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Acquisition and retention of *Clostridium difficile* by *Musca domestica* larvae and pupae during metamorphosis.

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SUMMARY

Background: Transfer of *Clostridium difficile* by *Musca domestica* has been demonstrated, revealing their potential for disseminating infection in the hospital environment.

Aim: To determine the ability of *M. domestica* larvae to acquire and retain *C. difficile* throughout their metamorphosis into adult flies.

Methods: Larvae were exposed to spores of *C. difficile* in a faecal emulsion and examined externally and internally to determine carriage and internalisation of spores through their development to adults.

Findings: Larvae harboured *C. difficile* externally, with means of 21.56 +/- 5.76 CFUs at day zero, 22.44 +/- 9.90 after two days, decreasing to 0.56 +/- 0.34 at day four, with no *C. difficile* isolated thereafter. The same larvae harboured *C. difficile* internally, with means of 587.33 +/- 238.29 CFUs at day zero, decreasing to 297.44 +/- 155.21 after two days, decreasing further to 73.67 +/- 46.74 after four days, with no *C. difficile* isolated thereafter. The zero recovery of *C. difficile* coincided with the development of *M. domestica* larvae into pupae. From day six onwards, all larvae had developed into the pupal stage and no *C. difficile* was recoverable from any pupae. No *C. difficile* was recovered from adult flies (emerged on day twelve), or empty puparia.

Conclusion: Although *C. difficile* spores are readily acquired and internalised by larvae during feeding, they are not retained through development to adults. Adult flies therefore acquire *C. difficile* contamination as adults. The potential antimicrobial action of *M. domestica* larvae and their extracts against *C. difficile* spores warrants further investigation.

INTRODUCTION

The contribution of adult *Musca domestica* to environmental persistence and spread of *Clostridium difficile* in hospitals has been demonstrated previously, highlighting flies as potential vectors of this microorganism in clinical areas.[1] *M. domestica* adults may not be the only life stage able to transfer *C. difficile*, as housefly larvae can also carry pathogenic bacteria, sometimes throughout their development.[2]

Larvae are an active, feeding, mobile stage of the fly life cycle and could therefore present infection risks if they are acquiring and subsequently transferring bacteria from breeding sites that may be present in hospitals. Such breeding sites could include excrement, rotting organic matter associated with drains, animal carcasses and food spillage.[3] Indeed, housefly larvae have been shown to harbour *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Providencia rettgeri* and many other species of bacteria.[4,5]

Evidence shows that the vast majority of larval gut microorganisms are destroyed during metamorphosis and that at the point of emergence, approximately 20% of adult houseflies are sterile.[6] In addition, the adult fly has been proposed to offer the most likely potential for disease transmission, as the maggot has limited motility and possesses autosterilization mechanisms which limit its capacity to carry pathogens over into the adult stage.[6] In contrast, a number of authors have shown that housefly larvae acquire and retain a considerable number of bacteria acquired at this stage through to the pupal stage and finally adulthood.[2,7]

Although *Clostridium* species have been recovered from fly larvae, there is no evidence in the current literature of *C. difficile* being isolated from the larvae of any flies. For example, *Clostridium* spp. have been found in association with non-biting midge larvae *Chironomus plumosus*,[8] and *Clostridium botulinum* with *Lucilia caesar* larvae.[6, 9] As Gram-positive spore-forming bacteria such as *Bacillus anthracis* can be acquired by fly larvae, retained through metamorphosis and isolated from adult flies,[10] it is reasonable to hypothesise that the same may also hold true for *C. difficile*, especially as members of the same genus have been isolated from fly larvae.

In this study the acquisition and retention of *C. difficile* through the life stages of *M. domestica*, from larvae to pupae to adults, was explored. If *C. difficile* is acquired by *M. domestica* larvae and retained through to adulthood, the identification and elimination of larval development sites as potential sources of contamination should become increasingly important in fly control programmes in hospitals.

METHODS

Larvae

Laboratory reared housefly larvae, *M. domestica*, were provided by the Insect Supplies Unit at the Food and Environment Research Agency (FERA, York, UK). Only third instar larvae that were observed to feed were used in the experiments. The larval medium supplied by and produced to a GLP Standard Operating Procedure by FERA consisted of bran, grass meal, dried brewer's yeast, malt extract, dried milk powder and water.

Faecal emulsion

A faecal emulsion was prepared by suspending a fresh human faecal sample in sterile distilled water (SDW) at a ratio of 1:20 w/v.[11, 12] This emulsion was seeded with *C. difficile* spores NCTC11204 PCR ribotype 001 TOX A/B + (Anaerobe Reference Laboratory, Cardiff, UK) using a 1 x 10^6/mL suspension prepared as described by Shetty *et al*,[13] with the presence of spores verified by microscopy and quantified using a haemocytometer. The 1 x 10^6/mL suspension was chosen as a realistic level for fly exposure because *C. difficile* can be excreted by a human patient at levels of 1 x 10^4 to 1 x 10^7 per gramme of faeces.[14]

Pre-treatment control

A pre-treatment control sample of housefly larvae (n=3) were macerated individually in 1ml of sterile Phosphate Buffered Saline (PBS) (Sigma Aldrich, Poole, UK), using the end of a sterile plate spreader and mixed by vortexing for 30 seconds. The homogenate was serially diluted down to 1 x 10^{-3} and 0.1 mL of each dilution was inoculated onto the surface of a CCFA plate + Tc selective supplement (Oxoid Ltd, Basingstoke, UK) and 7% v/v defibrinated horse blood (Southern Group Laboratories, Corby, UK). The plates were incubated for 48 hours at 37°C under anaerobic conditions in a MiniMACS anaerobic workstation (Don Whitley Scientific, UK) and subsequently observed for characteristic *C. difficile* colony morphology.

Identification of *C. difficile* colonies

Following incubation of the CCFA + Tc plates, colony counts were made. Presumptive *C. difficile* colonies demonstrating characteristic colonial morphology were sub-cultured on to Columbia blood agar (Oxoid Ltd, Basingstoke, UK) and were subsequently identified by macroscopic morphology, Gram staining, microscopic examination of morphology,[15] characteristic 'farmyard' smell,[16] and rapid ID 32A API tests (bioMérieux, Marcy l'Etoile, France).

Isolation of *C. difficile* from the external structures of *M. domestica* larvae

Housefly larvae (n=3) were exposed to *C. difficile* for 30 minutes, by being allowed to move over a sterile Petri dish containing 200µL of the faecal emulsion. Following exposure, individual larvae were transferred to their own sterile Petri dish and cooled to 4°C in a refrigerator, to aid subsequent handling and ligation. Fly larvae mouthparts and anus were then ligated with superglue (Loctite super glue liquid, Henkel, Hempstead, UK), to prevent expulsion of gut contents during subsequent vortexing. Larvae were washed individually in 1 mL of sterile PBS in a sterile 1.5 mL universal micro test tube (Eppendorf, Stevenage, UK) and mixed by vortexing for 30 seconds. The resulting PBS wash was serially diluted down to 10^{-3} in sterile PBS and 0.1 mL of each dilution used to inoculate the surface of a CCFA + Tc agar plate. Plates were incubated and colonies identified as described. This experiment was performed in triplicate.

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

Housefly larvae (n=3) were exposed to *C. difficile* in a faecal emulsion as described previously. Each larva was subsequently washed as described and this was repeated four times. This process of repeated washing was undertaken to remove *C. difficile* from external surfaces, to avoid issues of contamination when attempting to isolate *C. difficile* from internal structures. The final set of PBS washings was retained, serially diluted down to 10^{-3} in sterile PBS and 0.1 mL of each dilution used to inoculate the surface of a CCFA+Tc agar plate. The plates were incubated anaerobically as described previously. The larva alimentary canal and crop were dissected aseptically, macerated, mixed and analysed for the presence of *C. difficile* as described. This experiment was performed in triplicate.

Dissection of fly larvae

A dissection microscope (Stereo Zoom Model GXM XTL 3101, GX Optical, Haverhill, UK), iridectomy scissors (Surgins Surgical Ltd, Birmingham, UK), wax from ‘tea light’ candles (Morrisons, Wakefield, UK), 0.19 mm entomological pins (Watkins and Doncaster, Kent, UK) and fine entomological forceps (Watkins and Doncaster, Kent, UK) were used for dissection. The exposed gut was then removed with the iridectomy scissors and then transferred to 1 mL of PBS in a sterile 1.5 mL universal micro test tube.

This alimentary canal was analysed as per the pre-treatment control. Plates were incubated and colonies identified as described.

Retention of *C. difficile* through the life stages of *M. domestica*

Housefly larvae (n=24) were split into three groups of eight individuals and exposed to *C. difficile* for 30 minutes, by being allowed to move freely within a sterile Petri dish containing 600 µL of the *C.*

difficile faecal emulsion. Then, one larva from each Petri dish was washed by vortexing for 30 seconds in 1 mL PBS, macerated with the end of a sterile plate spreader, serially diluted down to 10^{-3} in sterile PBS and 0.1 mL of each dilution used to inoculate the surface of a CCFA + Tc agar plate. Plates were incubated anaerobically and colonies identified as described. This sample was considered as Day 0 and was used to confirm the ingestion of *C. difficile* spores.

The remaining larvae were incubated at 30°C in darkness in an incubator (Sanyo Gallenkamp, Loughborough, UK) in the same faecal emulsion dishes to allow metamorphosis to proceed. Sterile substrate (sawdust) (FERA, York, UK) to allow burrowing and aid successful pupation was included in the sterile Petri dishes, as well as larval medium. The substrate was moistened daily with 0.1 mL SDW to prevent desiccation of the larvae. These larvae were not ligated as this would prevent development.

From each group, one larva was extracted and examined for *C. difficile* carriage both externally and internally as described previously every alternate day during development. Plates were incubated anaerobically and colonies identified as described.

As pupation occurred, the pupae were removed into fresh sterile Petri dishes and stored at 30°C in darkness in an incubator (Sanyo Gallenkamp, Loughborough, UK). The pupae were extracted (n = 3) each day post-pupation, for microbiological examination externally via maceration as per the larvae, to determine whether *C. difficile* was present. The plates were incubated and subsequently observed for characteristic *C. difficile* colony morphology.

Pupae were retained in separate sterile Petri dishes and stored at 30°C in an incubator until adult emergence. Adult flies and empty puparia were examined microbiologically by the described external washing and maceration techniques. The plates were incubated and subsequently observed for characteristic *C. difficile* colony morphology. This method was designed to determine whether adult flies emerge with external contamination of *C. difficile* obtained from the puparium, or *C. difficile* had been retained throughout development. This experiment was repeated in triplicate to give n=9 observations for each life stage of *M. domestica*.

RESULTS

Pre-treatment control

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

Isolation of *C. difficile* from the external and internal structures of *M. domestica* larvae

M. domestica larvae (n = 9) exposed to the faecal emulsion and then washed, harboured the following mean *C. difficile* CFUs externally; 222.5 +/- 87.03 first wash, 22.5 +/- 3.88 second wash, 16.67 +/- 1.72 third wash and 10 +/- 0 for the fourth wash. Further washes were negative for *C. difficile*. The mean of the total *C. difficile* CFUs isolated from external structures of the larvae was 262.5 +/- 91.79 and 306.25 +/- 103.77 internally.

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

The mean *C. difficile* CFUs isolated from the alimentary canals of *M. domestica* larvae (n = 18) exposed to a 4 x 10^6/mL spore suspension for 30 minutes were 56.36 +/- 21.56.

Retention of *C. difficile* through the life stages of *M. domestica*

M. domestica larvae (n = 9) exposed to the faecal emulsion harboured *C. difficile* externally, with means of 21.56 +/- 5.76 CFUs initially at day zero, 22.44 +/- 9.90 after two days, decreasing to 0.56 +/- 0.34 at day four, with no *C. difficile* isolated thereafter (Figure 1). The same *M. domestica* larvae harboured *C. difficile* internally, with means of 587.33 +/- 238.29 CFUs initially at day zero, decreasing to 297.44 +/- 155.21 after two days, decreasing further still to 73.67 +/- 46.74 after four days, with no *C. difficile* isolated thereafter (Figure 1). The zero recovery of *C. difficile* coincided with the development of *M. domestica* larvae into pupae. From day six onwards, all larvae had developed into the pupal stage and no *C. difficile* was isolated from any pupae (Figure 1). Adult flies emerged on day twelve, from which no *C. difficile* was recovered (Figure 1). Empty puparia from which the flies emerged were negative for *C. difficile* spores.

DISCUSSION

M. domestica larvae, via contact with their external surfaces, may be able to mechanically transfer *C. difficile* after initial exposure to a source of spores, as they harboured the bacterium externally, following contact with a seeded faecal emulsion under experimental conditions. *C. difficile* was isolated specifically from the alimentary canal of *M. domestica* larvae allowed to feed on a seeded faecal emulsion, demonstrating that ingestion of the spores also occurs.

Carriage of *C. difficile* continued both externally and predominantly internally through the larval stage. The level of *C. difficile* was at its greatest following initial exposure to the spores, decreasing up to the end of the larval stage at which point none could be isolated from pupae that developed thereafter and adult flies emerged free of *C. difficile*. These observations suggest that *C. difficile* spores associated with immature stages of *M. domestica* may be destroyed due to changes which

occur during metamorphosis, by fly antimicrobial peptides, by other aspects of the fly immune system, or simply excreted.

The observation that *C. difficile* is not retained beyond the larval stage is not unexpected because it is known that larval gut microorganisms are destroyed during metamorphosis and that at the point of emergence, a percentage of adult flies are sterile.[6] From the mature larval stage to the prepupa stage of houseflies, there is a greater than 90% reduction in numbers of bacteria, mainly due to feeding ceasation and evacuation of bacteria from the larval gut.[17] A further reduction in the number of bacteria occurs when the larval foregut and hindgut are shed during pupation and become deposited in the puparium upon adult fly emergence. It is also thought that destruction and synthesis of structures and general reorganisation of tissues in metamorphosis probably reduces the amount of bacteria present and these factors all contribute to approximately 20% of houseflies being sterile upon emergence.[6]

Competition with normal housefly gut flora appears to be a main reason why experimentally introduced bacteria are not retained during metamorphosis. For example, bacteria such as salmonella and shigella, when introduced to larvae with normal gut flora, were unable to survive pupation and were not isolated from any adults that emerged.[17] In the same experiment, the introduced bacteria could not be isolated from the majority of the fly larvae. Only by using aseptic rearing techniques and gnotobiotic flies could experimentally introduced bacteria at the larval stage be recovered from pupae and adults. Even then, although the bacteria survived metamorphosis, there was still a reduction in numbers. The conclusion of the study was 'the adult fly has the most potential for disease transmission, as the maggot has limited motility and possesses autosterilization mechanisms which limit its capacity to carry pathogens over into the adult stage.'[17]

Of the 'autosterilization mechanisms' described,[17] secretions of fly larvae and specifically antibacterial peptides from *M. domestica* and *L. sericata* have been shown to be active against bacteria.[18-20] Larval secretions of the blowfly *L. sericata* have a bactericidal property against *Staphylococcus aureus,* haemolytic streptococci and *Clostridium perfringens.*[21] An antibacterial peptide, seraticin, has been extracted from larvae of *L. sericata* and shows activity against MRSA as well as a range of Gram-positive (e.g. *Bacillus cereus*) and Gram-negative (e.g. *Escherichia coli*) bacteria.[22] It is possible that the presence of antibacterial peptides in *M. domestica* larvae could be influential in the lack of retention of *C. difficile* through metamorphosis and although yet to be discovered in *M. domestica,* some insects (the Korean dung beetle, *Copris tripartitus*) do possess antimicrobial peptides (coprisin) with activity against vegetative cells of *C. difficile*.[23]

CONCLUSION

Although *C. difficile* spores are acquired and internalised by *M. domestica* larvae, the spores are not retained through development to the pupal and adult stages. Therefore adult *M. domestica* can be considered the most important life stage in the acquisition and transfer of *C. difficile* in source environments such as hospitals. This is partly due to their ability to act as mobile fomites, travelling up to five miles in a day in search of breeding material.[6] The potential anti-sporicidal action of *M. domestica* larvae and their extracts against *C. difficile* warrant further study.

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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. Retention of *Clostridium difficile* throughout *Musca domestica* life cycle (Mean ± SE CFUs isolated per insect), corresponding with life stages. Numbers above the columns are numbers of positive individuals / number of individuals tested in terms of external and internal isolation of *C. difficile*. The corresponding life stages were: 9 larvae at days 0 & 2; 4 larvae & 5 pupae at day 4; 9 pupae at days 6, 8 & 10; 9 adults at day 12.