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The housefly *Musca domestica* as a mechanical vector of *Clostridium difficile*

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Running title: The housefly *Musca domestica* as a mechanical vector of *Clostridium difficile*

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SUMMARY

Background: *Clostridium difficile* is a bacterial healthcare-associated infection, which houseflies *Musca domestica* may transfer due to their close ecological association with humans and cosmopolitan nature.

Aim: To determine the ability of *M. domestica* to transfer *C. difficile* both mechanically and following ingestion.

Methods: *M. domestica* were exposed to independent suspensions of vegetative cells and spores of *C. difficile* then sampled on to selective agar plates immediately post-exposure and at one hour intervals, to assess mechanical transfer of *C. difficile.* Fly excreta was cultured and alimentary canals dissected to determine internalisation of cells and spores.

Findings: *M. domestica* exposed to vegetative cell suspensions and spore suspensions of *C. difficile* were able to mechanically transfer the bacteria for up to four hours upon subsequent contact with surfaces. The most colony forming units (CFUs) per fly were transferred immediately following exposure (mean CFUs 123.8 +/- 66.9 for vegetative cell suspension and 288.2 +/- 83.2 for spore suspension). After one hour this had reduced (21.2 +/- 11.4 for vegetative cell suspension and 19.9 +/- 9 for spores). Mean *C. difficile* CFUs isolated from the *M. domestica* alimentary canal was 35 +/- 6.5; and per faecal spot was 1.04 +/-0.58. *C. difficile* could be recovered from fly excreta for up to 96 hours.

Conclusion: This study describes the potential for *M. domestica* to contribute to environmental persistence and spread of *C. difficile* in hospitals, highlighting flies as realistic vectors of this microorganism in clinical areas.

INTRODUCTION

The housefly, *Musca domestica*, presents a significant worldwide threat to public health due to its close ecological association with humans.[1] *M. domestica* breed in faecal matter, move from filth to food indiscriminately,[2, 3] and are therefore implicated in the spread of many diseases.[4-6] *M. domestica* has been sampled from hospitals previously and was shown to carry potentially pathogenic bacteria in the clinical environment, including *Bacillus* spp.,[7] *Escherichia coli*,[8] *Klebsiella pneumoniae*,[9] meticillin-resistant *Staphylococcus aureus* (MRSA)[10] and *Salmonella* sp.[11]

C. difficile infection (CDI) is the leading cause of infectious nosocomial diarrhoea worldwide.[12] It has serious implications, potentially resulting in the isolation of patients, closure of wards and hospitals and even the death of infected individuals.[12] CDI typically affects elderly patients exposed to antimicrobials, and can cause severe disease such as pseudomembranous colitis (PMC) via toxins that affect intestinal cells.[13] While it is generally thought that *C. difficile* is commonly passed from person-to-person nosocomially via the faecal-oral route, most newly ocurring cases cannot be explained by potential contact with known infected individuals. Although the main routes of transmission are unknown transmission from unidentified symptomatic carriers and/or asymptomatic carriers is likely to be important.[14-16]

We postulated that flying insects such as *M. domestica* could play a role in the transmission of *C. difficile* in hospitals. To our knowledge *C. difficile* has not previously been isolated from flying insects in hospitals. However, flies collected from pig farms have been found to harbour ribotype 078,[17] suggesting that there is potential for insects to be mechanical vectors of *C. difficile* in other environments where a source of contamination exists.

In this study, the ability of *M. domestica* to transfer *C. difficile* mechanically and via ingestion and excretion following exposure to vegetative cell and spore suspensions was determined. Subsequent isolation from the alimentary canal and excreta, duration of excretion and whether the *C. difficile* was excreted as spores or vegetative cells were also investigated.

METHODS

Flies

Laboratory reared, mixed-sex adult houseflies (*M. domestica)* were provided by the Insect Supplies Unit at the Food and Environment Research Agency (FERA, York, UK).

C. difficile inocula

C. difficile NCTC11204 PCR ribotype 001 TOX A/B + was used in this study (Anaerobe Reference Laboratory, Cardiff, UK). A 1 x 10^6/ml culture of *C. difficile* vegetative cells was prepared in 15ml Wilkins Chalgren broth (Oxoid Ltd, Basingstoke, UK) by inoculation with 10 colonies previously cultured on Wilkins Chalgren Agar (Oxoid Ltd, Basingstoke, UK) incubated anaerobically for 48 hours at 37°C. 1 x 10^6/ml suspensions of *C. difficile* spores were prepared as described by Shetty *et al.*[18] 1 x 10^6/ml quantities were chosen as realistic proxy for fly exposure in faeces given that *C. difficile* may be found at levels of 1 x 10^4 to 1 x 10^7 per gram of human faeces.[19]

Mechanical transfer of *C. difficile* by *M. domestica*

Houseflies were inactivated by incubation in a sterile Petri dish in a -18°C freezer (Beko, Watford, UK) for two minutes. Inactivated houseflies were taken from the freezer and both wings removed by dissection with sterilized entomological spring scissors and fine entomological forceps (Watkins and Doncaster, Kent, UK) to prevent escape by flight. *M. domestica* wings do not play an important role in the mechanical transmission of bacteria.[20] The flies were stored at 4°C in a refrigerator until required.

Pre-treatment control

A pre-exposure control sample of houseflies (n=5) was macerated individually in 1ml of sterile Phosphate Buffered Saline (PBS) (Sigma Aldrich, Poole, UK), using the end of a sterile plate spreader. The homogenate was serially diluted to 1 x 10^{-3} and 0.1ml of each dilution was inoculated on to the surface of a CCFA plus sodium taurocholate (Tc) plate (Oxoid Ltd, Basingstoke, UK). The plates were incubated anaerobically for 48 hours at 37°C. Colonies with typical morphology were sub-cultured on to Columbia blood agar (Oxoid Ltd, Basingstoke, UK) and identified rapid ID 32A API test strips (bioMérieux, Marcy l'Etoile, France).

Mechanical transfer of vegetative cells

To confirm the fly was clear of *C. difficile* carriage prior to the experiment, a single fly was transferred from the sterile holding dish on to the surface of a CCFA plate (no spore germinant) and

allowed to walk around the plate for six minutes. Following this it was transferred to a CCFA plus Tc plate for a further six minutes. The same fly was then transferred to a 'donor' CCFA plate that had been inoculated with 0.1ml of the *C. difficile* vegetative cell culture immediately before the fly was introduced. After exposure to the 'donor' plate for six minutes, the fly was transferred to a fresh CCFA plate ('recipient' plate), and then a CCFA plus Tc plate (second 'recipient' plate) (six minutes on each). The six minute contact times were chosen to reflect observations by the authors of how long adult houseflies have contact with foodstuffs and surfaces. The fly was then transferred to a sterile empty Petri dish ('resting' plate) for one hour. The fly then underwent three further cycles of transfer to CCFA the CCFA plus Tc plates separated by one hour periods in 'resting plates.' These experiments were replicated nine times.

Mechanical transfer of spores

This experiment used the same methodology as described above but with a 1 x 10^6/ml *C. difficile* spore suspension rather than a vegetative cell culture. Additionally, only CCFA plus Tc plates were used.

Isolation of *C. difficile* from *M. domestica* alimentary canal

Five houseflies were exposed to *C. difficile* for 30 minutes, by being allowed to walk over a CCFA agar plate inoculated with 0.1ml of the 1 x 10^6/ml spore suspension. Flies were then killed by incubation in a sterile Petri dish at -18°C for five minutes. Each fly was subsequently removed from frozen storage and washed five times in PBS.

The fly alimentary canal (including crop) was dissected aseptically. The fly alimentary canal was added to 1ml PBS in a sterile 1.5ml universal micro test tube, macerated with the end of a sterile plate spreader and mixed by vortexing for 30 seconds to release bacteria into the PBS wash. Of this PBS wash, 0.1ml was then inoculated on to the surface of a CCFA plus Tc agar plate. The PBS wash was diluted ten-fold in sterile PBS and 0.1ml of this 10^{-1} dilution inoculated on to a further CCFA plus Tc agar plate. The plates were incubated and any presumptive *C. difficile* colonies identified as described above.

Initial isolation of *C. difficile* from *M. domestica* excreta

Five houseflies were exposed to *C. difficile* as in the alimentary canal experiment. Inactivated flies were individually washed washed five times in 1 ml volumes of sterile PBS. The flies were then introduced onto invidivual Petri dishes containing with 1ml of 5% sterile sucrose solution to encourage feeding. Flyspots (material deposited from the gut) on the surface of the Petri dishes were sampled immediately using a sterile swab that was used to directly inoculate a CCFA plus Tc

agar plate. Each time a flyspot was removed the fly was transferred to a new Petri dish; sampling continued for a three hour period.

Isolation of *C. difficile* from *M. domestica* excreta over time

Twenty five houseflies were exposed to *C. difficile* spores for 30 minutes by being allowed to walk over filter paper inoculated with 0.6ml of the 1 x 10^6 CFU/ml spore suspension. Inactivated flies were transferred, washed and fed as described above. Flyspots were sampled for four hours, and then every 24 hours for four days.

RESULTS

Pre-treatment control

No colonies were present on the pre-treatment control plates, confirming that the houseflies were not contaminated with *C. difficile* prior to being exposed to the bacterial suspensions.

Mechanical transfer of vegetative cells

The most colony forming units (CFUs) per fly were transferred immediately and one hour following exposure to the vegetative cell suspension and this transfer continued, albeit with low numbers of CFUs transferred, up to four hours following exposure (Figure 1.).

The mean number of CFUs of *C. difficile* transferred immediately by *M. domestica* (n=9) to the recipient CCFA plates without a germinant (therefore likely to represent vegetative cell transfer), was 10.2 +/- 4.3. After one hour this had reduced to 6.7 +/- 3.9 (Figure 1.).

The mean number of CFUs of *C. difficile* transferred immediately by *M. domestica* (n=9) to the recipient CCFA plates incorporating the germinant Tc (therefore likely to represent combined spore and vegetative cell transfer) was 123.8 +/- 66.9. After one hour this had reduced to 21.2 +/- 11.4 (Figure 1.).

Mechanical transfer of spores

The most CFUs per fly were transferred immediately and one hour following exposure to the spore suspension, with minimal transfer after two hours, three hours and no transfer apparent after 4 hours (

Figure 2.). The mean number of CFUs of *C. difficile* transferred immediately by *M. domestica* (n=9) to the recipient CCFA+Tc plates was 288.2 +/- 83.2; after one hour this had reduced to 19.9 +/- 9 (Figure 2.).

Isolation of *C. difficile* from *M. domestica* alimentary canal

The mean number of *C. difficile* CFUs isolated from the *M. domestica* alimentary canal (n=20) was 35 +/- 6.5.

Initial isolation of *C. difficile* from *M. domestica* excreta

The mean number of *C. difficile* CFUs isolated per *M. domestica* faecal spot was 1.04 +/-0.58, over a three-hour period. *C. difficile* spores were recovered from *M. domestica* excreta for 96 hours (Figure 3). *C. difficile* was isolated on CCFA plus Tc plates from *M. domestica* excreta, with means of 4.16 +/- 0.59 CFUs per fly at day one, decreasing to 1.35 +/- 0.27 after two days, decreasing further still to 0.64 +/- 0.19 after three days and 0.38 +/- 0.14 at four days (Figure 3.). No growth was observed on CCFA plates without germinant.

DISCUSSION

Our laboratory based study shows that adult houseflies, via direct contact with their external surfaces, are able to mechanically transfer *C. difficile* for up to four hours after initial exposure to vegetative cells or spores. Whilst our laboratory studies may not exactly replicate the behaviour of flies in the hospital environment our results do show the potential infection risk to patients posed by flies in hospital. The contaminated hospital environment is a recognised risk factor in the acquisition of *C. difficile.* [21] Cleaning and disinfection strategies remove or reduce this contamination to a level which reduces the risk.[22] However the findings of this study demonstrating that flies are able to move this contamination around from previously clean surfaces, highlight their potential as vectors. Moreover, as *M. domestica* is often the most common fly in human occupied premises, and can disperse for miles,[3, 23,24] it could also be implicated in community associated *C. difficile* cases.

Although the infectious dose of *C. difficile* in humans is unknown, it is believed to be low (of the order of tens of spores). Indeed ingestion of only one or two spores has been found to colonise hamsters exposed to antibiotics.[25] Thus, while the numbers of *C. difficile* transferred per fly were low, these could still be significant especially taking into account that hundreds of flies can be present in hospitals at any one time.[8, 9]

C. difficile was isolated specifically from the alimentary canal of adult *M. domestica*, showing that ingestion of the bacteria occurs in addition to contamination of the body surfaces. Excretion of *C. difficile* spores, but not of vegetative cells occurs, which suggests that germination does not take place in the fly. Lack of *C. difficile* spore germination may be due to absence of bile salts in the fly digestive system, which are known to be required for germination.

There appears to be a 'timeline of transfer' which involves initial transfer of *C. difficile* via direct contact of external surfaces of the fly and which is highest. This decreases over time with bacteria in excreta probably becoming responsible for continuing the transfer. The reasons for the low recovery of *C. difficile* from adult *M. domestica* alimentary canals and excreta are unclear; possibly antimicrobial peptides that have previously been identified in this species could have a role.[26,27]

Identical *C. difficile* 078 isolates are increasingly encountered in pigs and humans leading researchers to the conclusion that a common origin of animal and human strains should be considered.[28] It seems plausible that *M. domestica* may have a role in interspecies transmission of *C. difficile*,[22] as has been shown in other flies collected from pig farms and shown to be positive for *C. difficile* 078.[17]

In conclusion, *M. domestica* may harbour *C. difficile* for extended periods of time and transfer low numbers in the environment, potentially presenting a reservoir and infection risk to patients due to the likely low infective dose. This study describes the potential for *M. domestica* to contribute to environmental persistence and possible spread of *C. difficile* in hospitals, and even in the community.

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Conflict of interest statement

Dr Matthew Davies and Professor Moray Anderson are employed by Killgerm Chemicals Ltd, a manufacturer and distributor of pest control products. Professor Anthony Hilton has no conflicts of interest to declare.

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Figure 1. Vector potential of *C. difficile* by *M. domestica*, after exposure to vegetative cells. Mean number (± Standard Error (SE)) of *C. difficile* cells disseminated per fly (n=9), over time, after exposure to a 1 x 10^5 suspension of vegetative cells. Numbers above the columns are numbers of positive flies / number of flies tested. 'CCFA' is the recovery of *C. difficile* from Cycloserine Cefoxitin Fructose Agar without a germinant, which is likely to represent vegetative cell transfer by *M. domestica*. 'CCFA+Tc' is the recovery of *C. difficile* from Cycloserine Cefoxitin Fructose Agar with the germinant sodium taurocholate, which is likely to represent combined spore and vegetative cell transfer by *M. domestica*.

Figure 2. Vector potential of *C. difficile* spores by *M. domestica*. Mean number (± SE) of *C. difficile* CFUs disseminated per fly (n=9), over time, after exposure to a 1 x 10^5 suspension of spores. Numbers above the columns are numbers of positive flies / number of flies tested.

Figure 3. Isolation of *C. difficile* from *M. domestica* excreta over time. Mean number (±SE) of *C. difficile* CFUs isolated per *M. domestica* (n=25) from pooled flyspots sampled over time, after flies were exposed to a 1 x 10^5 suspension of spores. Numbers above the columns are numbers of positive flies / number of flies tested.