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Surveillance of antimicrobial resistant bacteria in flies (Diptera) in Rio de Janeiro city

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ABSTRACT

Antimicrobial-resistant bacteria were isolated from muscoid dipterans collected at five different areas of Rio de Janeiro city, in proximity to hospitals. Extracts obtained by maceration of flies were diluted and used as inocula for different culture media, with or without antibiotic (ceftriaxone 1 mg/L) supplementation. Purified isolates were submitted to antimicrobial susceptibility testing (AST). Bacterial identification was performed by MALDI TOF Microflex LT (Bruker Daltonics). A total of 197 bacterial strains were obtained from 117 dipterous muscoids. Forty-two flies (35.9%) carried bacteria resistant to at least one antimicrobial, while 7 insects (5.9%) carried multidrug-resistant bacteria (MDR), which were all members of the family Enterobacteriaceae. Among 10 MDR bacteria (5%), 5 strains (2,5%) were positive by PCR for one or more of the following antibiotic resistance genes: *aac(6')-Ib*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$. Analysis of variance (ANOVA) and cluster analysis compared the number of resistant isolates per collection point and showed that a single location was statistically different from the others with regard to resistance. Although there are still no criteria to determine the environmental contamination by resistant bacteria the fact that they have been isolated from flies is an indication of a disseminated contamination. As such, these insects may be useful in monitoring programs of antibiotic resistance in non-hospital environments, where they could function as sentinels.

Introduction

The projections of annual global deaths from antibiotic-resistant infections are estimated to grow from 700,000 in 2014 to 10 million by 2050, resulting in extensive financial burdens for health services worldwide (He et al., 2020). The production of livestock is estimated to be responsible for more than half of all the antibiotics used globally. However, it has been reported that only 10% of studies on the topic of antibiotic resistance have considered the potential contribution from animal husbandry (He et al., 2020). The concept of One Health may be defined as a collaborative effort between multiple disciplines and achieve optimal health for humans, animals, and the environment (Collignon and McEwen, 2019). Thus, research that integrates these three fields is essential for tracking the emergence, persistence, and dissemination of antimicrobial resistance and for developing prevention and control strategies. Although it is generally recognized that antimicrobial resistance is a natural and inevitable phenomenon, there is clearly an urgent need to develop and adopt measures to curb the indiscriminate use of antimicrobials and consequently try to prevent the advance of antimicrobial resistance mechanisms.

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In this context, antibiotic resistance is disseminated between and within diverse interconnected environments, including animals, farms, soil, sewage, residences, restaurants, and the community in general, including hospitals. Importantly, muscoid dipterans are frequently found in each of those environments and as such they are considered to act as one of the main agents of dissemination and dispersion of resistant bacteria, both pathogens and commensals (Kappel et al., 2013; Chaiwong et al., 2014) and are myiasis causing agents (Azevedo et al., 2015). The synanthropic flies from the families Muscidae and Calliphoridae are particularly involved in public health problems, mainly affecting poorer human with deficient sanitary conditions (Blaak et al., 2014; Ranjbar et al., 2016; Songe et al., 2016 and Zhang et al., 2018).

The study of Nazari et al. (2017) reported that flies from hospital settings showed greater levels of contamination with pathogens than did the same insects collected from non-hospital environments. Those authors proposed that flies could be used as indicators of contamination including by antimicrobial resistant bacteria. However, it has been argued that more studies should be conducted to endorse the "sentinel role of flies for antimicrobial resistance monitoring" (Poudel et al., 2019). In response to that recommendation, the present investigation sought to evaluate the potential of flies (collected from environments in proximity to hospitals in Rio de Janeiro, Brazil), as a vehicle for the transport of bacterial strains of concern to public health. In addition, the study classified the levels of phenotypic resistance towards multiple antibiotics and determined the presence of antibiotic resistance determinants of clinical importance.

2. Material and methods

2.1. Areas of collection, capture and identification of muscoid dipterans

This study was conducted in the city of Rio de Janeiro, Brazil. Collections were made during 2014, 2015, 2016 and 2017 at 5 different sampling points throughout the city. Collection points were selected based on their proximity to hospitals: **Point 1**- Amorim Community (-22.875775,-43.250680); **Point 2** - Fiocruz Institute campus (-22.878228,-43.245142); **Point 3** - Quinta da Boa Vista (-22.906929,-43.222696); **Point 4** – a garbage dumpster on the property of the Hospital da Piedade (-22.891561,-43.309761) and **Point 5** - 100 meters from Point 4, where there was a dumpster of household waste, used by local residents (-22.892372,-43.309836). Flies were captured with black plastic traps designed to avoid the passage of light. An attractive bait (putrefied beef, which was allowed to decompose for more than 24 h at room temperature) was placed inside the traps. Importantly, the bait was covered by sterilized nylon nets in order to avoid contamination of the flies with meat-associated bacteria. Upon entering the trap through openings on the side, seeking light (positive phototaxis), flies became trapped within a plastic bag located on the top of the trap. Examination of the traps and removal of captured flies was conducted 24 h after installation. The average temperature during the collection period was 29 ºC, with no precipitation. Captured flies were placed in individual sterilized test tubes and ascribed a code. Upon arrival in the laboratory flies were anesthetized, by placing the tubes on ice, and identified using dichotomous keys for South American Diptera (Carvalho and Mello-Patiu, 2008).

2.2. Isolation and storage of bacteria

All 117 dipterans were macerated with sterile pestle in 1 ml of sterile saline, vortexed and serially diluted to 10^{-4}, then each was used as inocula for plates of Nutrient Agar (NA) and NA supplemented with ceftriaxone (1 mg/L).In addition, 22 insects were also plated on Sheep Blood Agar and MacConkey Agar. The plates were all incubated at 25 and 37°C. The number of colony forming units (CFU/mL) was determined directly on the plate after 24 h of incubation at 25 and 37 ºC. Representatives of the different colony morphologies were sub cultured on fresh plates of the appropriate isolation medium, to obtain pure cultures with subsequent storage in Brain Heart Infusion Broth (BHI - Merck) supplemented with 20% glycerol (Sigma) at -20 ºC.

2.3. Antimicrobial sensitivity testing (AST)

AST was performed by the Kirby-Bauer disc diffusion method on plates of Mueller-Hinton Agar and interpreted according to the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2017). The following antibiotics were used: cefepime (30 μg), ceftazidime (30 μg), cefoxitin (30 μg), meropenem (10 μg), gentamicin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg) and trimethoprim-sulfamethoxazole (2375/125 μg) (Sensifar). Determination of the antimicrobial resistance profile employing the automated system (BD Phoenix Automated Microbiology System (BD Diagnostics) was performed with bacterial isolates which demonstrated a MDR profile and that were positive for amplification of at least one of the resistance genes (Winstanley and Courvalin, 2011). The criteria used for characterization of strains as multidrug resistant were those reported by Magiorakos et al. (2012).

2.4. Bacterial identification by MALDI-TOF-MS

Isolates which showed resistance to at least one of the antibiotics tested were examined by MALDI-TOF-MS (Bruker Daltonics).

2.5. Bacterial identification through partial 16S rRNA gene sequencing

Isolates not classified using MALDI-TOF-MS were identified by sequencing PCR amplicons, generated using the primer pair 515F/806R, corresponding to the variable region 4 (V4) of the gene encoding 16S rRNA as reported previously (Zahner et al., 2008).

2.6. Molecular detection of resistance genes

A battery of polymerase chain reaction (PCR) assays were used to screen phenotypically resistant bacterial isolates for the following resistance determinants; colistin (*mcr-1* and *mcr-2*) (Liu et al., 2016; Xavier et al., 2016), β-lactamases genes (bla_{SHV}, bla_{TEM}, bla_{SPM}, bla_{CTX-M}, bla_{NDM}, bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{GES}, bla_{SIM}, bla_{OXA-23}, bla_{OXA-48}, $bla_{OXA-143}$) (Poirel et al., 2001; Pagani et al., 2003; Dallenne et al., 2010; Higgins et al., 2010; Poirel et al., 2011), enzyme-modifying aminoglycoside encoding gene (*aac*-(6)-Ib) (Warburg et al., 2012) and integrase class 1 integron gene (*Int1*) (Gillings et al., 2015). Amplicons were sequenced at the DNA-PDTIS sequencing platform (IOC-FIOCRUZ), sequences were aligned by BioEdit Program Version 7.0 and entered into the BLAST search algorithm and the NCBI nucleotide database to determine gene identity.

2.7. Statistical analysis

Analyses of variance (ANOVA) were performed to compare sample population averages and determine statistical differences. The number of resistant isolates per collection point was used. In addition, Student's T test was applied to perform comparisons between the data derived from individual points. Both tests were performed with Excel data analysis supplement, with a value of $p < 0.05$ considered as significant. Cluster analysis, which compared the resistance data for each collection point, was performed using Statistica software version 7.0, and weighted data were used (considering the total number of isolates per collection point). The grouping method configured in the Statistica program was that of complete linkage, which is based on the greatest distance between the elements. It is important to note that only strains obtained from NA plates, a non-selective culture medium (with or without ceftriaxone supplemention at 1 mg/L) were considered in the analysis. Strains were classified according to established criteria (CLSI, 2017).

Intrinsic resistance bacteria were not considered.

3. Results

3.1. Collection of flies

A total of 117 muscoid dipterans were collected. Details of the flies identified from each point of capture are shown in Table 1, while data in relation to the correlation between bacterial strains isolated at each collection point and the corresponding species of fly, from which they were isolated, are presented as Supplementary data (Table S1). Details of bacterial identification at the species level are provided as Supplementary data (Table S2). The information provided in Supplementary data Table S3, illustrates the correlation between the point of collection and the presence of bacteria exhibiting phenotypic resistance towards the different antimicrobials tested. Among the 117 dipteran specimens collected, 50 (42%) were identified as members of the family Calliphoridae: *Chrysomya megacephala* (Fabricius, 1794) (n = 44; 37.6%), *Chrysomya putoria* (Wiedemann, 1818) (n = 3; 2.56%) and *Lucilia cuprina* (Wiedemann, 1830) (n = 3; 2.56%). The major representative from the Muscidae family was *Musca domestica* (Linnaeus, 1758) (n = 18; 15.4 %), being collected at all points (except Point 3). The species *Ophyra chalcogaster* (Wiedemann, 1824) and *Synthesiomyia nudiseta* (Wulp, 1883) were also captured infrequently (n = 2,1,7% and n = 3,2,6%, respectively). Fourteen (11,9%) *Atherigona orientalis* (Schiner) were captured. Representatives of the Sarcophagidae family were: *Tricharaea* (*Sarcophagula*) *occidua, Peckia* (*Sarcodexia*) *lambens* and *Malacophagomya filamenta* (n = 2, 1,7%; n = 2, 1,7% and n = 1,0,85%, respectively).

3.2. Bacterial identification and prevalence of AMR isolates in relation to collection point

Intially a total of 238 bacterial colonty types were recovered from the 117 flies. However, during the process of subculture 41 bacterial colonies became non-viable resulting in a final total of 197 isolates that were employed in the subsequent analayses. The use MALDITOF-MS resulted in the identification of 175 strains at the species or genus levels. Supplementary data Table S1 provides details of bacterial identifications according to the MALDITOF-MS scores. Bacterial genera labeled with (*) means probable genus level identification with scores between 1.7–2.0, while bacterial species with (**) means certainty of genus and possible species, with scores between 2.0–2.3, and bacterial species with (***) denote confident identification, with scores above 2.3. The most frequently recorded taxonomic groups were: the order Enterobacterales (n = 54,27,4%), including the families Enterobacteriaceae, Morganellaceae, Erwiniaceae, Yersiniaceae and Hafniaceae (Adeolu et al., 2016), secondly the Pseudomonadaceae (n = 37,18,7%) followed by the Bacillaceae (n = 20,10,15%), Staphylococcaceae (n = 14,3%), Enterococcaceae (n = 14,3%) and Moraxellaceae (n = 11,5, 6%).

Forty two of the 117 flies (35.9%) carried bacteria that demonstrated phenotypic resistance to at least one antimicrobial compound. Based upon the criteria established by Magiorakos et al. (2012) 10 (5%) isolates, identified as *Escherichia coli* (n = 5), *Serratia marcescens* (n = 2), *Klebsiella quasipneumoniae* (n = 1), *Raoultella ornithinolytica* (n = 1) and *Enterobacter cloacae* (n = 1) were classified as MDR (Table 2). The MDR strains were isolated from 7 to 117 (5.9%) the flies. A common feature recorded for the MDR isolates was the detection of resistance or intermediate resistance to at least one of the broad-spectrum cephalosporins examined (cefepime or ceftazidime).

Table 1
Species of muscoid dipterans identification and number of individuals captures at each of five collection points.

Collections Points	Identification of muscoid dipteran species and the number (n) of individuals collected by species
Point1	*Chrysomya megacephala* (31), *Chrysomya putoria* (3), *Musca domestica* (7) **Total: 41 muscoid dipterans**
Point2	*Musca domestica* (2), *Lucilia* sp. (1), *Ophyra chalcogaster* (2), *Atherigona orientalis* (2), *Synthesiomyia nudiseta* (2), Sarcophagidae (2) **Total: 13 muscoid dipterans**
Point3	Sarcophagidae (1), Muscidae (1), *Chrysomya megacephala* (5) **Total: 7 muscoid dipterans**
Point4	*Chrysomya megacephala* (3), *Lucilia cuprina* (3), *Musca domestica* (7), *Atherigona orientalis* (9), *Synthesiomyia nudiseta* (1), *Tricharaea* (*Sarcophagula*) *occidua* (1), *Fannia pusio* (1), *Fannia* sp. (2), *Peckia Sarcodexia lambens* (1), *Sarcophaga* (*Liopygia*) *ruficornis* (1), *Euxesta* sp. (2), *Sarcophagidae* (2), *Fannidae* (2), **Total: 35 muscoid dipterans**
Point5	*Chrysomya megacephala* (5), *Lucilia cuprina* (1), *Musca domestica* (2), *Tricharaea* (*Sarcophagula*) *occidua* (1), *Malacophagomya filamenta* (1), *Atherigona orientalis* (3), *Peckia lambens* (1), *Sarcophagidae* (2), *Phoridae* (1), *Chloropidae* (4) **Total: 21 muscoid dipterans** **Total: 117 muscoid dipterans**

3.3. Statistical analysis of phenotypic resistance in relation to collection point location

Student T test and cluster analysis (Fig. 1) demonstrated that collection Point 5 was the only location which differed statistically from the others ($p < 0.05$) in terms of the number of resistant isolates recovered. The similarity measured for cluster analysis was the frequency of resistant bacteria to at least one antimicrobial, weighed by the total number of isolations at that point. The number of resistant isolates for each antimicrobial in relation to the location of the collection points are presented in Supplementary data Table S3.

3.4. Molecular detection of resistance genes

The results obtained for the PCR assays are provided in Table 3 which

Table 2
Details of the resistance phenotypes recorded for MDR Enterobacterales isolated from flies.

Origin	Strain (collection number) Species identification	antimicrobial resistance profile
Point 4	(14) *Escherichia coli*	Tetracycline, cephalosporins, ciprofloxacin, trimetoprim-sulfametoxazol
	(104) *Escherichia coli*	Cephalosporins, chloramphenicol, ciprofloxacin
	(105) *Escherichia coli*	Meropenem, cephalosporins, ciprofloxacin
Point 5	(23) *Klebsiella quasipneumoniae**	Meropenem, cephalosporins, gentamicin, ciprofloxacin, cefoxitin, trimetoprim-sulfametoxazol
	(25) *Escherichia coli*	Tetracycline, meropenem, cephalosporins, trimetoprim-sulfametoxazol
	(26) *Escherichia coli*	Tetracycline, meropenem, cephalosporins, gentamicin, cefoxitin, trimetoprim-sulfametoxazol
	(53) *Enterobacter cloacae*	Tetracycline, meropenem, cephalosporins, gentamicin, ciprofloxacin
	(71) *Raoultella ornithinolytica***	Tetracycline, cephalosporins, gentamicin, ciprofloxacin, trimetoprim-sulfametoxazol
	(119) *Serratia marcescens*	Cephalosporins, gentamicin, cefoxitin
	(134) *Serratia marcescens*	Tetracycline, meropenem, cephalosporins, gentamicin

Additional details were provided in *Carramaschi et al. (2021), **Carramaschi et al. (2019).

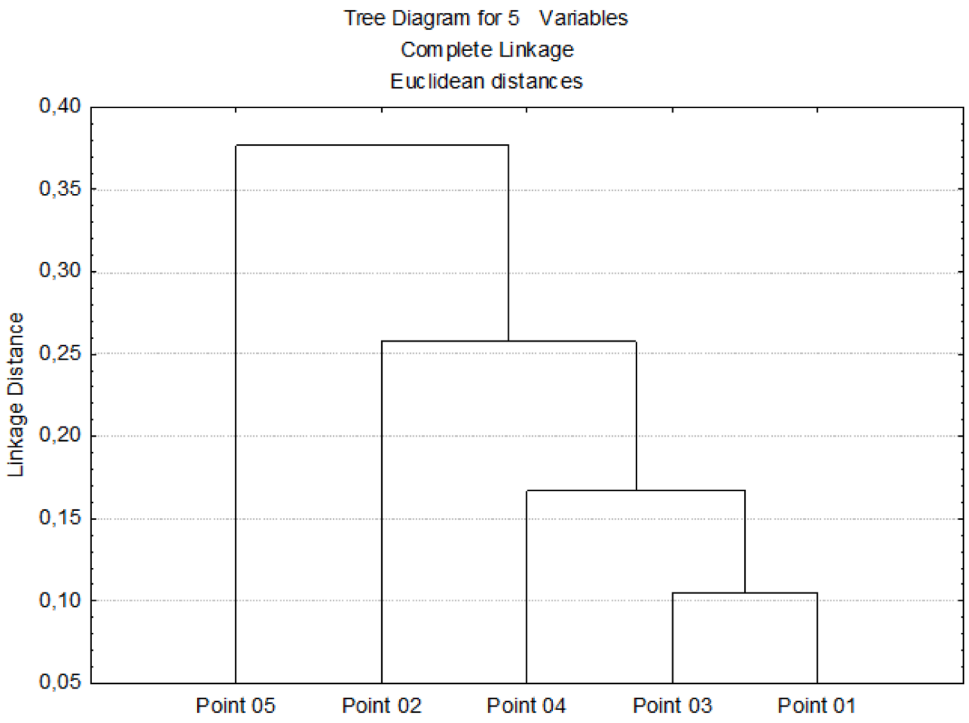

Fig. 1. Cluster analysis generated by the Statistica version 7.0 program, considering the number of resistant isolates per collection point and the tested antimicrobials, weighting the total number of isolates per point.

Table 3
Strains carrying any resistance gene, collection point, fly species and AST profile.

Strain	Collection Point	Origin (Fly species)	PCR Resistance Profile	Antimicrobial resistance profile (*Phoenix*)
JC3 *Bacillus* sp.	P1	*Chrysomya megacephala*	*int1*	ND
Lemef105 *E. coli* (MDR)	P4	*Lucilia cuprina*	bla_{KPC-2}, *aac(6')-Ib.*	AMP, ASB, CPM, CAZ, CRO, CIP, ETP, PPT
Lemef23 *K.quasipneumoniae** (MDR)	P5	*Musca domestica*	bla_{NDM-1}, bla_{TEM-1}, $bla_{CTX-M-15}$, *int1*, aac(6')-Ib,	AMP, ASB, CPM, CFO, CAZ, CRO, CIP, ETP, GEN, IPM, MEN, PPT, TGC
Lemef71 *R. ornithinolytica*** (MDR)	P5	*Malacophagomya filamenta*	$bla_{KPC-2,}$ bla_{TEM-1}, *int1, aac(6')-Ib,*	AMP, ASB, CPM, CAZ, CRO, CIP, ETP, GEN, PPT, TGC
Lemef17 *K. ascorbata*	P5	*Chrysomya megacephala*	$bla_{CTX-M-15}$	AMP
Lemef26 *E. coli* (MDR)	P5	*Musca domestica*	bla_{NDM-1}, *int1, aac(6')-Ib*	AMP, ASB, CPM, CFO, CAZ, CRO, CIP, ETP, GEN, IPM, MEN, PPT

AMP (ampicillin), ASB (ampicillin-sulbactam), CPM (cefepime), CFO (cefoxitin), CAZ (ceftazidime), CRO (ceftriaxone), CIP (ciprofloxacin), ETP (ertapenem), GEN (gentamicin), IPM (imipenem), MEN (meropenem), PPT (piperacicllin-tazobactam) and TGC (tigecycline). More details were provided in *Carramaschi et al. (2021) and **Carramaschi et al. (2019).

also gives details of the antimicrobial resistance profiles determined according to BD Phoenix Automated Microbiology System (BD Diagnostics). It was observed that resistance genes were amplified only in Enterobacteriacea. In addition, *Bacillus* sp. (JC3) isolated from a specimen of *C. megacephala,* was positive for the *int1* gene (Table 3).

4. Discussion

To evaluate the possibility of using flies as sentinel organisms in relation to antibiotic resistance, an enhancement in our knowledge concerning the presence of MDR bacteria associated with these insects is clearly essential. Although muscoid dipterans are the predominant insects in the environment (Kappel et al., 2013) studies reporting the microbial communities associated with insects in hospitals settings and/or in surrounding environments have commonly focused on cockroaches (Prado et al., 2006), ants (Fontana et al., 2010) or specifically on the species *Musca domestica* (Rahuma et al., 2005; Ranjbar et al., 2016; Sobur et al., 2019; Akter et al., 2020). However, as shown in the present study and in a recent investigation of hospitals in the United Kingdom (Boiochi et al., 2019), MDR bacteria are present in a diversity of fly species.

Flies were identified at the species level, with the exception of individuals belonging to the genera *Lucilia, Euxesta* and *Fania* as well as some individuals belonging to the families Sarcophagidae, Phoridae and Chloropidae. This difficulty occurred due to the impossibility of handling them, since they were placed in sterile glass test tubes and identified only by viewing through the tube wall, in order to avoid possible contamination in the laboratory.

Flies have bristles on their legs and pulvili that facilitate adherence to surfaces and also serve to increase colonization by bacteria and other microorganisms present on contaminated surfaces (Graczyk et al., 2001). Furthermore, flies carry pathogens in their alimentary tract which may be transmitted during feeding, regurgitation, and defecation. In addition, horizontal transmission of antibiotic resistance has been reported to occur specifically in the crop of this insects (Stoffolano, 2019).

It has been shown that 'filth flies', specifically Muscidae and Calliphoridae, are involved in the transmission of a variety of veterinary-medically important pathogens (Onwugamba et al., 2018), contributing to their dissemination due to the ability of these insects to fly long

distances between habitats (Braack et al., 1990; Nazni et al., 2005). As such, it has been postulated that 'filth flies' may also play an important role in the spread of antimicrobial resistance (AMR) between animals and humans.

The data presented in Table 1 demonstrated that *C. megacephala* (Calliphoridae) was the principal 'filth fly' encountered. This synanthropic species was introduced in the American continent in 1970, dispersing it self quickly and causing a population decline of native species (Guimarães et al., 1978). According to Leandro and D'Almeida (2005), *C. megacephala* is an r-strategist species and food generalist, which adapts to environments with different landscapes, such as forest fragments and even urban regions. It is worth mentioning that representatives of *C. megacephala* were recovered from all the points analyzed, with the exception of point 2, which corroborates its classification as synanthropic fly (Guimarães et al., 1978).

Musca domestica (Linnaeus, 1758) was the major representative of the family Muscidae, being registered at each of the five collection points. It is a cosmopolitan species, considered a problem in urban areas without adequate sanitary management (Muñoz and Rodríguez, 2015).

In relation to the resistance rate per collection point it was not considered surprising that points 4 and 5 showed the highest resistance rates in terms of absolute number of bacterial isolates (Table 1). Interestingly, point 5 (next to a hospital) was shown to be statistically different from the other collection points (Fig. 1). It is pertinent to note that this location, differed from the others owing to the practice of improper disposal of solid waste, such as household waste, soiled diapers, and food scraps. The presence of such materials contributes to the proliferation of disease-transmitting vectors, such as mice, which are reservoirs of different diseases, cockroaches and flies, increasing the risk of human exposure to a wide variety of pathogens (Mucelin and Bellini, 2008; Cardozo et al., 2009). In addition, the proximity to the hospital may contribute to the carriage, by the flies, of bacteria that possess different resistance mechanisms, making point 5 a potential hotspot for resistance dissemination. It is well established that flies are able to fly 5–7 Km, or even more, however when provided with favorable environment conditions and with sufficient food resources they tend to persist at such sites (Stoffolano, 2019).

Flies captured from within hospital settings or from animal production facilities frequently carry antimicrobial resistant bacteria. More worryingly, when collected inside hospitals they may also be associated with the transmission of agents associated with nosocomial infections and demonstrate elevated levels of pathogen carriage when compared to flies collected from external environments (Zurek and Ghosh, 2014; Nazari et al., 2017). Thus based on our data, it may be hypothesized that the higher levels of antibiotic resistance recorded at point 5 may have emerged owing to the flies transiting between the hospital garbage and the external areas. In addition to phenotypic resistance, collection point 5 was also the location where the greatest diversity of resistance genes, $bla_{\text{KPC-2}}$, *aac(6')-Ib,* $bla_{\text{NDM-1}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$ and *int1* (Table 3), was detected. Those sets of genes are circulating in the hospital next to this point (data not shown).

The flies examined in this investigation carried a wide variety of environmental bacterial species (Supplementary data Tables S1 and S2) including bacteria which may acquire multidrug resistance traits and are part of the acronym ESKAPE: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp. These bacteria are characterized by their important virulence, resistance, and prevalence in hospital environments (De Olveira et al., 2020). In addition, it is shown in Tables 2 and 3 that important resistance genes ($bla_{\text{KPC-2}}$, *aac(6')-Ib,* bla_{NDM}, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$) were detected in *E.coli* and *K. pneumoniae* highlighting the importance of flies as dissemination vectors of antimicrobial resistance.

In relation to the Gram-positive species, *E. faecium* was recovered from collection point 2, while *E. faecalis* was isolated from points 1, 4 and 5, with one strain (Lemef64) resistant to tetracycline, chloramphenicol and ciprofloxacin. It is well known that *E. faecium* and *E. faecalis* are prominent causes of health care-associated infections globally especially vancomycin resistant strains (De Oliveira et al., 2020). These pathogens also represent reservoirs of virulence and resistance genes that can be transmitted from animals to humans via the food chain (Thu et al., 2019). Another group of Gram-positive bacteria, regarded as of great concern, are hospital or community acquired methicillin resistant *Staphylococcus aureus* (Kourtis et al., 2019), which were not isolated in the present study. However, *Staphylococcus sciuri* and *Staphylococcus succinus* both important emerging pathogens and recognized reservoirs for resistance and virulence genes (Nemeghaire et al., 2014; Rossi et al., 2020) were isolated. In contrast to our findings, Onwugamba et al. (2020) described a high prevalence of enterotoxin producing *S. aureus* from 'filth flies' in Nigeria.

Pseudomonas spp. are nonfermenting opportunistic bacteria, able to acquire multiple resistance genes, with *P. aeruginosa* the most frequently reported pathogen in this group (Lupo et al., 2018). As shown in Supplemental data Table S1, members of this genus including *P. aeruginosa, P. fluorescens, P. fragi, P. ludensis, P. putida* and *Pseudomonas* sp., were recorded in flies from all collection points, but no isolates were considered as MDR. In accordance with the date presented in Supplemental data Table S3, strains Lemef107, Lemef50 and LemefJO115 of *P. aeruginosa* were resistant to a variety of antimicrobials, including meropenem, while other non-*P. aeruginosa* species were resistant to at least one antimicrobial. Liu et al. (2013) reported an association between *P. aeruginosa* and flies collected at a Chinese airport, but they provided no evidence for meropenem resistance. More recently, Hemmatinezhad et al. (2015) detected bla_{TEM} positive *P. aeruginosa* from flies in Iran.

The order Enterobacterales was the predominant taxonomic group recorded in the present study. As shown in Table 2, isolates identified as *E. coli, K. quasipneumoniae, R. ornithinolytica, S. mascescens* and *E. cloacae* were classified as MDR (Magiorakos et al., 2012). As reported previously, *K. quasipneumoniae* (Lemef23) carries important carbapenemase ($bla_{\text{NDM-1}}$) and cefotaximase ($bla_{\text{CTXM-15}}$) genes as well as the $bla_{\text{TEM-1}}$ resistance determinant (Table 3) (Carramaschi et al., 2021). The isolate Lemef71 is a MDR strain of *R. ornithinolytica* $bla_{\text{KPC-2}}$ positive but interestingly is not resistant to carbapenens (Carramaschi et al., 2019). The presence of carbapenemase resistance determinants in a strain with reduced susceptibility suggests that the genes may be spreading silently and draws attention to the need for both molecular and phenotypic assessment of antibiotic resistance in environmental bacteria.

The *E. coli* strains, Lemef26 and Lemef105 were $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ positive (Table 3), respectively. In Brazil $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ are considered as the most important carbapenemase genes in hospital settings and are often associated with mobile genetic elements (Reyes et al., 2020). Importantly, our results provide robust evidence for the dissemination of these determinants to non-hospital environments. Based on an analysis of the culturable intestinal microbiota of flies, (Onwugamba et al., 2020) described a low colonization rate (0.8%) by ESBL positive Enterobacterales albeit with no evidence for isolates resistant to cabapenems. In contrast, 3,4% of the flies examined herein carried bacteria positive for carbapenem and β-lactam resistance genes.

The widely disseminated $bla_{\text{CTXM-15}}$, which originated in *K. ascorbata* as a chromosomally located gene (Sampaio et al., 2016, Bevan et al., 2017) was detected in an isolate of *K. ascorbata* recovered from *C. megacephala* collected at point 5. This observation is intriguing and raises the question as to whether this bacteria species is actually a natural carrier of $bla_{\text{CTXM-15}}$ or if the gene was acquired from the neighboring hospital environment?

When considered in combination with previous reports, our data indicated that the resistance determinants and phenotypic resistances recorded in our study population were location dependent, suggesting that in some cases (specifically collection point 5), the acquisition of antibiotic resistance was a consequence of selective pressure imposed by the use of antibiotics in the near-by hospital settings. Yet, in other areas

it was more likely that the resistance genes were simply circulating randomly within the environmental bacteria (Martinez, 2009), wherein their carriage may have conferred some advantage to the bacteria, other than resistance to the antibiotics (Cases et al., 2003).

The decision to examine the bacteria for the presence of the class 1 integron integrase genes (*int1*) was based on the fact that they may represent a genetic marker of pollution in the environment (Gillings et al., 2015). This gene is associated with elements that confer resistance to antimicrobials, heavy metals, and pollutants. In addition, it has been found in a wide variety of bacterial species and can be disseminated through horizontal gene transfer, providing new hosts with the ability to respond quickly to different environmental pressures. Four isolates (*E. coli, K. rizophila, K. pneumoniae* and *R. ornithinolytica*) recovered from flies at point 5, tested positive for the *int*1 gene and in addition, they each showed phenotypic resistance to broad-spectrum cephalosporins. As stated above, point 5 is the only location which differed statistically from the others ($p < 0.05$) (Fig. 1), reinforcing the suggestion that it may represent a potential hotspot for resistance dissemination.

Given that various abiotic factors may influence the community structure of flies in certain areas, the current study did not attempt to ascribe any strict correlation between the bacterial isolates and the species of fly from which they were collected. In addition, maceration of the whole insect body was carried out, without distinction between internal and external structures, in order to more fully evaluate the diversity of the culturable bacterial species carried by the insects. The findings of that analysis, showing the origin of each bacterial isolate i.e. the fly species from which it was recovered, are presented in Supplemental data Table S1 and confirmed that there was no clear correlation between any particular fly and bacteria species. Nonetheless, it can be stated that from 117 insects, 42 (35,9%) presented at least one species of bacteria which in turn were resistant to at least one antibiotic and that 5,9% of the flies carried MDR bacteria. As such, our findings are compatible with those of Poudel et al. (2019) who reported antimicrobial-resistant bacteria in 35.3% of the flies collected from farm environments, wherein 9.0% of the flies carried isolates classified as MDR. However, the significance of our data in terms of risk analysis, remains to be elucidated.

The high bacterial diversity found in the present study, together with the presence of clinically important resistance determinants in 5% of the isolates suggests that flies offer conditions suitable for the flow of resistance genes within and between different environmental compartments. The role of selective antibiotic pressure as a driver for the acquisition of resistance was suggested at a single site, while data from the other sites raised the possibility that the carriage of resistance genes provided the bacteria with some physiological or adaptative advantage. It is well documented that hospitals are environments with high selection pressure due to the use of antimicrobials, and in this context, the presence of resistant bacteria in environmental strains (isolated near to the hospital), could be considered as representing an accelerating agent for the acquisition and dissemination of resistance genes.

This work represents an important inventory with respect to bacteria carried by flies and provides valuable information on this subject of their potential role in inter-antimicrobial resistance. It should not be overlooked that microbial transmission to food or hosts is a problem as important as antibiotic resistance gene (Onwugamba et al., 2018). When our data are considered in combination with the findings of Poudel et al. (2019), it can be suggested that flies hold potential as indicators for environmental contamination of antimicrobial resistance. Nevertheless, we agree with those authors that more extensive studies will be necessary to determine under which circumstances a sentinel role of flies would be most appropriate for the surveillance of antimicrobial resistance.

5. Conclusions

This work represents an important inventory with respect to bacteria carried by flies and provides valuable information on the subject of their potential role in the dissemination of antimicrobial resistance. The flies examined carried a wide variety of environmental bacterial species, with the order Enterobacterales being the predominant taxonomic group. The high bacterial diversity found in the present study, together with the presence of clinically important resistance determinants in 5% of the isolates suggests that flies offer conditions suitable for the flow of resistance genes within and between different environmental compartments.

CRediT authorship contribution statement

Isabel Nogueira Carramaschi: Conceptualization, Methodology, Writing – original draft, Visualization. **Jonathan Christian O Lopes:** Investigation, Methodology. **Jéssica Albuquerque Leite:** Investigation, Methodology. **Marcos Tavares Carneiro:** Formal analysis, Writing – review & editing. **Rodrigo Rocha Barbosa:** Investigation, Methodology. **Maria Helena Villas Boas:** Investigation, Methodology. **Karyne Rangel:** Investigation, Methodology. **Thiago Pavoni Gomes Chagas:** Investigation, Methodology, Writing – review & editing. **Margareth MC Queiroz:** Project administration, Funding acquisition, Resources. **Viviane Zahner:** Conceptualization, Resources, Data curtion, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

All authors hereby declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2021.105962.

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