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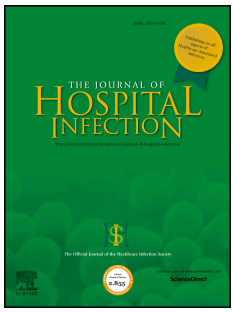
DOI:
10.1016/j.jhin.2017.05.014

Document Version
Peer reviewed version

Citation for published version (Harvard):
Manzoor, SE, McNulty, CAM, Nakiboneka-Ssenabulya, D, Lecky, DM, Hardy, K & Hawkey, P 2017, 'Investigation of community carriage rates of Clostridium difficile and Hungatella hathewayi in healthy volunteers from four regions of England', The Journal of hospital infection, vol. 97, no. 2, pp. 153-155. https://doi.org/10.1016/j.jhin.2017.05.014

Accepted Manuscript



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S.E. Manzoor, C.A.M. McNulty, D. Nakiboneka-Ssenabulya, D.M. Lecky, K.J. Hardy, P.M. Hawkey

PII: S0195-6701(17)30284-0

DOI: 10.1016/j.jhin.2017.05.014

Reference: YJHIN 5110

To appear in: *Journal of Hospital Infection*

Received Date: 14 March 2017

Accepted Date: 18 May 2017

Please cite this article as: Manzoor SE, McNulty CAM, Nakiboneka-Ssenabulya D, Lecky DM, Hardy KJ, Hawkey PM, Investigation of community carriage rates of *Clostridium difficile* and *Hungatella hathewayi* in healthy volunteers from four regions of England, *Journal of Hospital Infection* (2017), doi: 10.1016/j.jhin.2017.05.014.

Investigation of community carriage rates of *Clostridium difficile* and *Hungatella hathewayi* in healthy volunteers from four regions of England.

S.E. Manzoor[1], C.A.M. McNulty[2], D. Nakiboneka-Ssenabulya[2], D.M. Lecky[2], K.J. Hardy[1,3] and P.M. Hawkey[1,3].

Public Health Laboratory Birmingham, Heart of England Foundation Trust, Birmingham, B9 5SS[1].

Primary Care Unit, Public Health England, Gloucester, Gloucestershire Royal Hospital, Gloucester GL1 3NN[2].

Institute of Microbiology and Infection, University of Birmingham, B15 2TT, UK[3].

Key words: Clostridium difficile, Hungatella hathewayi, Community carriage

Summary

Faecal samples from 1365 healthy asymptomatic volunteers from four regions in England were screened for the presence of *Clostridium difficile* between December 2013 and July 2014. The carriage rate of *C. difficile* in healthy patients was 0.5%, which is lower than previously reported. This study demonstrates that the true community reservoir of *C. difficile* in the healthy UK population is very low and is, therefore, unlikely to be a reservoir for infections diagnosed in the hospital setting.

Introduction

Clostridium difficile infection (CDI) is primarily considered to be a hospital-associated infection most prominent amongst the elderly; it is a major problem in health care settings and nursing homes, causing a wide spectrum of illness ranging from mild diarrhoea to pseudomembranous colitis[1]. Use of broad spectrum antibiotics alters the commensal bacterial flora allowing *C. difficile* spores to germinate, colonise the gut and produce enterotoxins. Numerous studies have investigated the carriage rate of *C. difficile* in subjects in the community; however, they have primarily looked at elderly subjects, who are more prone to acquiring CDI due to increased hospital visits and antibiotic usage, or have looked at samples from subjects that have diarrhoea[2-4]. Two large studies looking at the incidence of infectious intestinal diseases in the community showed low incident rates of CDI, however, patient samples were only screened when there were symptoms of sickness or diarrhoea[5;6]. Other Clostridia species are present in faeces, one of which, *Clostridium hathewayi* was first isolated from human faeces and described as a new species in 2001 and then reclassified as *Hungatella hathewayi* in 2014 [7] Unlike *C. difficile*, there have been very few reported cases of human infection caused by *Hungatella hathewayi* [8] and little is known about the incidence

of carriage and the potential for infection. In this structured and stratified study we aimed to determine the carriage rate of *C. difficile* and observe the prevalence of *H. hathewayi* in healthy volunteers from four demographically diverse regions of the United Kingdom.

Methods

Subject recruitment

Randomly selected patients aged 18 plus registered with fourteen community healthcare practices within four National Health Service (NHS) Primary Care Trusts (PCTs) in England were invited by post to submit a faecal sample to "culture the different bacteria in our gut". The PCTs were non-randomly selected, each to represent a section of the population with a different ethnic composition: Newham (in London with one of the most mixed ethnic compositions in the UK), Heart of Birmingham (predominantly Asian population), Shropshire (rural, almost entirely white British population) and Southampton City (mixed ethnicity). Patient lists from the fourteen community healthcare practices were randomised and stratified according to antibiotic use in the previous twelve months, gender and ethnic groups; patients were then invited in order from these lists to participate. A total of 42,355 invitations were sent out to patients on the lists up to the end of July 2014, with 2865 accepting the invitation to participate. Between December 2013 and July 2014, faecal samples from 1365 invitees (44% men; 56% women), aged 18-97 with an average age of 58 (Aged 18-49: 30%; 50-65: 32%; 66-75: 23% and 15% were ≥75 years), were screened for the presence of *C. difficile* by culture. Antibiotic usage was obtained by questionnaire from each patient. This study was approved by The National Research Ethic committee, reference number 13/SW/0017.

C. difficile culture and identification

A pea-sized amount of stool, which had previously been frozen at -80°C, was treated with 0.5ml of 100% ethanol, homogenised using a vortex mixer and incubated at room temperature for 30 mins. One hundred microliters of the treated specimen was inoculated onto *C. difficile* ChomID media (BioMerieux). Plates were incubated at 37ºC under anaerobic conditions for 24hrs read and then reincubated for a further 24hrs (48hrs in total). Multiple colony picks of presumptive positive isolates, identified by colony colour and morphology, were purity plated onto Columbia blood agar and incubated for 48hrs at 37°C anaerobically. Each of these colonies were then identified from a single colony pick using MALDI-TOF (Bruker Daltonik MALDI Biotyper).

PCR-Ribotyping

All isolates confirmed as *C. difficile* were ribotyped. Crude DNA extracts of *C. difficile* were prepared using the chelex extraction method. For PCR-ribotyping two microliters of extracted DNA were added to 18µl PCR mastermix to give final concentrations of 1x Qiagen HotStar Taq Plus master mix (Qiagen) and 0.1pmol/µl each primer as described by Janezic *et al*[9] .

The reaction mixes were subjected to an initial polymerase activation step at 95°C for 5 min followed by 26 cycles of 95°C for 1 min, 55°C for 1.5 min and 72°C for 1 min followed by 95°C for 1 min, 55°C for 45 sec and a final elongation step of 72°C for 30 min. PCR products were diluted 1 in 20 in molecular grade water and 1µl aliquots of the DNA mixes were mixed with 9µl aliquots of HiDi formamide-LIZ600 size standard (Applied Biosystems) at 44:1 (vol/vol), denatured by heating to 95°C for 5 min, and transferred to a 3130xl genetic analyzer (Applied Biosystems) for PCR product size determination by capillary electrophoresis. PCR fragment profiles were analyzed using GeneMapper v4.0 software

(Applied Biosystems) and fragment sizes exported to BioNumerics v5.1 (Applied Maths NV, Sint-Martens-Latem, Belgium) for ribotype assignment by comparison with a library of known PCR fragment profiles.

Results

Based on colony colour there were 39/1365 (2.8%) presumptive positives after 24hrs incubation and 393/1365 (28.8%) after 48hrs, of which 5 did not grow on Columbia blood agar. Of the remaining 388 isolates, MALDI-TOF identified 360 isolates as members of the Clostridia family, with the exception of 1 isolate which was *Dichelobacter nodus* (Table I). The other 28 isolates could not be identified by MALDI-TOF. Seven isolates were *C. difficile*, (0.5% of total screened) with the majority of isolates (337/360) being *Hungatella hathewayi*. Ribotyping of the seven isolates identified two isolates with an indistinguishable ribotype, ribotype 026, which is a non-toxigenic strain. The five other isolates all had distinct ribotypes, four of which were common UK strains, ribotypes 002, 014, 015 and 020. The final isolate did not match any of the profiles in our locally held database, and is, therefore, not likely to be a commonly occurring UK ribotype. Six of the seven *C. difficile* isolates were from the Newham region of London, but all had distinct unrelated ribotypes. Four of the seven patients were in their 40s with the other three patients being in their 60s. In the preceding year, five of the seven patients had been treated with antibiotics, including amoxicillin, whilst the other two patients did not know if they had taken antibiotics.

Discussion

The point prevalence of *C. difficile*, within the healthy community population observed in this study was 7/1365 (0.51%), with only 5/1365 (0.37%) being toxigenic *C. difficile*. Similar low rates have also been observed in the UK infectious intestinal disease (IID) population

cohort studies that took place in 1999 and 2008/9. In 1999 only 6 cases of *C. difficile* were detected from 9,776 patients recruited [5] and none in 2008/09 [6]. The IID studies included healthy people from community healthcare practices, but unlike the current study samples were only sent when the patient had symptoms of diarrhoea or vomiting.

The main differences between this study and those that have shown a higher prevalence of *C. difficile* is the age of the study population (27% ≥70 years with a median age of 60 in our study compared to 100% ≥70 years with a median age of 81 in the Miyajima *et al*. study[2]) and the inclusion of asymptomatic healthy volunteers[2-4]. Previous community studies where the age of the study population was more elderly [2] have reported carriage rates of 4% (6/149)and 1.6% respectively[4]. In a study which screened samples from community patients with diarrhoea, which would include community cases of CDI, the rate was 2% [3]. *C. difficile* infection is much more common in the elderly population, due to the increased likelihood of antibiotic usage, hospital admissions, nursing home residence and loss of gut microbiota diversity[4].

ChromID media provides a rapid, sensitive medium for screening for *C. difficile*, due to the formation of black/grey colonies, allowing easy detection within 24 hours. However, similar to the study by Eckert and colleagues [10], the sensitivity of the media was improved by reading at 48hrs as three of the seven *C. difficile* isolates were detected, but did result in a much reduced specificity with a tenfold increase in the number of other Clostridium species being isolated (Table 1). This has been described in previous studies, where they also show a lower specificity in comparison to other media used to isolate *C. difficile*[10]. *Hungatella hathewayi* was the predominant organism isolated, with approximately 25% of the 1365 volunteers carrying it; accounting for 87% of the presumptive positives identified. There are a few reports of *Hungatella hathewayi* causing clinical disease, including bacteraemia[8], but no

studies of its prevalence in the faecal flora. Whilst it rarely causes disease it appears to be a common faecal flora commensal.

Conclusion

This study shows that the prevalence of *C. difficile* carriage in the asymptomatic healthy population is very low. Previous studies have shown higher carriage rates but this is probably due to sample groups being comprised of only elderly volunteers or samples from symptomatic patients. This suggests that the likelihood of the healthy community being a reservoir for infection is low unless the individual has been on a course of antibiotics or had a recent admission to hospital, predisposing them to increased chance of *C. difficile* colonising the gut.

Acknowledgements

The sample collection part of this work was funded by the Department of Health Policy Research programme project number 041/0038S.

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Table I. MALDI-TOF identification of the 388 positive isolates isolated from ChromID media after 24 and 48 hours incubation.

Organism identified	**Initial No of isolates after 24hr**	**Further No of isolates after 48hr**	**Total number of isolates 48hr**
Clostridium difficile	4	3	7
Clostridium baratii	0	1	1
Clostridium disporicum	2	3	5
Hungatella hathewayi	19	318	337
Clostridium perfringens	3	0	3
Clostridium tertium	0	3	3
Clostridium species	2	1	3
Dichelobacter nodosus	1	0	1
No peaks or Unidentifiable	7	21	28
Total	**38**	**350**	**388**