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ORIGINAL ARTICLE

Wild mice in and around the city of Utrecht, the Netherlands, are carriers of *Clostridium difficile* but not ESBL-producing *Enterobacteriaceae*, *Salmonella* spp. or MRSA

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Significance and Impact of the Study: This study shows that mice in buildings can carry *Clostridium difficile* ribotypes that are associated with clinical disease in humans. Whether the mice are the source or whether they picked up these bacteria from the human environment has not been investigated. Either way, mouse droppings in the indoor environment are a hazard for transmission of *C. difficile* to humans.

Keywords

Apodemus sylvaticus, *Clostridioides difficile*, *Clostridium difficile*, house mouse, *Mus musculus*, wood mouse.

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Abstract

Mice in buildings are a hygiene hazard because they harbour several zoonoses and animal diseases. The aim of this study was to gather information on specific bacteria in house mice caught in the urban environment. Mice caught in snap traps during pest control activities were collected in and around the city of Utrecht, the Netherlands, during May-June 2014, October-November 2015 and September-November 2016. The gut contents were analysed for ESBL/AmpC-producing *Enterobacteriaceae*, *Salmonella* spp., and *Clostridium difficile* and the buccal cavities were swabbed for methicillin-resistant *S. aureus* (MRSA). In total, 109 house mice (*Mus musculus*) and 22 wood mice (*Apodemus sylvaticus*) were examined. One mouse was found positive for *Enterobacter* spp. *Salmonella* spp. and MRSA were not found. Of $n = 80$ mice, 35·0% carried *C. difficile* (ribotypes in descending order of frequency: 014/020, 258, 002, 005, 013, 056, 081 and two unknown ribotypes). In conclusion, mouse droppings are a hazard for transmission of *C. difficile* to humans and their environment.

Introduction

Globally, the house mouse (*Mus musculus*) is the most widely spread mammal apart from Man (Global Invasive Species Database, Last accessed: 13 January 2017). Classed as a commensal, the species lives close to humans and their kept animals, benefitting from the food and shelter available. House mice in buildings are a hygiene hazard because they harbour several zoonoses and animal diseases, such as *Salmonella* spp. (Davies and Wray 1995), *Campylobacter* spp. (Meerburg *et al.* 2006), *Leptospira* spp. (Williams *et al.* 2018), *Neospora caninum* and *Toxoplasma gondii* (Meerburg *et al.* 2012). Although laboratory mice are commonly used as disease models for infection studies with *Clostridium difficile* (Yang *et al.* 2015) (*Clostridioides difficile* proposed as new name (Lawson *et al.* 2016)) and *Staphylococcus aureus* (Schulz *et al.* 2017), studies on carriage of these bacteria by wild house mice are limited and mostly carried out in rural or farm populations (Mrochen *et al.* 2017). Information on the possible carriage of antibiotic-resistant bacteria by mice is also scarce (Williams *et al.* 2018).

Since rodent infestations in homes appear to be relatively common (Lipman and Burt 2017), more knowledge of specific zoonotic hazards, including antibiotic-resistant bacteria would be valuable in assessing the hazards

associated with mice and their droppings in the human environment. In this study, therefore, we aimed to obtain more information on specific groups of bacteria in urban mice. The aim of this study was to investigate the prevalence of ESBL/AmpC producing *Enterobacteriaceae*, *Salmonella* spp., methicillin-resistant *S. aureus* (MRSA) and *C. difficile* in mice caught during pest control operations in and around the city of Utrecht.

Results and discussion

In total, 131 mice were examined. The majority were house mice (*Mus musculus*) and the rest (16·8%) were wood mice (*Apodemus sylvaticus*). An overview of the species and sex of the mice examined is presented in Table 1. None of the mice showed specific pathological symptoms but a few had begun to show signs of autolysis due to the length of time since death.

The results for the bacteriological analyses during the three sampling periods are presented in Table 2. The frequencies for *C. difficile* positive mice caught in urban and rural settings and for house mice vs. wood mice are presented in Table 3. The prevalence of *C. difficile* was higher in urban mice than in rural mice and higher in house mice than wood mice, although these differences were not significant ($P > 0·05$). For *C. difficile* positive mice of both species the ratio of males to females was about equal. The ribotypes (RT) identified by PCR analysis are presented in Table 4. RT014/020 was most often isolated and was found in urban and rural samples. Most of the *C. difficile* isolates originated from house mice; only two wood mice carried *C. difficile* (RT014 and RT258) and these were both caught in urban locations. On two occasions, two *C. difficile* isolates were found to contain unidentified but identical ribotypes (Table 4). In one of these cases, the mice originated from the same urban location on the same day. In the second case, the mice originated from an urban location and a rural location 40 km apart.

ESBL/AmpC-producing *Enterobacteriaceae* were isolated from only 1 of 42 house mice and 0 of 9 wood mice in this study and that sample concerned *Enterobacter* spp., which is intrinsically AmpC-producing. Little data are available in the literature on antibiotic-resistant *Enterobacteriaceae* carried by mice. In a study carried out in Ivory Coast, 2 of the 4 nests of house mice were found positive for ESBL-producing *Citrobacter freundii* or *Enterobacter asburiae* in a village where the human population had a high percentage of faecal carriage of multiresistant ESBL-producing *Escherichia coli* (Albrechtova *et al.* 2014).

Salmonella spp. were not isolated from the intestines of the 51 mice examined in this study. A recent study in New York city found 13 of 416 (3·1%) mice positive for *Salmonella* spp. (Williams *et al.* 2018) and a study of urban mice in India found 11/109 (10·1%) positive (Singh *et al.* 1980). The cause of this difference in outcome may be the smaller sample size in the present study or methodological differences; e.g. the New York study used PCR analysis for the *invA* gene from anal swabs, whereas the present study used culture methods on the faecal pellet. However, it is also possible that the lack of *Salmonella* in Utrecht mice is a reflection of the *Salmonella* status of the human population. It has been suggested that *Salmonella* prevalence in mice in farm buildings is a reflection of the prevalence in the farm animals kept in the same building. This idea originated from the finding that on poultry farms where the prevalence of *Salmonella* spp. is very low, there is also an absence or very low prevalence in farm mice (Backhans *et al.* 2013). Similarly, it could be that the mice in Utrecht have a similar status to the human population, whose buildings they inhabit.

Table 1 Overview of species and sex of mice caught during pest control activities in buildings in and around the city of Utrecht

Species	Sex	2014	2015	2016	Total
Mus musculus (house mouse)	Male	4	25	22	109
	Female	5	14	18	
	Undetermined (juvenile)	7	14	–*	
Apodemus sylvaticus (wood mouse)	Male	1	–	4	22
	Female	1	–	9	
	Undetermined (juvenile)	7	–	–	
Total		25	53	53	131

*None of this category was available for analysis.

Methicillin-resistant *S. aureus* was not isolated from any of the 109 house mice and 22 wood mice examined. Although several species of wild rodents and shrews are naturally colonized with *S. aureus*, it appears that previously only one instance of *S. aureus* (in Germany) and no instances of MRSA in house mice or wood mice have been recorded (Mrochen *et al.* 2017). Our findings are in accordance with these results. However, in a study of free ranging rodents and shrews in southern Spain 2/29 (6·9%) wood mice were found to be positive for MRSA carrying the *mecC* gene (Gómez *et al.* 2014).

Clostridium difficile ribotypes associated with *C. difficile* infection (CDI) were isolated from more than a third of the mice examined (Table 2). This proportion is much higher than was found in a recent study of mice in New York city, where 18 of 416 (4·3%) were found positive for *C. difficile* (Williams *et al.* 2018). The difference in prevalence may be caused by methodological differences. The New York prevalence was based on PCR analysis of

Table 2 Results of bacteriological analyses of mice caught during pest control activities in buildings in and around the city of Utrecht

	2014	2015	2016	Overall
ESBL/AmpC *Enterobacteriaceae*	0/25	1/26 (3·8%)	n.d.*	1/51 (2·0%)
Salmonella spp.	0/25	0/26	n.d.	0/51
MRSA	0/25	0/53	0/53	0/131
Clostridium difficile	n.d.	10/27 (37·0%)	18/53 (34·0%)	28/80 (35·0%)
Number of mice examined	25	53	53	131

*Not done.

Table 3 Prevalence of *Clostridium difficile* in the intestinal contents of mice caught during pest control operations in and around the city of Utrecht. Differences in prevalence between locations (mice caught in urban *vs* rural areas) and between species (house mice *vs* wood mice) are not significant ($P > 0·05$)

Location	*n*	*C. difficile* positive	95% CI	Species	*n*	*C. difficile* positive	95% CI
Urban	53	39·6%	26·5–52·8%	House mice	67	38·8%	27·1–50·5%
Rural	27	25·9%	9·4–42·5%	Wood mice	13	15·4%	−4·2–35·0%
Total	80	35·0%					

Table 4 *Clostridium difficile* ribotypes confirmed in samples of the intestinal contents of mice caught during pest control activities over 2 years and in urban and rural settings in and around the city of Utrecht

	2015		2016		
Ribotype (RT)	Urban	Rural	Urban	Rural	Overall
002	2	n.d.*	1	–†	3
005	1	n.d.	–	–	1
013	1	n.d.	–	–	1
014/020	6	n.d.	1	7	14
056	–	n.d.	1	–	1
081	1	n.d.	–	–	1
258	–	n.d.	4	–	4
Unknown identical ribotype RTx	–	n.d.	1	1	2
Unknown identical ribotype RTy	–	n.d.	2	–	2

*Not done.
†Samples were negative for *C. difficile*.

anal swabs, whereas the present study cultured *C. difficile* from intestinal contents. The mice positive for *C. difficile* originated from several different areas of Utrecht. Nine different ribotypes were identified, six of which have been associated with CDI in humans. Most often found (Table 4) was RT014/020 (also referred to as RT014/020/295), which was isolated in 12-17% of CDI patients diagnosed in the Netherlands between 2009 and 2015 (van Dorp *et al.* 2017), 15% of patients diagnosed between May 2016-May 2017 (Leiden University Medical Center 2017) and from patients with suspected CDI in Qatar (Al-Thani *et al.* 2014). RT014/020 has also been isolated from brown rats in Canada (Himsworth *et al.* 2014), and from cattle in Belgium (Rodriguez *et al.* 2017) and Slovenia (Bandelj *et al.* 2016). RT014 has also been isolated from 3/90 (3·3%) of diarrhoeic pet dogs in Spain (Andrés-Lasheras *et al.* 2018) and from 6/437 (1·4%) of dogs and 4/403 (1·0%) of cats tested in Germany (Rabold *et al.* 2018). As cats (and some dogs) are prone to catching mice, it is possible that there could be transmission of *C. difficile* between the species, all of which live in the indoor human environment. The second most frequently isolated ribotype was 258, which was the most commonly isolated ribotype in 6 out of 1532 patients (7·6%) with suspected CDI in Qatar (Al-Thani *et al.* 2014). The third most frequently identified ribotype was 002, which was isolated from 8% of Dutch CDI patients in 2016–2017 and is frequently found in patients in England, the Czech Republic, Lebanon, the USA and Korea (Wilcox *et al.* 2012; Kim *et al.* 2013; Tickler *et al.* 2014; Beran *et al.* 2017; Leiden University Medical Center, 2017; Berger *et al.* 2018). RT002 has also been isolated from brown rats in Canada (Himsworth *et al.* 2014) and calves' faeces in Slovenia (Bandelj *et al.* 2016). RT005 has earlier been isolated from mice in New York city (Williams *et al.* 2018), mice, rats and pigeons on pig farms in north-eastern Spain (Andres-Lasheras *et al.* 2017), brown rats (Himsworth *et al.* 2014) and cattle (Bandelj *et al.* 2016). It has also been found in patients suspected for CDI, as have RT056 and RT081 (Al-Thani *et al.* 2014). No references to RT013 could be found in the literature.

In an earlier study, we showed that mice on a pig farm carried ribotype 078, which is associated with piglet diarrhoea and human disease (Burt *et al.* 2012). None of the ribotypes identified in these two studies overlap.

However, the studies were conducted 4-6 years apart and mice have small territories — just large enough to procure sufficient food — so the lack of overlap in ribotypes is not unreasonable.

Our findings suggest that wild mice may function as a reservoir for *C. difficile* ribotypes associated with CDI in humans and contribute to the spread of *C. difficile* in the human environment. In a recent online survey just over 60% of respondents reported rodent sightings in or around the home during the previous year (Lipman and Burt 2017). This, in combination with the fact that mice produce 70-100 droppings per day (Aulicky *et al.* 2015), would indicate that there is potential for transmission from mice to the human environment. To test this theory, a larger scale analysis of mouse droppings in residential homes could be carried out to compare the ribotypes with those found in humans living in those houses. Additionally, patients with community-acquired CDI could be asked if they have mice and/or pets in the home and, if so, the mouse droppings as well as those of any pets should be examined.

Limitations of the study

This study was carried out in and around a city with mice available from pest control operations. Since mice generally have a small territory it is possible that the mice sampled are not representative for populations in other parts of the country. The fact that some samples (15%) were frozen prior to analysis should not have influenced the harvesting of MRSA or *C. difficile*, but may have damaged some *Enterobacteriaceae* present. This may have contributed to the lack of *Enterobacteriaceae* found.

In conclusion, this study shows that mice caught in and around the city of Utrecht are not important carriers of ESBL/AmpC-producing *Enterobacteriaceae*, *Salmonella* spp. or MRSA but that more than a third of the mice carried in their intestines *C. difficile* ribotypes known to cause clinical disease in humans. A mouse infestation or direct contact with mouse droppings may therefore be considered a risk factor for transmission of *C. difficile* to the indoor environment and to humans.

Materials and methods

Mice caught in snap traps in the course of pest control were collected from pest control companies and householders in and around the city of Utrecht, the Netherlands, during May-June 2014, October-November 2015 and September-November 2016. Mice were transferred directly to the laboratory for analysis in a refrigerated box or, if handed in shortly before the weekend, were stored at −80°C for up to three days before analysis.

Sample preparation

The species and sex of the mice were recorded and the origin was recorded as urban or rural, based on the locations where they were caught. All mice were sampled for MRSA by buccal swab and the faecal pellets were removed by section. In the 2014 study period, the intestinal contents of all mice were analysed for ESBL/AmpC-producing *Enterobacteriaceae* and *Salmonella* spp. In the 2015 research period the intestinal contents were analysed alternately for either (i) ESBL/AmpC-producing *Enterobacteriaceae* and *Salmonella* spp. or (ii) *C. difficile*. It was not possible to analyse the gut contents for three groups of bacteria because only one or at most two faecal pellets per mouse were available. In the 2016 research period, all mice were sampled for MRSA and *C. difficile* only.

Sample analysis

ESBL/AmpC-producing Enterobacteriaceae

Detection of ESBL/AmpC-producing *Enterobacteriaceae* was carried out using the method of Dierikx *et al.* (2012) with selective enrichment in Luria Bertani broth and plating out onto MacConkey agar (MAC, Oxoid, Basingstoke, UK) and in parallel on Tryptone Bile X-glucuronide agar (TBX, Oxoid), all containing 1 mg l^{-1} cefotaxime. For MAC: sharply defined pink colonies were counted as suspected *E. coli*; slimy pink colonies were counted as suspected *Klebsiella*; other pink colonies were counted as suspected *Enterobacter*. For TBX green colonies were scored as *E. coli*. All suspected colonies were streaked onto Tryptone Soya Agar (TSA, Oxoid) and tested for the indole and oxidase reactions.

For suspected ESBL/AmpC-producers susceptibility to five antibiotics was determined with the BD Sensi Disk® method. For this a fresh culture on TSA was adjusted to a turbidity of 0·5 McFarland in 0·85% physiological saline solution and spread onto a Müller-Hinton agar plate (Oxoid) using a sterile swab. Five discs containing antibiotics (cefotaxime, cefotaxime + clavulanic acid, ceftazidime, ceftazidime + clavulanic acid and cefoxitin) were dispensed by the Sensi Disk® apparatus and incubation was 18 h at 37°C. Inhibition zones were measured with a digital calliper and the phenotype (ESBL- or AmpC-producing) was determined by reference to breakpoints according to the CLSI standards (CLSI, 2010). *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as controls.

Salmonella spp.

Detection of *Salmonella* spp. in the faecal pellets was carried out according to the method described by Kilonzo *et al.* (Kilonzo *et al.* 2013) with one modification. Each

faecal sample was pre-enriched overnight in 10 ml buffered peptone water (BPW) (Biokar Diagnostics, Pantin, France) at 37°C followed by placing three droplets onto modified semi-solid Rappaport-Vassiliadis agar (Oxoid) and incubation for 24–-48 h at 41·5°C. A white swarm was a typical reaction for *Salmonella* spp. Further selective plating of suspect colonies was carried out on both xylose lysine deoxycholate agar (Oxoid) and brilliant green agar (Oxoid) for 24 h at 37°C.

MRSA

Detection of MRSA in the buccal swabs was according to the method of Graveland *et al.* by selective enrichments and plating out onto Brilliance 2 MRSA agar (Oxoid) (Graveland *et al.* 2009). Denim blue colonies on agar typical for MRSA were streaked onto TSA, incubated overnight at 37°C and subjected to coagulase and catalase tests.

Clostridium difficile

Detection of *C. difficile* in faecal pellets was according to the method described by Hopman *et al.* (2011) with two modifications. *C. difficile* enrichment broth (CDEB, Mediaproducts, Groningen, the Netherlands) was used for the enrichment phase. Also, after transferral of a 2 ml portion of sample in enrichment broth to a sterile tube for the ethanol shock the remainder of the sample was incubated a further 5 days. Samples were classed as positive for *C. difficile*, if one of the portions produced colonies of Gram-positive rods with a characteristic odour of horse manure and typical morphology (grey colonies with an uneven edge). Isolates were further identified and characterized at the National Reference Laboratory at Leiden, The Netherlands by capillary ribotyping (Fawley *et al.* 2015).

Statistical analysis

The results of the analyses for *C. difficile* were compared for mice caught in urban and rural areas and for house mice compared to wood mice using the *z*-test on the Epitools website http://epitools.ausvet.com.au/content.php?page=StatisticsHome.

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Conflict of Interest

No conflict of interest declared.

Ethics

No ethical permissions were required since the mice were killed during standard pest control activities and the cadavers were otherwise destined for disposal.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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