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An examination of flying insects in seven hospitals in the United Kingdom and carriage of bacteria by true flies (Order: Diptera)

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Abstract

Insects are efficient vectors of bacteria and in the hospital environment may have a role in spreading nosocomial infections. This study sampled the flying insect populations of seven hospitals in the United Kingdom (UK) and characterised the associated culturome of Diptera, including the antibiotic-resistance profile of bacterial isolates. Flying insects were collected in seven UK hospitals between the period March 2010 to August 2011. The bacteria carried by Diptera were isolated using culture-based techniques, identified and characterised by antimicrobial susceptibility testing. A total of 19,937 individual insects were collected with Diptera being the most abundant (73.6% of the total), followed by Hemiptera (13.9%), Hymenoptera (4.7%), Lepidoptera (2.9%) and Coleoptera (2%). From Diptera, 82 bacterial strains were identified. The majority of bacteria belonged to the Enterobacteriaceae (42%), followed by *Bacillus* spp. (24%) and *Staphylococcus* spp. (19%). Less abundant were bacteria of the genus *Clostridium* (6%), *Streptococcus* (5%) and *Micrococcus* (2%). A total of 68 bacterial strains were characterised for their antibiotic resistance profile; 52.9% demonstrated a resistant phenotype to at least one class of antibiotic. *Staphylococcus* spp. represented the highest proportion of resistant strains (83.3%), followed by *Bacillus* spp. (60%) and Enterobacteriaceae (31.3%). Diptera were the predominant flying insects present in the UK hospital environments sampled and found to harbour a variety of opportunistic human pathogens with associated antimicrobial resistance profiles. Given the ability of flies to act as mechanical vectors of bacteria they present a potential to contribute to persistence and spread of antimicrobial-resistant pathogenic bacteria in the hospital environment.

Keywords: Flying insects, Mechanical vector, Nosocomial infection, Antimicrobial resistance bacteria.

Introduction

Insects are efficient vectors of bacteria and other pathogenic agents (Mchugh 1994). Although hospital environments and other health care facilities implement controls for pest and non-pest insect species, their continued presence in hospital wards is reported from around the world (De Castro et al. 2015; M. Faulde & Spiesberger 2013; Faulde et al. 2001; Gliniewicz et al. 2003; Kappel et al. 2013; Máximo et al. 2014; Menasria et al. 2014).

Ants and cockroaches are common pests found in hospitals worldwide. Ants are prevalent pests in the urban environment and easily colonize buildings, nesting in inaccessible places such as wall cavities and foundations. According to a review of ants in Brazilian hospitals (De Castro et al. 2015), 59 different species of ants were recorded in health-care settings in Brazil between 1993 and 2014, with *Tapinoma melanocephalum* being the most common. In the United States, in Texas and Florida, legal action against physicians and health care facilities has been taken as a result of fire ant attacks on patients in nursing homes (deShazo et al. 2004). In the United Kingdom (UK), a study by Edwards and Baker (1981) reported 11.6% of NHS hospitals were infested with tropical ants, *Monomorium pharaonis*.

Cockroaches are highly successful pests, they breed indoors and share human food and shelter. *Blattella germanica* is the most predominant cosmopolitan pest in the world (Menasria et al. 2014) and reference to its presence in health care facilities are numerous. *Blattella germanica* has been collected from long-term care facilities and nursing homes in Taiwan (Pai 2013) and from hospitals in Poland (Gliniewicz et al. 2006), Algeria (Menasria et al. 2014), Cuba (Oliva et al. 2010), Japan (Saitou et al. 2009) and Ethiopia (Tachbele et al. 2006) .

Synanthropic Diptera are ubiquitous pests that similarly pose concern in health-care facilities. Filth flies (such as Muscidae, Sarcophagidae, Calliphoridae) and Psychodidae,

have been reported in hospital environments (Faulde & Spiesberger, 2013; Kappel et al., 2013; Kassiri et al. 2015; Ranjbar et al. 2016) and infestations documented (Faulde et al. 2001; Schouest et al. 2017).

Few published studies have considered the wider insect population present in the hospitals rather than focusing on a specific group of insects. Sramova et al. (1992) collected 161 arthropods specimens from a hospital in Warsaw. Almost half of the arthropod population collected was represented by *Blattella germanica*, the remaining insects were ants, flies, non-biting midges, spiders and casual intruders associated with the outdoor environment. A similar study by Kappel et al. (2013) investigated non-biting flying insects in a hospital environment reporting the majority to be Diptera, followed by Hymenoptera, Coleoptera, Lepidoptera, Orthoptera, Hemiptera and Trichoptera.

The public health significance of insects and other arthropods in healthcare settings is related to the risk they present as vectors of nosocomial infections. Insects including cockroaches, ants and flies may act as mechanical vectors of pathogens in the hospital environment. Although nosocomial myiasis are rare, they have been noted, especially in case of immobile, debilitated or disabled patients (Batista-da-Silva et al. 2011, Salmanzadeh et al. 2018). These insects move indiscriminately between filth and food and may therefore acquire and carry, on their external and internal integuments, bacterial contamination throughout their life cycles. It has been reported that 98% of cockroaches found in healthcare facilities harboured pathogens on their exoskeleton or in their gut (Cloarec et al. 1992). Among the bacteria isolated from cockroaches, many were recognised pathogens including *Escherichia coli*, *Enterobacter cloaceae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella* spp., *Salmonella typhi* and *Shigella dysenteriae* (Cloarec et al. 1992, Rivault and Cloarec 1993, Tachebe et al. 2006, Salehzadeh and Tavacol 2007).

From ants collected in the hospital environment, Lima et al. (2013) isolated several pathogens including coagulase-negative *Staphylococcus* spp., *Acinetobacter baumannii*, *Acinetobacter lwoffii* and *Staphylococcus aureus*, and additional bacterial species related to nosocomial infections including *Serratia marcescens*, *Citrobacter freundii*, *Klebsiella ozaenae*, *Enterobacter aerogenes*, *Proteus mirabilis* and *Staphylococcus epidermidis*.

The risk associated with *Musca domestica* as a vector of pathogenic microorganisms in hospitals has been described by Fotedar et al. (1992) and more recently by Borges Kappel et al. (2013), the latter study describing *Bacillus* spp., coagulase-negative Staphylococci, *Micrococcus* spp, *Pseudomonas* spp., *Proteus mirabilis* and *Enterobacter gergoviae* from flying insects collected inside a paediatric ward and neonatal-intensive care unit of a Brazilian hospital. In Germany, *Pollenia rudis s.s*. (Diptera: Calliphoridae) collected in a hospital following an infestation were revealed to harbour opportunistic aerobic mesophilic *Bacillus* spp., *Erwinia* spp. and *Erwinia amylovora*, *Stenotrophomonas malthophila*, *Staphylococcus lugdunensis*, *Pseudomonas aeruginosa* and *Flavobacterium odoratum*. All the bacterial strains isolated represented opportunistic agents of nosocomial infection (Faulde et al. 2001). In a later study by the same authors, several pathogenic bacterial strains were isolated from *Clogmia albincutata* (Diptera: Psychodinae) in different hospital settings across Germany (Faulde et al., 2013).

The causal link between the presence of insects in hospitals and nosocomial infection is not fully established however *Musca domestica* is the only currently recognised vector of human pathogens in hospitals (Graczyk et al. 2001). The vector potential of *Musca domestica* is enhanced through their ability of flight, however the fundamental mechanisms at the basis of the mechanical spread of bacteria by insect vectors are equivalent. The

risks related to flying insects in hospitals may be higher than crawling insects, due to their ability to fly long distances (Olsen 1998).

Although studies reporting the species of insects in international hospitals exist, few have been undertaken in the UK. Of the UK studies that have been reported, none focus on flying insects and the data are in some cases over 30 years old. The aim of this study was to undertake a detailed description of flying insects populations in seven UK hospitals collected with ultra-violet (UV) light flytraps and professional sticky traps. Furthermore, to determine the internal and external bacteria harboured by the Diptera collected and characterise the bacterial isolates in terms of their antimicrobial resistance profile.

Materials and Methods

Ethics and research and development approval from the UK National Health Service (NHS)

National Health Service Ethics approval was sought for the study. An ethics application was prepared using the Integrated Research Application System (IRAS) and submitted to the NHS Research Ethics Committee (REC) for review. Following NHS Ethics review, a Research and Development (R&D) application was submitted via IRAS to the R&D department for each NHS trust in which the study hospital resided. The study was approved under reference 09/H0408/99.

Summary description of hospital locations

Seven hospital locations in the North-West of England, Yorkshire and the Midlands of the United Kingdom were recruited into the study. Location 1 provided in- and out-patient services with expertise in maternity care and neonatal services. The hospital location was

adjacent to fields, water sources and a farm. Location 2 featured a major trauma centre, A&E services and other acute general provisions, intensive care in addition to units for high dependency patients and a maternity suite with a city centre location. Location 3 provided acute services and specialist services including trauma, neuroscience, infectious diseases and cancer with an urban location. Location 4 featured an A&E department and a major trauma centre with an urban location. Location 5 offered acute services with a large A&E and outpatient department in an urban area. Location 6 secialised in elective care and a site for rehabilitation and diagnostics in an urban area. Location 7 was a district general hospital providing all major specialist services and A&E in an urban area.

Collection and identification of flying insects from hospital locations

Flying insects were collected from pre-existing ultra-violet (UV) light flytraps in the form of Electronic Fly Killers (EFKs) and professional sticky traps located throughout the seven hospital locations within the period March 2010 to August 2011. Traps were located in ward kitchens, catering units, cafés, café kitchens, restaurants, coffee shops, cooked food stores, dry food stores, raw food stores, reception areas, laundry, leisure centre, maternity wing, neonatal, mental health wing kitchens, mortuary, nursery, patient hotel kitchen, plant room, theatre waiting room, wards, ward toilets and a maintenace room. Traps were wall mounted or ceiling suspended at a height of approximately two metres, sited away from sources of competing light and in a position to maximixse the interception of insects at potential ingress points. Catch trays of EFKs were emptied and cleaned without disinfection two weeks prior to sample collection. Glue boards from professional sticky traps were replaced with fresh boards every 2 weeks. Sample seasons were designated as experienced in the UK as follows: spring (March to May), summer (June to August), autumn (September to November) and winter (December to February). The contents of the

EFK's were recovered into sterile bags (Stomacher 400 Classic bags, Seward, UK). The glue boards from the sticky traps were aseptically removed and covered with a sterile plastic bag. The samples were transported to the laboratory and stored at 4°C pending identification and microbiological analysis. The insects were identified to species level where possible and to genus or family otherwise using a dissection microscope (Stereo Zoom Model GXM XTL 3101, GX Optical, Haverhill, UK) and entomological references (Colyer, C. N. & Hammond 1951, Unwin 1981, Chinery 1993, Chinery and Falk 2007, Chinery 2012). The insects were manipulated aseptically using entomological tweezers which were sterilised by submerging in ethanol (70% v/v) and subsequently passed through the flame of a Bunsen burner. Depending on classification, insects were sorted into 30ml screw-top sterile sample tubes (King Scientific, Liversedge, UK) and stored at 4°C, prior to microbiological analysis.

The grouping of arthropods proposed in the hospital study by Sramova et al. (1992) was applied in the currently described study; Parasites (insects that live on another organism causing it some harm, such as mosquitoes, sand flies, black flies, etc), Eusynanthropic (insects that are permanently synanthropic such as synanthropic flies, wasps, stored product insects), Hemisynanthropic (insects that are synanthropic on occasion such as ants and spiders) and 'occasionally encountered insects' (non-biting midges and other flies, moths, beetles).

Microbiological analysis of Diptera collected from the hospital locations

Individual Diptera assigned to the same identification and collected from the same flytrap were pooled according to identification into sterile phosphate-buffered saline (PBS) and washed / mixed by vortexing for 30 seconds. This method of pooling occurred in most

193 cases, however some pooling from different flytraps occurred when the number of
194 identified individuals at a particular hospital location was low. Larger flies were handled
195 similarly in that *Musca domestica*, *Calliphora vicina*, *Musca autumnalis, Lucilia sericata*,
196 *Phaonia* sp., *Helina* sp. were pooled into 1ml of PBS per fly i.e. 10 flies of the same
197 identification were pooled into 10ml of PBS. Flies of a medium size such as *Fannia*
198 *canicularis* were pooled into 0.5ml PBS per fly. The pooling of smaller flies such as those
199 of the families Psychodidae, Sphaeroceridae, Phoridae and Drosophilidae varied from six
200 flies to eighty per 1ml of PBS. The pooling of flies of the family Dolichopodidae varied from
201 seven to thirteen flies per 1ml of PBS. The pooling of *H. punctatissima* varied from one to
202 fifteen individuals per 1ml of PBS. Chironomidae x 10 were pooled into 1ml of PBS. *Culex*
203 *pipiens*, *Pollenia rudis* and *Sarcophaga carnaria* were pooled into 1ml of PBS per
204 individual. Both the external and internal parts of the flies were microbiologically analysed.
205 For purposes of this study, external structures were defined as the outer surface of the
206 insect exoskeleton including wings. Internal structures were defined as all other areas of
207 the insect excluding the outer surface of the exoskeleton. To minimise the risk of affecting
208 the internal bacterial population in the event an externally applied biocide were to
209 penetrate the insect, a non-biocidal approach was adopted for the removal of the
210 externally-carried bacterial population. Sample washings for microbiological analysis were
211 processed without delay and maintained at chilled temperatures on ice to minimise
212 changes in bacterial population number during sample preparation. Following the pooling
213 and vortexing, these external washings were then diluted by serial passage down to 10^{-6}
214 achieved by taking a 0.1ml volume of the washing PBS sample into 0.9ml of fresh PBS
215 and by thorough mixing to create a 10^{-1} dilution. Similarly, a 0.1ml volume of the resulting
216 10^{-1} dilution was taken into 0.9ml fresh PBS to create a 10^{-2} dilution. The serial dilution
217 process was repeated until a 10^{-6} dilution of the original sample was achieved. A 0.1ml
218 volume of each dilution was inoculated onto the surface of Cycloserine-Cefoxitin Fructose

Agar plus sodium taurocholate (Tc) (CCFA+Tc) for the selective isolation of *Clostridium difficile*, Nutrient Agar (NA) to determine the aerobic and anaerobic colony count Mannitol Salt Agar (MSA) for the selective culture of *Staphylococcus epidermidis* and *Staphylococcus aureus* and Violet Red Bile Glucose (VRBG) agar for the enumeration of the Enterobacteriaceae. All the culture media was obtained from Oxoid Ltd. (Basingstoke, UK). Nutrient agar, MSA and VRBG Agar plates were incubated at 37°C for 24 hours under aerobic conditions. The CCFA+Tc agar and NA plate to establish the anaerobic colony count were incubated at 37°C for 24 hours under anaerobic conditions. The number of bacterial cells recovered from each pooled sample was calculated as colony forming units (cfu) per ml accommodating for the different volume of PBS into which the insects were pooled, any dilution factor applied in the preparation of the serial dilution and x10 to correct for the 0.1ml volume inoculated onto the surface of the agar plate.

The pooled samples were washed four times further, with the same amount of PBS as the initial wash (fresh PBS added with each wash), in order to mechanically remove external bacteria and avoid contamination when examining macerates for bacteria as described previously by Davies (2015) and Davies et al. (2017). The flying insects were then macerated with the end of a sterile plate spreader in the same amount of PBS as for the initial external washing and the above process of sample dilution, inoculation onto microbiological culture media and incubation repeated for the macerated sample as described above. Bacterial colonies were initially identified and grouped together by characteristic macroscopic morphology. Further characterisation to the Genus and species level where appropriate was achieved by Gram staining, microscopic examination of cell morphology, oxidase and catalase tests, API 20E test kits, API Staph test kits, rapid ID 32A API test kits (BioMérieux, Marcy l'Etoile, France) and Bacillus-ID test kits (Microgen

Bioproducts Ltd, Camberley, UK) and for Streptococcaceae by reference to the UK Public England Standard for Microbiology Investigation (Public Health England, 2014). Calculation of the descriptive statistics of the bacterial loading on each fly (minimum, maximum, median and standard deviation) was performed using Microsoft Office 365 Excel version 1902.

Disk diffusion method for antimicrobial susceptibility testing of bacterial strains isolated from hospital true flies

The isolates were tested according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion method (EUCAST, 2017). Since disk diffusion criteria for antimicrobial susceptibility testing of anaerobes have not yet been defined, a minimum inhibitory concentration (MIC) method using a commercial MIC test strip (Liofilchem, Italy) was applied for testing *Clostridium* species according to the manufacturer instructions. The MIC of each isolate was determined in triplicate and a mean value calculated.

Results

Collection and identification of flying insects from hospitals

A total of 19,937 individual insects (and other arthropods) were collected from seven UK hospital locations within the period March 2010 to August 2011 in this study. Of these individuals, 114 arthropod species were identified. Table 1 shows that insects of the order Diptera were the most commonly identified of all flying insect orders sampled from hospitals, accounting for 76.3% of all samples. This was followed by Hemiptera (13.9%), Hymenoptera (4.7%), Lepidoptera (2.9%) and Coloeptera (2%). The remainder of the sample population was composed of Neuroptera, Thysanoptera, Psocoptera,Trichoptera,

Symphyta and Araneae. Among Diptera, the family Chironomidae represented the most numerous family accounting for 55.5%. Calliphoridae were the most common synanthropic fly, comprising 13.6% of all Diptera samples. Psychodidae, Phoridae, Sphaeroceridae and Cecidomyiidae, contributed 8.6%, 7.4% , 4.7% and 3.5% of Diptera respectively. Sciaridae accounted for 1.5% of Diptera population and Fanniidae for 1.1%. The remaining 4.1% of Diptera was represented by 25 less abundant families.

According to the synanthropy classification of Sramova et al. (1992), the 'occasionally encountered insects' were the group most commonly collected from the hospital locations, accounting for 64.9% of all insects. Eusynanthropic insects comprised 31.2% of the sample, while hemisynanthropic insects and parasites contributed 3.7% and 0.2% respectively.

Seasonal species abundance for all insects

Details of the abundance of insect Orders across the different seasons are reported in Table 2.

Microbiological analysis of true flies (Order Diptera) collected from hospitals

From the flying insects examined microbiologically, 86 different bacterial strains were identified by culture. Details of the source insect, hospital location, anatomical site and bacterial loading per fly is presented in table 2. Of the samples, 71% of occurrences of bacterial isolation were from internal structures, 16% from external structures and in 13% of cases, no bacteria were recovered. Among the bacterial species of interest due to their potential as opportunistic pathogens, the majority of Enterobacteriaceae species, including *Escherichia coli*, *Klebsiella pneumoniae* and several species of *Enterobacter* were isolated

mainly from the common housefly *Musca domestica*, but to a lesser extent also from *Calliphora vicina*, *Musca autumnalis* and *Lucilia sericata*. *Staphyloccocus aureus* was the main species of Staphylococci isolated (84.6 % of all *Staphylococcus* spp.) from *Musca domestica*, *Calliphora vicina*, *Musca autumnalis*, *Fannia canicularis*, *Lucilia sericata*, Psychodidae, Sphaeroceridae and *Phaonia* sp.

A summary description of the bacterial load (cfu) per fly per ml is presented in table 3. The highest median bacterial load was recovered externally from *C. vicina* at $1.4x10^6$ cfu fly^{-1} ml^{-1}, however this was not characteristic of the general pattern of bacterial loading across the dataset which tended to be higher from internal samples compared with external samples. Second highest was *L. sericata* at $3.1\ x10^4$ cfu fly^{-1} ml^{-1}, third highest in *M. autumnalis* at $4.6x10^3$ cfu fly^{-1} ml^{-1} and a variety of insect categories returned a bacterial load which was unrecoverable below the detection limit of 10 cfu fly^{-1} ml^{-1}. In one exceptional circumstance the level of *Enterobacter cloacae* and *Pantoea* sp. recovered from an internal sample of *M. domestica* returned at $1.0\ x\ 10^{10}$ cfu fly^{-1} ml^{-1}. However, as a general observation the standard deviation of the bacterial load was large and highly variable across the sample set.

Disk diffusion method for antimicrobial susceptibility testing of bacterial strains isolated from hospital flying insects

Of the 86 bacterial strains isolated from Diptera, 68 were further characterised for antibiotic susceptibility profiling using a standard disk-diffusion assay. An antimicrobial resistant phenotype was observed in 52.9%, that being defined as a bacterial strain resistant to at least one class of antibiotic according to EUCAST breakpoints. Enterobacteriaceae isolated from flying insects were susceptible to the majority of antibiotics included in the

study. Penicillin was the least effective antibiotic against the Enterobacteriaceae; 20% of *Pantoea* spp., 100% of *Raoultella terrigena* and 40% of *Escherichia* spp. were resistant to ampicillin and 40% of *Pantoea* spp. strains were resistant to Amoxicillin-Clavulanic acid. *Staphylococcus* species were mainly resistant to Penicillin with 80% of *S. aureus* isolates demonstrating a resistant phenotype. In table 4, resistance profiles of *Bacillus* spp. isolates could not be classified according to EUCAST breakpoints because of the lack of current guidelines, therefore MICs are reported as millimetres of inhibition. In this study, *Bacillus* spp. were considered as resistant if the diameter of the inhibition zone was less than 11mm. All the *Bacillus* spp. strains were susceptible to Imipenem, Ciprofloxacin, Levofloxin, Gentamicin, Tetracycline and Chloramphenicol at the concentrations detailed in table 4. Only two strains of *Bacillus sphaericus* were resistant to Streptomycin. Regarding *Enteroccoccus* sp., *Streptococcus* spp. and *Clostridium* sp., all the isolates tested showed resistance towards Penicillin G and Clindamycin. Only *Enterococcus* sp. was resistant to Fluoroquinolones, and Erythromycin was not efficacious on the β-haemolytic *Streptococcus* strain. *Enterococcus* sp. and *Clostridium* sp. showed resistance towards Vancomycin. The isolates were assessed for multi-drug resistance (MDR), that being defined as resistance to equal or greater than two classes of antibiotics. Among the Enterobacteriaceae there was the lowest incidence of MDR, recorded at 3.1%. The highest rate of MDR was found in *Staphylococcus* spp., among the 12 strains tested, five showed to be MDR. All the isolates of *Enterobacter* sp., *Streptococcus* spp. and *Clostridium* sp. were MDR (Table 4).

Discussion

Studies on hospital insects and their potential for dissemination of bacterial pathogens have been conducted worldwide, however the majority have focused on a single group of

insects, in particular crawling insects such as cockroaches or ants. In this study UV light flytraps in the form of Electronic Fly Killers and professional sticky traps were used to collect a wider range of flying insects in order to explore the composition of the flying insect populations in seven UK hospitals. Additionally, bacteria isolated from Diptera were characterised for antibiotic resistance to further assess their potential risk as vectors of antimicrobial-resistant bacterial pathogens. Recognising that not all insects will be captured using the traps employed in this study, it was demonstrated that Diptera were the most abundant of all the flying insects across the seven UK hospitals sampled. These findings correspond with previous studies which also showed that flies were the predominant insects in hospitals (Da Silva et al. 2011, Kappel et al. 2013). Chironomidae was found to be the most abundant fly family. This observation is consistent with a previous study undertaken in an hospital in Prague, where Chironomidae represented 12.4% of the total Diptera collected (Sramova et al. 1992). In the current study no bacteria were isolated from Chironomidae, however a recent study reported *Aeromonas* spp., an opportunistic pathogen, to be the most abundant genera in larvae of Chironomidae (Halpern and Senderovich 2015). The same study proposed Chironomidae as a potential natural reservoir for *Vibrio cholerae*, being detected in eggs and larvae in low abundance. Other studies of bacteria isolated from Chironomidae reported *Achromobacter, Acinetobacter, Bacillus, Citrobacter, Clostridium, Corynebacterim, Edwardsiella, Enterobacter, Escherichia, Klebsiella, Micrococcus, Pseudomonas, Serratia, Providenia, Yersinia* and *Staphylococcus* (Rouf 1993). Their true role as a vector of disease is still unknown, however in consideration of the pathogenic bacteria they have been demonstrated to carry, they may represent a public health risk.

Calliphoridae were the second most frequently encountered Dipteran Family in this study. Similarly to other 'filth flies', the potential health risk associated with their presence is high since they move indiscriminately between filthy, decaying, organic matter and human food or surfaces frequented by humans. Several papers have demonstrated that Calliphoridae act as mechanical vectors of bacteria acquired from the surrounding environment (Chaiwong et al. 2014, Russell et al. 2017, Pace et al. 2017). Like the majority of insects, flies have adhesive pads, or pulvilli, that facilitate their adherence to surfaces but also increase their ability to retain bacteria (Graczyk et al. 2001). In this study the majority of bacteria isolated from *C. vicina* sampled from hospitals were of the family Enterobacteriaceae, followed by Staphylococci. The association of *C. vicina* and Enterobacteriaceae, which are commonly isolated from the gut of animals, is unsurprising as these flies typically develop on animal carcasses and can feed on faeces, which are recognised sources of such bacteria (Erzinclioglu, 1996).

The next most commonly encountered flies were of the family Psychodidae. Several previously published studies (Pelli et al. 2007, Nmorsi et al. 2007, Faulde and Spiesberger 2013) reported that Psychodidae were an emerging problem in hospitals. Drain flies were of particular note with respect to public health as recognised carriers of *Clostridium difficile* (Burt et al. 2012). Faulde and Spiesberger (2013) showed that *Clogmia albipunctata* isolated in the hospital environment should be considered as a potential vector for pathogenic bacteria associated with nosocomial infections. In this study *C. difficile* was not isolated, however other pathogenic bacteria isolated from these flies were *Bacillus cereus* and *Staphylococcus aureus. Bacillus cereus* has previously been isolated from Psychodidae as described by Faulde and Spiesberger (2013), but there appears no currently published record of *S. aureus* isolated from these flies.

Considering the seasonal abundance of arthropods collected in this study, the highest was found to be in the summer months, followed by spring, autumn and winter. Overall, arthropod abundance in spring and summer is likely to be explained by the strong influence that the higher ambient temperature, typically experienced in these seasons, has on arthropod physiology. For example, the notable summer peak of Hemiptera may be explained by the observation that 71% of the Hemiptera collected were Aphididae which in their adult form reproduce extensively in summer while they spend the cooler months as eggs (Skaljac 2016).

A similar seasonality likely linked to typical ambient temperature was not observed in the Diptera, which were most represented across all seasons; greatest in abundance in spring, followed by autumn, summer then winter. This different trend may be explained by the fact that several Diptera species are synanthropic and the year-round availability of their breeding media and the constant temperatures provided by the centrally heated hospital institutions provide the requisite conditions for their survival throughout the year. Of particular note was a peak in isolation of *Calliphora vicina* in autumn which may be explained by increased availability of their preferred breeding matter, such as carrion.

The numbers of Lepidoptera and Hymenoptera were consistently represented across the seasons, however a small peak was observed in the summer months, again most likely attributable to higher ambient temperatures facilitating an increased rate of development and growth, and a higher number of generations per year (Dale and Frank 2017). Coleoptera were similarly represented across the seasons, however with a small peak in the number of *Harmonia axyridis* during the winter months, most likely a consequence of

this coleopteran overwintering in aggregated communities in the indoor environment, emerging in spring (Knapp et al. 2018).

Whilst determining the bacterial numbers recovered from the external sample sites of the Diptera it remains possible that a small proportion of the total number resulted from environmental contamination whilst the insects were in the trap, however this would be anticipated to be minimal and not contribute to the bacterial populations recovered from the internal sample sites. Enterobacteriaceae were the group of bacteria most commonly isolated from flying insects, followed by *Bacillus* spp., Staphylococci, Clostridia, Streptococci and *Micrococcus* spp. These findings are in subtle contrast to those of a similar study (Kappel et al. 2013), where Gram-Positive bacilli represented 68.2% of bacterial strains isolated from non-biting flying insects collected in a Brazilian hospital. It is likely this difference is attributable to the prevailing environmental conditions in Brazil compared with the UK. Another possible reason may be a difference in the kind of patients and diseases of the sampled hospital wards in the two countries.

All Enterobacterial strains isolated in this study were opportunistic human pathogens. The antimicrobial susceptibility testing revealed that Enterobacteriaceae were susceptible to the majority of antibiotics tested. Members of the Enterobacteriaceae are a common cause of nosocomial infections and antibiotic-resistant strains in clinical settings are a public health concern worldwide (Perez 2018, Ruppé et al. 2018).. In several studies antibiotic-resistant Enterobacteriaceae have been isolated from hospital insects (Gliniewicz et al. 2003, Davari et al. 2010, Faulde and Spiesberger 2013, Pai 2013, Loucif et al. 2016). In the current study a strain of *Enterobacter cloaceae* resistant to Ertapenem and with an intermediate resistance to Imipenem was identified. This bacterium is considered by the

WHO as a priority pathogens for Research and Development of new antibiotics indeed, according to WHO, *Acinetobacter baumannii*, *Pseudomonas aeuroginosa* and carbapenem-resistant Enterobacteriaceae are considered as the first priority (WHO 2017). With the exception of this resistant strain of *E. cloaceae*, overall it appeared that the Diptera collected in this study carried bacterial pathogens capable of causing infections in humans which would be considered treatable on the basis of their antimicrobial susceptibility patterns.

Staphylococcus aureus was the most common Staphylococci isolated from Diptera and the incidence of resistance in this genus was high, in particular towards β-lactam antibiotics. This is not surprising as according to Lowy (2003) more than 90% of staphylococcal isolates are now resistant to penicillin driven by exposure to β-lactam antibiotics in multiple environments including environmental, clinical and veterinary (Miragaia 2018). The incidence of penicillin resistance observed in *Staphylococcus* isolates in this study is similar to those of Teixeira et al. (2009), where the microbiota of Trump ants and the antimicrobial susceptibility patterns of isolates was investigated.

The link between the presence of insects in hospitals and the incidence of infection is not fully established, however it is a recognised risk factor. During investigation of infectious disease it is not uncommon for the source and routes of transmission to remain unidentified and in these circumstances, risk factors that might have led to exposure and subsequent infection are considered as predisposing factors. Dipterans in both the domestic and hospital environment have been implicated as specific risk factors for the development of a variety of infectious diseases. Knight et al. (1992) identified Calliphoridae and Muscidae as risk factors for acute diarrhoea in Malaysian children.

Households that did not use fly covers to protect stored food were twice as likely to be 'case' households. The risk factor attributed to flies was almost equivalent to that of the children's carers not washing their hands. Sengupta et al. (1995) recovered *Vibrio cholerae* O139 from Calliphoridae and Muscidae associated with families of patients hospitalised due to cholera infection. The level of recovery of *Vibrio cholerae* from Calliphoridae and Muscidae was comparable to the level of recovery from the washings of the hands of contacts of the index cases. It is accepted that thorough hand-washing is an essential component of infection prevention and control policies (Loveday et al. 2014) and the risk factor studies by Knight et al. and Sengupta et al. suggest that the failure to control flying insects carries an infection risk comparable to that associated with a lack of hand-washing.

The current study sampled the flying insect populations over a 16-month period of seven UK hospitals and investigated the associated culturome of Diptera, further characterising the antibiotic-resistance profile of bacterial isolates. This contributes to a growing body of evidence that seeks to identify the human infection risk factors associated with flying insect populations and the potential positive impact of controlling these to reduce infectious disease burden by informing infection risk assessments and prevention control strategies.

Conflict of interest statement

None declared.

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Figure Legends

Table 1 Arthropod orders sampled from seven UK hospital locations within the period March 2010 to August 2011. The Arthropod Orders fall into three categories of abundance with Diptera and Hemiptera representing the most abundant, followed by Hymenoptera, Lepidoptera and Coleoptera in the second category and the remaining orders representing <0.1% of those individuals isolated.

Table 2 United Kingdom seasonality of the five most abundant insect orders from seven UK hospital locations within the period March 2010 to August 2011, presented as mean number of insects per hospital per sampling occasion. Diptera dominate in abundance across all four seasons with Hemiptera showing a marked increase in the summer months.

Table 3 Details of the source insect and sample site (internal or external), pool identifier including number of individuals in that pool, sample location within the hospital, bacterial identification and cfu isolated per fly per ml. Bacteria were recovered from 71% of internal structures, 16% from external structures and in 13% of cases no bacteria were recovered. The sample pool identifier enables cross reference to the individual bacterial isolate antibiotic susceptibility patterns detailed in table 4.

Table 4 Summary descriptive statistics of the bacterial cfu per fly per ml recovered from the internal and external samples of Diptera. Internal samples tended to yield a greater bacterial load than external, however the variance associated with the levels of bacteria recovered was large across the data set. Samples recorded as <10 cfu fly^{-1} ml^{-1} were below the detection threshold of the microbiological assay.

Table 5 Antibiotic susceptibility patterns of bacterial isolates expressed as resistant (R), Intermediate (I) or Sensitive (S) according to EUCAST breakpoint guidelines. *Bacillus* spp. are expressed in millimetres zones of inhibition. The MIC of each strain was examined in triplicate. An antimicrobial resistant phenotype to at least one class of antibiotic was observed in 52.9% of cases. Multi-drug resistance was recorded in 19.1% of cases, the

715 highest rate being observed in *Staphylococcus* spp. The sample pool identifier enables
716 cross reference to the source of the bacterial isolate as detailed in table 2.
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