BACTERIAL ECOLOGY

The distribution of *Clostridium difficile* in the environment of South Wales

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A large study of the distribution of *Clostridium difficile* in the environment of the Cardiff area of South Wales was performed with a methodology designed to maximise recovery. A total of 2580 samples was taken, with 184 (7.1%) yielding isolates. The highest yield for *C. difficile* was obtained from river waters, with 14 (87.5%) of 16 samples from four rivers positive, and from sea water samples with 7 (44%) of 15 positive from six beaches on the Bristol Channel. In addition, 7 (46.7%) of 15 samples of lake water were also positive. Twenty-two (21%) of 104 soil samples, taken from random sites in Cardiff, were positive, as were 20% of environmental samples from four Cardiff hospitals. *C. difficile* was also isolated from 50% of eight swimming pool waters examined and 1 (5.5%) of 18 of mains tap water. Carriage of *C. difficile* in 524 faecal samples of assorted farm animals was c. 1%, and was 10% in dogs and 2% in cats. In private residences, the organism was present in 12 (2.2%) of 550 samples. While 2.4% of 300 raw vegetable samples were positive, none of 107 assorted fish gut contents was. These findings indicate that *C. difficile* may be more widely distributed in the general environment, particularly water, than was previously thought.

Introduction

Clostridium difficile is a nosocomial pathogen responsible for many cases of antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis [1, 2]. Several studies have revealed the widespread presence of the organism in hospital wards and on the hands of nursing personnel [3–5], and infection is generally believed to be acquired nosocomially. Fewer studies have been directed at the environment outside hospitals and reports of *C. difficile* in the environment suggest either that its geographical distribution may be patchy, or that the different methodologies used were responsible for different isolation rates. For example, Hafiz from Sheffield, England [6] reported the organism in soil, sand and mud, and other workers in Korea [7] and Poland [8] have also found it in soil. However, one study in Perth, Western Australia [9], and a study of 20 random soil samples from Michigan, USA [4] both gave negative results.

Little is known about the prevalence of *C. difficile* in the domestic environment. Kim *et al.* [4] reported positive cultures from 12.2% of samples from floors and random surfaces in several rooms within a house occupied by a case of *C. difficile* diarrhoea. As a

control, the home of a healthy person was similarly sampled and no positive cultures were reported.

The total number of potential animal reservoirs of *C. difficile* is large [9] but no studies have proved that they are the source of infection in man. Riley *et al.* [10] showed that *C. difficile* was the most commonly isolated organism in a survey of patients with diarrhoea presenting to general practices in Western Australia. These findings suggested exposure to the organism in the community.

The distribution of *C. difficile* in the general environment of the UK is largely unknown. A large study of the distribution of *C. difficile* in the environment of the Cardiff area of South Wales is described. The aim was to demonstrate how healthy individuals in the community may be exposed to *C. difficile* and to promote an understanding of the ecology of this organism.

Materials and methods

Cultural methodology

A commercially-prepared basal medium for the isolation of *C. difficile* (Brazier's Medium, Lab M 160-A; Lab M, Bury) [11], containing a selective supplement of cefoxitin 8 mg/L and cycloserine 250 mg/L, was used. This medium incorporates the sodium salt of cholic acid to enhance the germination of spores;

prolonged incubation of samples on this medium was required to maximise the recovery of *C. difficile* from the environment. The medium was prepared according to the manufacturer's instructions and poured in either 5.5-cm contact plates (Bibby-Sterilin, Stone) for sampling directly from surfaces, or in 9-cm petri dishes for routine culture. All incubations were performed in an anaerobic chamber with an atmosphere of H₂ 10%, CO₂ 10%, N₂ 80% at 37°C. Cultures were incubated initially for 48 h and, if negative at this time, were re-incubated for up to a further 72 h. *C. difficile* was recognised by screening typical colonies under long-wave ultra-violet light for characteristic yellow-green fluorescence. These colonies were subcultured on non-selective Fastidious Anaerobe Agar (FAA) (Lab 90; Lab M) supplemented with horse blood 6% and incubated overnight. Confirmation of identification of *C. difficile* was by agglutination of colonies with the Microscreen *Clostridium difficile* latex reagent (Microgen Bioproducts Ltd, Camberley) and noting the characteristic odour of cultures.

Testing isolates for toxin A production

Environmental isolates of *C. difficile* were tested for their ability to produce toxin A (enterotoxin) by an enzyme immunoassay kit (TOX-A TEST, TechLab, Blacksburg, VA, USA). Isolates were inoculated on FAA supplemented with horse blood 6% and incubated anaerobically for 48 h. A heavy suspension of the growth of each isolate was made in the diluent buffer provided with the kit. The assay instructions were then followed as if performing the test with a faecal suspension. Positive and negative controls were included in each batch.

Water samples

Samples of untreated water from rivers, lakes, drainage channels and the sea, in a 20-mile radius of Cardiff, South Wales, were collected. Treated water samples from swimming pools and tap water from domestic supplies were also collected. Each sample was collected in 500-ml sterile bottles with collection techniques recommended for standard water microbiological examination. Cultures for *C. difficile* were made by the membrane filtration method, passing 100-ml volumes of each water sample through a 0.45- μ m pore size cellulose membrane filter (Millipore, Bedford). Membranes were placed on Brazier's selective agar, incubated anaerobically for up to 5 days, and the number of colonies was recorded as cfu/100-ml. For the detection of organisms present in lower numbers, enrichment cultures were performed on all samples by placing three membranes of 100-ml filtrate into cooked meat broth containing the selective agents. Each selective broth was incubated for 48 h before sub-culture on to selective agar. Direct cultures that were negative after 48 h were incubated for a further 72 h before being discarded.

Faeces of dogs and cats

Stool samples from 100 cats and 100 dogs were collected from two veterinary clinics and an animal shelter. Faeces were collected on a swab and placed immediately in charcoal transport medium. In the laboratory an equal volume of stool sample was added to absolute alcohol [12], mixed and left to stand for a minimum of 30 min. The deposit was inoculated on selective agar and plates were incubated anaerobically for 48 h. Any plates negative at 48 h were re-incubated for a further 3 days before being discarded.

Faeces of assorted farm animals and fish gut contents

Animals from a cattle and sheep market and 45 farms were sampled over a 7-month period. In total, 524 faecal samples were taken from 104 cattle, 100 sheep, 100 horses, 100 pigs and 120 mixed poultry. Swabs of the gut contents of 107 assorted sea and fresh water fish were taken at a fishery store. Culture for *C. difficile* was performed as described above for domestic animals.

Soil samples

Soil samples (104) from public parks, gardens, playgrounds and fields from sites in the Cardiff area were collected in sterile universal containers; 5 g of each sample were treated by alcohol shock [12] and c. 0.5 g of deposit was inoculated on the selective medium.

Environmental surface samples in hospitals, veterinary clinics, nursing homes and private residences

The distribution of *C. difficile* in hospitals, veterinary clinics, nursing homes and private residences was examined either by the use of direct contact plate cultures or moist swabs. Surfaces sampled included floors, tables, work surfaces, chairs, toilets, commodes, baths, carpets, windows, curtains and collections of dust. Swabs were plated out on selective medium and contact plates of 23.7 cm² in area were applied to the surfaces under test.

Raw vegetable samples

A selection of 300 raw vegetables on sale in retail premises in Cardiff were cultured for *C. difficile*. The unwashed surfaces of the vegetables were sampled by contact plates. Vegetables included in the survey were cucumber, onion, tomato, potato, mushroom, cabbage, lettuce, carrot and radish.

Results

A total of 2580 samples was taken yielding 184 isolates

and an overall recovery rate of 7.1% (Table 1). Toxin A was produced by 76% of the isolates.

Water samples

Fig. 1 shows the sampling points on the four rivers and the coastline of the Bristol Channel. There was a high yield from the four rivers tested with 14 (87.5%) of 16 samples positive. Direct cultures that were negative were also negative by enrichment culture. Counts ranged from 1 to 5-cfu/100-ml. Seven (43.7%) of 15 sea water samples from six sites on the Bristol Channel coastline of South Wales were positive with counts ranging from 3 to 6-cfu/100-ml, as were 7 (46.7%) of 15 samples of lake water with counts ranging from 1 to 5-cfu/100-ml and 7 (27%) of 26 samples of inland drainage. Of the treated waters, 4 of 8 swimming pool and 1 of 18 tap water (5.5%) were positive with counts ranging from 1 to 3-cfu/100-ml. Overall, 84.6% of isolates from water samples produced toxin A.

Faecal specimens

C. difficile was isolated from 10 of 100 samples of dog faeces and from two of 100 samples of cat faeces. Of these, two of 10 and one of two were positive for toxin A, respectively. Of 524 samples of faeces from an assortment of farm animals, the results by animal type were as follows: cattle none of 100, sheep one of 100, pigs none of 100, poultry two of 120, horse one of 100. *C. difficile* was not isolated from 107 swabs of the gut contents of an assortment of fresh and sea water fish.

Table 1. Summary results for *C. difficile* in all samples

Source	n	Number (%) samples positive	Number (%) isolates toxin A positive
Dogs	100	10(10)	2(20)
Cats	100	2(2)	1(50)
Horses	100	1(1)	1(100)
Cattle	104	0	0
Sheep	100	1(1)	1(100)
Pigs	100	0	0
Fish	107	0	0
Poultry	120	2(1.6)	2(100)
Soil	104	22(21.4)	9(40.9)
Hospitals	380	76(20)	72(94.7)
Nursing homes	275	6(2.2)	4(66.7)
Family houses	350	8(2.3)	3(37.5)
Student residences	200	4(2)	3(75)
Veterinary clinics	30	5(16.7)	1(20)
Water samples	110	40(36)	36(90)
Vegetables	300	7(2.3)	5(71.4)
Total	2580	184(7.1)	140(76)

Soil

C. difficile was isolated from 22 (21%) 104 of soil samples taken from public parks, gardens, playgrounds, etc. in the suburbs of Cardiff. Less than half these isolates (40.9%) produced toxin A.

Environmental surface samples

A total of 1235 environmental samples was examined from hospital wards, veterinary clinics, nursing homes and private residences. Of these, 20% of 380 samples

Fig. 1. ● Sampling sites of river and sea water for *C. difficile*.

from hospitals were positive for *C. difficile*, as were 16.7% of 30 samples from the veterinary clinics, 2.6% of 275 samples from nursing homes and 2.2% of 550 samples from private residences. Nearly all (94.7%) of the isolates from hospitals produced toxin A, compared with 37.5% and 47% of isolates from private residences and nursing homes, respectively.

Raw vegetables

Three hundred cultures taken from assorted raw vegetables yielded seven isolates of *C. difficile*. These isolates were from potato (two), onion (one), mushroom (one), carrot (one), radish (one) and cucumber (one). Five of seven isolates produced toxin A.

Discussion

The purpose of this study was to determine the prevalence of *C. difficile* in foodstuffs, water and the general environment in an attempt to demonstrate how the population at large may be exposed to this organism during normal everyday life. As a spore former, *C. difficile* can theoretically survive indefinitely in the environment and could, therefore, be ingested by man *via* various routes. For example, if it were shown to be a common member of the faecal flora of a livestock animal, it could contaminate meat at slaughter; or, if it was widely distributed in soil it could contaminate raw food such as fresh vegetables. Despite isolated reports of *C. difficile* in two sick piglets [13] and in one of 162 Nigerian cattle [14], there has not been a large survey of the prevalence of *C. difficile* in the gut of animals in the human food chain. The results of the present study suggest that consumption of beef, pork or lamb is not likely to be an important source of exposure to *C. difficile*, as it was not part of the faecal flora of 104 cattle, 100 pigs and 1% of sheep. Likewise, with only 1.6% of poultry faeces containing *C. difficile*, this food source also appears to have a low risk potential. The absence of *C. difficile* from the gut contents of fish, despite the organism being present in sea water, is in agreement with other studies of the clostridial flora of fish and fish products in which *C. difficile* has not been reported [15–16]. All these data suggest that *C. difficile* does not readily colonise the normal gut of farm animals or fish, probably due to competitive inhibition of *C. difficile* by the resident microbiota.

In addition to the potential contamination of the human food chain, it follows also that the urban population could be exposed to *C. difficile* *via* water if the spores of the organism were not removed by the sewage and water treatment system. The samples of potable water that were examined in the present study were included to test this theory, and although only

one sample of chlorinated tap water was positive, the high percentage of river (81.2%) and lake water (40%) samples positive for *C. difficile* demonstrate the potential of water supplies as a source of infection should treatment fail. Although the presence of other clostridial species in water supplies has been demonstrated in several studies [17, 18], *C. difficile* has not been reported. To our knowledge, the present study is the first to report the presence of *C. difficile* in untreated fresh water, sea water and chlorinated water, and represents a significant contribution to our understanding of the ecology of this organism.

C. difficile has been found in the sediments beneath marine and fresh water, however, and two isolates were reported in the sea water in Puget Sound off Washington State, USA [19]. Willis [17] sampled 43 waters from consumer premises in the Leeds area of northern England and found 88% to contain various clostridia in numbers ranging from 2 to 30/40-ml of water. He found some seasonal fluctuations as the bacterial counts were generally higher in the colder months than in the summer. This was believed to be due to the algal/zoogeal layer in the sand filtration beds being less developed in the winter months, hence the filtration of micro-organisms by the sand beds is less efficient. It is of interest to note, therefore, that our sampling too was carried out over the autumn and winter months. The presence of *C. perfringens* in a treated water supply is thought to indicate a failure in the filtration process, and Nankivell [20] proposed that the sand beds themselves were a reservoir of clostridia, a theory that Willis [17] validated by isolating 'anaerobic bacilli' from 50 (91%) of 55 sand filters. One of the rivers sampled in the present study had a sewage treatment plant sited on its western bank *c.* 0.5 mile from the coast. Two samples of river water taken upstream from this treatment plant were positive for *C. difficile* and two samples downstream from the plant were also positive, indicating contamination above the outlet of the treatment plant. The high percentage (84.6%) of toxigenic isolates found in water is of interest, and typing studies are needed to establish the relationship between these strains and those from hospitals. The presence of *C. difficile* in swimming pool water is a finding that perhaps could be explained by a carrier, such as a young baby, using the pool. At this stage it is not possible to determine if the demonstration of an unexpectedly high prevalence of *C. difficile* in water is peculiar to the environment of South Wales, or due to the improved methodology used in this study. It was not surprising that samples from the Bristol Channel, into which the four culture-positive rivers flow, were also positive for *C. difficile* – although this finding does indicate that spores can survive the salinity of sea water.

Another potentially significant finding was the isolation of *C. difficile* from 21% of soil samples and

2.4% of unwashed raw vegetables on sale in retail outlets. To our knowledge this is the first report to demonstrate that root vegetables are a potential source of *C. difficile* infection in man.

The results from the environmental samples taken from hospital sites and veterinary clinics were not unexpected, as nosocomial distribution of *C. difficile* has been well documented. Perhaps of more interest was the isolation of the organism from c. 2% of private residences, indicating that some people are exposed to *C. difficile* in their own home. The findings of *C. difficile* in the faeces of dogs and cats corroborates earlier reports [21, 22], although the percentage of positive cultures in dogs (10%) and cats (2%) was lower than reported in some studies [23].

The proportionally high detection rates of *C. difficile* in this study compared to earlier environmental studies may be attributable to the large nature of this study plus the use of a medium with spore germinating properties with prolonged incubation times to maximise recovery. On several occasions cultures that were negative after incubation for 48 h became positive after 72–120 h. Typing studies are needed to establish whether the strains of *C. difficile* in the environment are similar to those found in hospitals, as well as further sampling to determine whether the organism is equally abundant in other regions of Britain, particularly in water supplies, rivers and the sea.

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