Microbiological testing for the presence of MRSA and *Clostridium difficile* in urban and rural mice

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Abstract
The house mouse (*Mus musculus*) is a species living in close proximity to humans and is capable of carrying and transmitting various pathogens. This study aims to determine the presence of MRSA and *Clostridium difficile* in mice and to investigate whether there is a difference in presence between urban and rural mouse. Mice (n=53), urban (n=26) and rural (n=27), were collected from pest control companies and oral and faecal swabs were taken to investigate the presence of MRSA and *C. difficile* respectively. No MRSA was found. A total of 21 (40%) samples were presumptive for *C. difficile.* From the urban mice 13 (50%) samples were presumptive positive and from the rural mice 8 (30%) were presumptive positive. No conclusion could be drawn on MRSA in urban and rural mice. There seems to be no difference in the presence of *C. difficile* between urban and rural mice

Index

Introduction

Rodentia is an abundant and diverse order of the class Mammalia, comprising about 43% of the mammalian species. Except for Antarctica rodents are found on all continents.[1]
The house mouse (*Mus musculus*) is a species of the genus *Mus* (subgenus *Mus*). It is worldwide spread and can be divided into several subspecies with different geographical locations. *Mus musculus domesticus*, known as the western European house mouse, is the subspecies found here in western Europe.[2] House mice are known as a commensal rodent because, and different from other species in the *Mus* genus, house mice primarily live in close proximity to humans (and their farm animals and pets).[2] Mice reproduction is short. Gestation time is approximately 3 weeks and litter sizes vary from 4 to 9 pups. The baby mice reach sexual maturity at 6 to 8 weeks[2] so mice populations have the ability to grow exponentially in a short time. Besides the more obvious damage such as pre-harvest damage, food spoilage and structural damage by gnawing, rodents can also pose a threat to public and veterinary health.[1] They can be a source of various pathogens which they can transmit directly (by biting, faeces/urine) and indirectly (by hosting arthropod vectors) to humans.[1] Several studies found evidence of the involvement of rodents in the dissemination of pathogens on farms. *Clostridium. difficile* was found in house mice on a Dutch pig farm.[3] On agricultural farms in California (USA) *Salmonella* was found in deer mice (*Peromyscus maniculatus*).[4] This may cause a health risk in the food chain (e.g. contaminated meat). Also pathogens such as Hantaviruses and *Campylobacter* have been found in mice.[1]

Methicillin-resistant *Staphylococcus aureus* (MRSA) acquired resistance to nearly all β-lactam antibiotics through the acquisition of a *mec* gene (mostly *mec*A).[5] This can cause a simple infection to become a complicated and difficult to treat infection. Transmission of *S. aureus* is primarily through physical contact e.g. contaminated faeces or dust.[5] Infection with MRSA can cause a range of problems from skin infections to sepsis. In the Netherlands MRSA has a high prevalence on pig farms. Black rats captured on pig farms also showed to be positive for MRSA. So rats, and possibly also other rodents, might play a role in the spread of MRSA, causing a public health risk.[6] On MRSA in mice currently very few data are available.

Clostridium difficile is a Gram-positive bacterium that is ubiquitous in the environment. By forming spores it can persist on inanimate surfaces several months. It is one of the most important nosocomial (hospital-acquired) pathogens in humans.[7] Symptoms include diarrhea and pseudomembranous colitis. Although *C. difficile* has not been confirmed as a zoonotic agent, human epidemic PCR-ribotypes (PCR-ribotype 027, PCR-ribotype 078) have been found in pets, food animals, horses and wild animals[7] and research on a Dutch pig farm showed captured mice to be contaminated with *C. difficile*[3] showing that these animals might be a potential risk for transmission.

The World Health Organization reports the parasitic load of rats to be much higher in rural than urban areas, which is explained by the lower population density in modern urban areas (through pest control programs and better sanitation) and on the other hand the livestock on farms which drive zoonotic cycles by contaminating soil and water with infected excreta hereby maintaining or initiating rodent reservoirs.[8] This may also be the case for mice populations.

The aim of this study is to test mice caught by pest control companies by performing isolation protocols for two specific bacteria: MRSA and *Clostridium difficile* and to compare two groups, urban and rural mice, to see whether there is a difference in the presence of MRSA and *C. difficile* in urban and rural mice.

Materials and Methods

Materials

Sample collection

Most mice were supplied by Rentokil Pest Control and Anticimex as a byproduct of their pest control activities. ABM Ongediertebestrijding supplied a few too. Also some mice caught in people's homes were brought in by individuals. Drop boxes cooled with ice packs were set up in the center in the city of Utrecht (Drift 6) and at the Uithof (Educatorium) in Utrecht. When caught, the mice were dropped into the drop boxes by Rentokil pest controllers where they were collected and then stored at -80°C. Also, a drop box was given to our contact person at Anticimex (Houten). Mice were handed over at an arranged time and place as soon as possible after being trapped and then stored at -80°C. Preferably, the mice would be killed without the use of any rodenticide. The methods of extermination used by the pest control companies consisted of asphyxiation by carbon dioxide, electrocution, snap traps and glue boards. Mice brought in by individuals were partly caught with snap traps and once by a cat, the other part is unknown. Although contact with rodenticides can never be ruled out completely, in a few cases it was clear the mice had been in contact with some kind of rodenticide (green paws/snout/intestines content). The mice were divided into two groups according to the location where they were caught:

- urban mouse (UM): large cities with a high building density (such as the city of Utrecht).
- Rural mouse (RM): smaller cities/villages with lower building density and a rural character (woods and pastures e.d.)

Methods

The mice were researched for two specific bacteria: *C. difficile* and MRSA.

MRSA

A swab of the nostrils and mouth of the mice was taken. After being plated straight onto Brilliance MRSA 2 agar plates (ready-made, Oxoid) the swab were inoculated into Müller-Hinton broth with 6,5% NaCl (MH). Plates and MH broth were incubated overnight at 37°C. The next day 1 ml of MH broth was transferred to 9 ml of Phenol Red Mannitol Broth (PHMB) with 135 µl aztreonam (AZT) stock (AZT dissolved in DMSO; concentration: 5 mg/ml) and 375 µl oxacillin (OXA) stock (OXA dissolved in MilliQ water; concentration 0,1 mg/ml). AZT and OXA were added to PHMB on the day of use. The PHMB was incubated overnight at 37°C. Also, the straight plated Brilliance MRSA 2 plates were read. The next day PHMB was streaked on Brilliance MRSA 2 agar which was incubated overnight at 37°C. Denim-blue colonies are presumptive for MRSA and were streaked on Tryptone Soya Agar (TSA) and incubated overnight at 37°C. A catalase and coagulase test were performed from the TSA culture.

Catalase slide method: a small amount of culture was suspended in a standard saline solution on a microscope slide and then a drop of hydrogen peroxide was added. The formation of gas bubbles was considered positive for the presence of catalase.

Coagulase tube test: to 0,5 ml rabbit coagulase plasma 0,1 ml of broth culture (culture inoculated in Brain Heart Infusion (BHI) broth and incubated overnight at 37°C) was added in a tube. This was incubated at 37°C and after 4 hours checked for gelling of the plasma, which remains in place even after inverting the tube. When gelling did not occur after 4 hours at 37°C the tube was kept overnight at room temperature and then checked again for gelling. Samples were noted as presumptive positive for MRSA when both the catalase test and coagulase test were positive. Conformation will be done at a later date by PCR for the *mecA* gene and *femA* gene.

Clostridium difficile
To obtain faecal samples, the mice were pinned down through the paws and cut open along the abdominal cavity. The intestines were taken out after which the contents were squeezed out. This was inoculated into 5 ml of *C. difficile* moxalactam norfloxacin (CDMN) broth (ready-made, Mediaproducts) and then incubated at 37°C for 48h anaerobically. Then the culture broth was homogenized and 2 ml of the broth was transferred to a sterile tube together with 2 ml 96% EtOH and mixed well. The remainder of the culture broth was further incubated for 5 days at 37°C anaerobically. The EtOH/culture broth mix was left at room temperature for at least 60 minutes. The tube was then centrifuged for 10 minutes at 4000 x g. The sediment was plated onto *C. difficile* agar (CLO) plates (ready-made, Biomérieux) and incubated at 37°C for 48h anaerobically. CLO plates were read; colonies of Gram-positive rods (and spore forming resembling tennis rackets) with a characteristic horse manure odour and typical morphology (swarming, rough, non-haemolytic) are presumptive for *C. difficile*. After incubation for another 5 days 2 ml of the remaining culture broth was taken and again processed as described above. Samples were considered positive when the first and/or second processing were characteristic for *C. difficile*.

Also, all presumptive positive isolates were transferred to a buffered peptone water (BPW) + 24% glycerol solution and stored at -80°C (suspected MRSA positive colony taken from TSA plate; *C. difficile* from CLO plate). One suspected *C. difficile* positive colony was also transferred to a TSA slant for ribotyping at UMC Leiden[9].

Several other samples were taken for other research purposes. The tails were cut off and stored in 99,8% ethanol (EtOH) at room temperature for research on rodenticide resistance (through mutations in the *Vkorc1* gene).[10] Heart, spleen and liver were taken out and stored at -80°C. Also, species and sex were determined.

Results

A total of 53 mice were tested for MRSA and *C. difficile* of which 36 were supplied by the pest control companies together, 10 mice were handed in by individuals and 7 were already available from earlier collections. From those 53 mice 26 (49%) were classified as UM and 27 (51%) were classified as RM. The results are shown in Table 1.

Table 1 Results of the *C. difficile* and MRSA analysis of the mice collected plus the determined species and gender and the location where the mice were caught.

Number of sample	Species	Gender	Location	MRSA	*C. difficile*
UM54	*Mus musculus*	Male	3584 ED	Neg	Neg
UM55	*Mus musculus*	Male	3512 HH	Neg	Neg
UM56	*Mus musculus*	Female	3512 BS	Neg	Neg
UM57	*Mus musculus*	Male	3512 BL	Neg	Pos
UM58	*Mus musculus*	Female	3584 CE	Neg	Neg
UM59*	*Mus musculus*	Female	3584 CE	Neg	Neg
UM60*	*Mus musculus*	Male	3584 CE	Neg	Pos
UM61	*Mus musculus*	Male	3512 BS	Neg	Pos
UM62	*Mus musculus*	Male	3512 BS	Neg	Pos
UM63	*Mus musculus*	Male	3584 CE	Neg	Neg
UM65	*Mus musculus*	Female	3512 BS	Neg	Neg
UM66	*Mus musculus*	Male	3512 BS	Neg	Neg
UM67	*Mus musculus*	Male	3512 BS	Neg	Pos
UM68	*Mus musculus*	Female	1118 AX	Neg	Pos
UM69	*Apodemus sylvaticus*	Male	1118 AX	Neg	Pos
UM70	*Mus musculus*	Female	1118 AX	Neg	Pos
UM71	*Apodemus sylvaticus*	Female	1118 AX	Neg	Pos
UM72	*Mus musculus*	Male	1118 AX	Neg	Neg
UM73	*Mus musculus*	Male	1118 AX	Neg	Neg
UM74	*Mus musculus*	Female	1118 AX	Neg	Pos
UM75	*Mus musculus*	Male	3584 EE	Neg	Neg
UM76**	*Mus musculus*	Female	3512 BS	Neg	Pos
UM77	*Mus musculus*	Male	3584 ED	Neg	Neg
UM78	*Mus musculus*	Female	3512 BS	Neg	Pos
UM79	*Mus musculus*	Male	3513 BB	Neg	Pos
UM80	*Mus musculus*	Male	3511 BR	Neg	Neg
RM2*/***	*Mus musculus*	Male	3831 VW	-	Neg
RM3	*Mus musculus*	Female	4191 GV	Neg	Pos
RM4	*Mus musculus*	Female	4191 GV	Neg	Neg
RM5	*Mus musculus*	Male	4191 GV	Neg	Neg
RM6	*Apodemus sylvaticus*	Male	3947 BD	Neg	Neg
RM7	*Apodemus sylvaticus*	Female	3947 BD	Neg	Neg
RM8	*Apodemus sylvaticus*	Male	3947 BD	Neg	Neg
RM9	*Apodemus sylvaticus*	Female	3947 BD	Neg	Neg
RM10	*Apodemus sylvaticus*	Female	3947 BD	Neg	Neg
RM11	*Apodemus sylvaticus*	Male	3947 BD	Neg	Neg
RM12	*Apodemus sylvaticus*	Female	3947 BD	Neg	Neg

RM13	*Mus musculus*	Male	3956 EM	Neg	Pos
RM14	*Mus musculus*	Female	3956 EM	Neg	Pos
RM15**	*Mus musculus*	Female	3956 EM	Neg	Neg
RM16	*Mus musculus*	Female	3956 EM	Neg	Pos
RM17	*Mus musculus*	Female	3956 EM	Neg	Pos
RM18	*Mus musculus*	Female	3956 EM	Neg	Neg
RM19	*Mus musculus*	Male	3956 EM	Neg	Pos
RM20**	*Mus musculus*	Female	3956 EM	Neg	Pos
RM21	*Mus musculus*	Male	3956 EM	Neg	Neg
RM22	*Mus musculus*	Female	3956 EM	Neg	Neg
RM23	*Apodemus sylvaticus*	Female	3731 EP	Neg	Neg
RM24	*Mus musculus*	Male	4191 NN	Neg	Neg
RM25	*Apodemus sylvaticus*	Female	3911 CL	Neg	Neg
RM26	*Apodemus sylvaticus*	Female	3991 CL	Neg	Neg
RM27	*Apodemus sylvaticus*	Female	3731 EP	Neg	Neg
RM28****	*Mus musculus*	Male	4191 NN	Neg	Pos

* frozen/thawed twice
** green/blue intestines
*** part of its head was missing (caught by cat)
**** code was changed from UM64 to RM28

In one case an oral swab was not possible because half its head was gone (caught by cat). Three mice were frozen and thawed twice. Also in three cases the intestines and their contents had a green/blue colouration and another five mice showed green/blue coloured paws/snouts.

MRSA

No samples were found to be presumptive for MRSA, see Table 2. From 52 samples 10 swabs streaked straight onto Brilliance MRSA 2 plates showed blue(ish) spots but none were positive for both catalase and coagulase test.

C. difficile

In total 21 of 53 faecal samples (40%) were found to be presumptive for *C. difficile* according to colony morphology and the characteristic smell of horse manure. Gram staining showed Gram-positive rods in all cases. Characteristic spore forming (rods resembling the shape of a ‘tennis racket’ or ‘drumstick’) was seen in all stains except four though in all four cases the stains of the colonies cultured the second time on CLO from these samples did show spore forming. Positive isolates were stored at -80°C for confirmation by ribotyping at a later date. In total 20 (38%) samples were presumptive for *C. difficile*. Of those, 12 (46%) were found in the UM group and 8 (30%) were found in the RM group, see Table 2.
The house mouse (*Mus musculus*) was the most common found species though a high frequency of the wood mouse (*Apodemus sylvaticus*) was found in the RM group, see Table 3. In total 26 (49%) males and 27 (51%) females were identified, see Table 4.

Table 2 Presumptive positive samples for MRSA and *C. difficile* in UM and RM

Bacteria	**UM (n=26)**	**RM (n=27)**	**Total (n=53)**
MRSA	0/26 (0%)	0/26 (0%)	0/52 (0%)
C. difficile	13/26 (50%)	8/27 (30%)	21/53 (40%)

Table 3 Identified species in UM and RM

Species	**UM (n=26)**	**RM (n=27)**	**Total (n=53)**
House mouse (*Mus musculus)*	24/26 (92%)	16/27 (59%)	40/53 (75%)
Wood mouse (*Apodemus sylvaticus)*	2/26 (8%)	11/27 (41%)	13/53 (25%)

Table 4 Male/female ratio in UM and RM

Gender	**UM (n=26)**	**RM (n=27)**	**Total (n=53)**
Male	16/26 (62%)	10/27 (37%)	26/53 (49%)
Female	10/26 (38%)	17/27 (63%)	27/53 (51%)

Discussion

Because consistently no MRSA was detected the question rose whether the freezing of the mice in -80°C would be of influence on the detection of MRSA in mice after thawing them. So when brought in an oral swab also taken from UM75-UM80 and RM6-RM27 before they were frozen at -80°C but still no presumptive positive samples were found. In Spain MRSA was isolated from two wild wood mice (out of 35 mice)[11] and in the Netherlands MRSA was found in black rats (*Rattus rattus*) from pig farms.[6] Although only in small numbers, given the impact on public health, these findings of MRSA in mice and rats support further research.

The green/blue colouration of the intestines in some mice indicates the ingestion of rodenticides. Although a possible interference of the rodenticide on the finding of *C. difficile* cannot be ruled out, the finding of several presumptive positive samples within the group of mice that clearly had been in contact with rodenticide (RM13-RM22) suggests that this interference may not be as big as expected.

Two different species were identified. The house mouse is known for its close association with humans so it is no surprise the UM group consisted largely of house mice. In the RM group a large part also consisted of wood mice whose habitat consists mainly of woodlands and fields. So finding more wood mice in a more wooded/fielded area was not unexpected.

Circumstances lead to the freezing and thawing of three mice twice which is unwanted because of the deterioration of the tissues and so the possible interference with MRSA/*C. difficile* detection. Still one of these samples did give a presumptive positive result for *C. difficile*.

Earlier research by Burt et al showed the finding of *C. difficile* in mice on a pig farm showing a possible role of mice in the transmission of *C. difficile* to pigs.[3] *C. difficile* was also found in urban rats[12] and the housefly (*Musca domestica*) has shown to be a potential vector of *C. difficile* in hospitals.[13] This research shows that urban and rural mice also carry *C. difficile* with them and so may act as a reservoir for *C. difficile*; a reservoir in close proximity to humans and thus a possible threat to public health.

PCR ribotyping of the samples found presumptive for *C. difficile* (which will be done at a later date) would give insight into whether the *C. difficile* strains found in mice are similar to strains found in production animals and humans (associated with disease) and thus whether there may be a likely transmission route from mice (to production animal) to people.

Conclusion

Out of 52 mice no MRSA was detected so no conclusion can be drawn on whether there is a difference in the carriage of MRSA between rural and urban mice. From a total of 53 mice 21 (40%) were presumptive positive for *C. difficile*. There was no distinct difference in the finding of *C. difficile* in urban mice compared to rural mice so there seems to be no difference in the carriage of *C. difficile* by urban and rural mice. But the number of mice tested is not significant enough to verdict a definite conclusion.

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References

1. Meerburg, B.G., Singleton, G.R. & Kijlstra, A. Rodent-borne diseases and their risks for public health. *Crit. Rev. Microbiol*. 35, 221-70 (2009)
2. Phifer-Rixey, M. & Nachman, M.W. Insights into mammalian biology from the wild house mouse *Mus musculus. eLife*. 4, e05959 (2015)
3. Burt, S.A, Siemeling, L., Kuijper, E.J., Lipman, L.J. Vermin on pig farms are vectors for *Clostridium difficile* PCR ribotypes 078 and 045. *Vet. Microbiol*. 160, 256-8 (2012)
4. Kilonzo, C., Li, X., Vivas, E.J., Jay-Russell, M.T., Fernandez, K.L., Atwill, E.R. Fecal shedding of zoonotic food-borne pathogens by wild rodents in a major agricultural region of the central California coast. *Appl. Environ. Microbiol.* 79, 6337-44 (2013)
5. Cuny, C., Wieler, L.H., Witte, W. Livestock-associated MRSA: The impact on humans. *Antibiotics (Basel).* 4, 521-43 (2015)
6. Van de Giessen, A.W., Van Santen-Verheuvel, M.G., Hengeveld, P.D., Bosch, T., Broens, E.M., Reusken, C.B. Occurrence of methicillin-resistant *Staphylococcus aureus* in rats living on pig farms. *Prev. Vet. Med.* 91, 270-3 (2009)
7. Rodriguez, C., Van Broeck, J., Taminiau, B., Delmée, M., Daube, G. *Clostridium difficile* infection: Early history, diagnosis and molecular strain typing methods. *Microb. Pathog.* 97, 59-78 (2016)
8. Bonnefoy, X., Kampen, H., Sweeney, K. Public health significance of urban pests. Copenhagen, World Health Organization Regional Office for Europe, 2008. Print
9. Hopman, N.E.M., Oorburg., D., Sanders, I., Kuijper, E.J. & Lipman, L.J.A. High occurrence of various *Clostridium difficile* PCR ribotypes in pigs arriving at the slaughterhouse. *Vet Q*. 31, 179-81 (2011)
10. Meerburg, B.G., Van Gent-Pelzer, M.P.E., Schoelitsz, B., Van der Lee, T.A.J. Distribution of anticoagulant rodenticide resistance in *Rattus norvegicus* in the Netherlands according to *Vkorc1* mutations. *Pest Manag. Sci.* 70, 1761-6 (2014)
11. Gómez, P. González-Barrio, D., Benito1, D., García, J.T., Viñuela, J., Zarazaga1, M. *et al*. Detection of methicillin-resistant *Staphylococcus aureu*s (MRSA) carrying the *mecC* gene in wild small mammals in Spain. *J Antimicrob. Chemother.* 69, 2061-2064 (2014)
12. Himsworth, C.G., Miller, R.R., Montoya, V., Hoang, L., Romney, M.G., *et al*. Carriage of *Clostridium difficile* by wild urban Norway rats (*Rattus norvegicus*) and black rats (*Rattus rattus*). *PLoS ONE* 9, e87983 (2014)
13. M.P. Davies, M.P., Anderson, M., Hilton, A.C. The housefly *Musca domestica* as a mechanical vector of *Clostridium difficile*. *J Hosp. Infect.* 94, 263-267 (2016)