkeys, cats, dogs, hamsters, a snake and a Weddell seal [3]. It has also been isolated from a number of environmental sources such as soils, marine sediments and peat [3].

Recent studies on patients with *C. difficile*-associated diarrhoea who appear to have relapsed suggested that some apparent relapses were due to reinfection with

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a different organism [4]. It is now generally accepted that most patients with *C. difficile*-associated diarrhoea acquire the organism from the environment. Borriello and colleagues [5] suggested that domestic pets were possibly a significant reservoir, when they detected *C. difficile* in 23% of animals, primarily cats and dogs, and an environmental contamination rate of 11·4% for the veterinary hospital which they sampled. In a similar study recently, we showed that *C. difficile* was carried by 39% of cats and dogs and that 68% of environmental sites at veterinary clinics were contaminated [6].

These findings suggested that the direct transmission from household pets, or contamination of the environment by them, may be a factor in the pathogenesis of *C. difficile*-associated diarrhoea. To investigate this possibility further, we compared isolates of *C. difficile* obtained from pets and the veterinary clinic environment with those obtained from patients and the hospital environment by restriction enzyme analysis (REA) of chromosomal DNA, restriction fragment length polymorphisms (RFLPs) in ribosomal RNA genes and cytotoxin production.

MATERIALS AND METHODS

Bacterial strains

A total of 116 isolates of *C. difficile* was studied. Twenty-five were obtained from the environment of the Karrinyup Small Animal Hospital (KSAH), 22 from animals in the KSAH, 8 from the environment of the Bassendean Veterinary Hospital (BVH) and 4 from animals in the BVH [6]. Twenty-eight isolates were from patients at Sir Charles Gairdner Hospital (SCGH) and 9 were obtained from patients at Royal Perth Hospital (RPH). These isolates were from patients who had undergone multiple infections with *C. difficile* [4]. A further 20 isolates were obtained from the environment of SCGH. The origins of these isolates are listed in Table 1. Methods for the isolation and identification of *C. difficile* [7–9], and for the environmental sampling [6] have been described previously. Isolates were stored in 15% glycerol in tryptone soya broth (TSB) at −70 °C.

Cytotoxin detection

Isolates were inoculated onto blood agar plates and incubated anaerobically for 48 h at 37 °C in an anaerobic cabinet (Don Whitley Scientific Ltd). Single colonies were then inoculated into 5 ml of pre-reduced supplemented brain heart infusion broth (BHIB-S) and incubated at 37 °C for 72 h. Sterile filtrates of the BHIB-S cultures were tested for cytotoxin as described previously [7].

REA

Extraction of chromosomal DNA, restriction enzyme digestion and gel electrophoresis for REA were performed as described previously [4]. The restriction enzyme *Hin*d III was used to generate the REA patterns.

RFLP

The procedure for identifying RFLPs in the ribosomal RNA genes of *C. difficile* has been described previously [11]. The restriction enzyme used to generate restriction fragments was *Hin*d III.

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Table 1. *Origins of the isolates of* C. difficile *used in the study*

Organisms	Source*	Number	Reference
Patient isolates	Inpatients and outpatients of SCGH and RPH	37	4
Hospital environmental isolates	Wards C14, G63, G72 and G73 of SCGH	20	—
Pet isolates	Isolation from pets at KSAH and BVH	26	6
Veterinary environmental isolates	The environment of KSAH and BVH	33	6

* SCGH, Sir Charles Gairdner Hospital; RPH, Royal Perth Hospital; KSAH, Karrinyup Small Animal Hospital; BVH, Bassendean Veterinary Hospital.

RESULTS

Hospital environmental isolates

The isolates obtained from the environment of SCGH and the sites from which they were isolated are listed in Table 2.

Cytotoxin profiles

A total of 63 (54·3%) of 116 isolates produced cytotoxin; 7 (28%) of 26 isolates from the environment of the KSAH, 12 (54·5%) of 22 isolates obtained from pets at KSAH, 4 (50%) of 8 isolates from the environment of BVH and 1 (25%) of 4 isolates obtained from the pets at BVH. Twenty-four (64·8%) of 37 isolates from the patients produced cytotoxin as did 15 (75%) of 20 isolates from the environment of SCGH. The results from the cytotoxin testing are included in Tables 3, 4 and 5.

REA profiles

Investigation of the isolates by REA revealed that four different patterns were present among the environmental isolates from KSAH. Two of these patterns accounted for 23 of the 25 strains examined. The patterns were designated arbitrarily types a–d. Seventeen isolates belonged to type a, 6 isolates belonged to type b and there was 1 isolate each of types c and d. Environmental isolates from BVH exhibited 3 REA patterns (e–g); 4 isolates belonged to type e, 1 isolate belonged to type f and 3 to type g. Pet isolates exhibited 6 REA patterns. These isolates belonged to REA types a, b, g, h, j and k. Nine isolates from KSAH and 1 isolate from BVH belonged to type a, 12 isolates from KSAH belonged to type b, 1 isolate from BVH belonged to type g, 1 isolate from BVH belonged to type h, 1 isolate from KSAH belonged to type j and 1 isolate from BVH belonged to type k. These results are summarized in Table 3.

Twenty-one different REA patterns (designated A–H, J–N and P–X) were identified among the isolates from the human patients. Three different REA patterns were found among the isolates obtained from the environmental sampling of SCGH. Fourteen of the isolates exhibited the same REA type, type B, and this type was also present among those isolates obtained from the patients. The other 2 REA patterns, designated Y and Z, were exhibited by 1 and 5 isolates

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Table 2. *The source of the hospital environmental isolates at SCGH**

Isolate	Source
E3	Ward C14, Room 2, Chair
E6	Ward C14, Room 2, Floor
E12	Ward C14, Room 2, Light
E19	Ward C14, Room 2, Bed A, Bedhead
E27	Ward C14, Room 2, Shelf
E38	Ward C14, Room 2, Sideboard
E42	Ward B11, Room 16, Basin tiles
E49	Ward C14, Room 2, Wardrobe
E61	Ward C14, Room 2, Bed A, Bedbase
E62	Ward C14, Room 2, Bed A, Bedbase
E69	Ward C14, Room 2, Shelf
E98	Ward C14, Room 2, Bed B, Bedbase
E100	Ward G73, Room 4, Toilet floor
E103	Ward G72, Room 4, Bedbase
E104	Ward G63, Room 8, Toilet floor
E108	Ward C14, Room 2, Bed B, Floor
E110	Ward G63, Room 19, Toilet 2, Floor
E113	Ward G63, Room 8, Chair
E114	Ward G63, Room 8, Carpet
E132	Ward G63, Room 19, Toilet 1, Floor

* C14, extended care; G72, general medicine; G73, gastroenterology; G63, general surgery.

Table 3. *Analysis of the patient and hospital environmental isolates by REA*

	REA type											
	A	B	C	D	E	F	G	H	J	K	L	M
Human	1	6	1	1	4	1	1	1	1	1	1	1
Environment	0	14	0	0	0	0	0	0	0	0	0	0
Cytotoxin	−	+	−	+	+	−	+	+	+	−	−	+
Total	1	20	1	1	4	1	1	1	1	1	1	1

	REA type											
	N	P	Q	R	S	T	U	V	W	X	Y	Z
Human	1	7	1	1	1	1	1	1	1	2	0	0
Environment	0	0	0	0	0	0	0	0	0	0	1	5
Cytotoxin	+	−	−	+	+	+	+	+	+	+	+	−
Total	1	7	1	1	1	1	1	1	1	2	1	5

Table 4. *Analysis of pet and veterinary environmental isolates by REA*

	REA type									
	a	b	c	d	e	f	g	h	j	k
Pets	10	12	0	0	0	0	1	1	1	1
Environment	17	6	1	1	4	1	3	0	0	0
Cytotoxin	−	+	+	−	−	+	+	−	−	−
Total	27	18	1	1	4	1	4	1	1	1

respectively. These results are summarized in Table 4. There were no similarities between the REA patterns found among the isolates from the pets and the veterinary environmental isolates and those found among the isolates from patients and hospital environment.

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Table 5. *Analysis of all isolates by RFLP*

	RFLP type							
	I	II	III	IV	V	VI	VII	VIII
Human	1	26	7	0	0	2	1	0
Hospital environment	0	20	0	0	0	0	0	0
Pets	1	24	0	0	0	0	0	1
Veterinary environment	4	29	0	0	0	0	0	0
Cytotoxin	+	±	−			−	+	−
Total	6	99	7	0	0	2	1	1

RFLP profiles

The RFLP profiles of the environmental isolates from BVH generated with the enzyme *Hin*d III produced two patterns designated types I and II. Four isolates belonged to type I and four belonged to type II. The RFLP profiles of the environmental isolates from KSAH were only of the type II pattern. Three different RFLP patterns occurred among the pet isolates. One isolate from BVH was type I, 24 isolates from BVH and KSAH belonged to type II and 1 isolate from BVH was type VIII. Five RFLP types were found among the patient isolates. One isolate belonged to type I, 26 isolates belonged to type II, 7 isolates belonged to type III, 2 isolates belonged to type VI and 1 isolate belonged to type VII. All the isolates from the environment of SCGH belonged to RFLP type II. The results from the RFLP typing are shown in Table 5.

DISCUSSION

This investigation seeks to address two important questions. First, to what extent is environmental contamination with *C. difficile* related to human and animal colonization or infection and, second, does animal colonization or infection with *C. difficile* pose a potential risk to humans?

A variety of techniques has been used to study the epidemiology of *C. difficile* infection including bacteriocin and bacteriophage typing [12], protein profiles [13], serotyping [14], plasmid analysis [15] and immunoblotting [16]. REA of chromosomal DNA has been shown to be a useful and highly discriminating tool for epidemiological studies of *C. difficile* [17–19]. We have reported previously on a method of typing based on RFLPs of ribosomal RNA genes of *C. difficile* [11]; however, this method has not been evaluated further. In the present study, REA was compared to RFLP typing using two groups of isolates that were thought to be related epidemiologically; isolates from human patients and the hospital environment, and isolates from pets and the veterinary clinic environment. RFLP typing was not as discriminatory as REA when *Hin*d III digests of chromosomal DNA were used in both methods. In total, 34 different REA patterns could be distinguished among 116 strains of *C. difficile* studied, while only 6 RFLP types were demonstrated. A disadvantage of REA is that very complicated patterns are produced which require some time to analyse and RFLP typing may overcome this difficulty [11]. It may be possible to improve the discrimination of the RFLP method by using a different restriction enzyme to digest the chromosomal DNA.

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The pet and environmental isolates from the KSAH showed considerable similarity. Four different REA types were found in the environment at KSAH, with types a and b accounting for the majority. Four different REA types were also found among the pet isolates from KSAH and again these were predominantly types a and b. However, the proportions of both types were reversed in the two groups. The environmental isolates were predominantly the type a, non-cytotoxigenic strain (68%) whilst the pet isolates were predominantly the type b, cytotoxigenic strain (54·5%). There were some strains present in the environment at KSAH which we did not isolate from pets attending the clinic and vice versa; however, these were in the minority. Those strains isolated only from pets may have been due to these animals being colonized with *C. difficile* outside the veterinary environment prior to their admission. Alternatively they may have been present in the environment in small numbers and consequently not isolated. Those strains isolated only from the environment were found in very low numbers which may not have been sufficient to compete with the two more common strains present in high numbers. It has been reported that some non-cytotoxigenic strains of *C. difficile* are capable of eliminating cytotoxigenic strains from the human intestine [20]. It is likely that certain strains of *C. difficile*, whether cytotoxigenic or not, can be dominant over others. In contrast to the situation at KSAH, the most common environmental type at BVH, the type e, non-cytotoxigenic strain, was not found in pets from BVH. However, the sample size from BVH was small and this discrepancy may not be significant. One pet isolate from BVH had an REA type identical to one found at KSAH; however, the remaining pet isolates and the isolates obtained from the environments of the two veterinary clinics showed little similarity in their REA profiles.

The isolates from the environment at SCGH were mainly from the extended care area and were obtained from a ward which had contained patients with *C. difficile*-associated diarrhoea. The REA type found in this ward is common in the extended care wards of the hospital suggesting that the area is permanently colonized by this strain. This supports the hypothesis that contamination of the environment may be responsible for the ongoing problems with *C. difficile* in these areas. An identical strain was also isolated from the oncology ward, several hundred metres from the extended care area suggesting, possibly, spread of this strain between wards. The other environmental isolates were from the gastroenterology ward in the hospital where heavy environmental contamination has been recorded previously. A relationship between hospital environmental contamination and human infection has been implied for some years [21]; however, it is only with the advent of suitable typing schemes that this relationship has been proved [22].

There was a very good correlation between the REA patterns found in pets and the veterinary clinic environment, demonstrating the usefulness of this method of typing. This was not as obvious for the isolates from patients and the hospital environment with a much greater range of REA types detected. One reason for this could be that the isolates from pets and the veterinary clinic environments were collected over a period of several days whereas the hospital environment was sampled up to several months after isolates were recovered from patients.

The REA patterns among isolates of *C. difficile* from pets and veterinary clinics

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and from hospital patients and environment showed no overlap. It would be tempting to speculate, therefore, that there is little likelihood of *C. difficile* from pets infecting humans. However, this conclusion may still not be justified. Various investigators have reported isolates from different geographical locations, both within a country [12] and within an institution [23], having different typing patterns. Hence variations between strains of *C. difficile* from different institutions within a city is likely to occur. In addition, Borriello and colleagues [5] tested 4 isolates of *C. difficile* from pets (2 dogs, 1 cat and 1 duck) for pathogenicity in their hamster model of infection. Two cytotoxigenic strains were lethal while two non-cytotoxigenic strains, although able to colonize the hamster gastrointestinal tract, were not lethal. Over 50% of our pet isolates were cytotoxigenic indicating that they may be pathogenic given the opportunity.

The large number of types of *C. difficile* demonstrated by REA, particularly those associated with infected patients and their environment, supports the hypothesis that infection with *C. difficile* is more a host-related phenomenon rather than being related to the characteristics of the organism. Similar conclusions were reached by McFarland and colleagues [24] in a study of acquisition of *C. difficile* during hospitalization.

In conclusion, the most important prerequisite to colonization with *C. difficile* is exposure to the organism [22]. Until such time as it can be shown that patients predisposed to infection with *C. difficile* have been exposed to *C. difficile* from veterinary sources and not been infected, pets should still be regarded as a potential reservoir of infection.

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