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Carrier flies of multidrug-resistant *Escherichia coli* as potential dissemination agent in dairy farm environment

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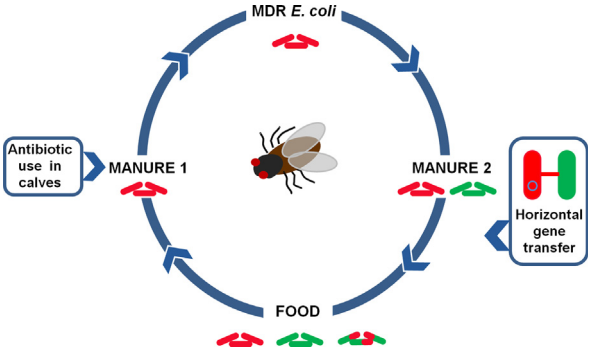
HIGHLIGHTS

- Multidrug-resistant *Escherichia coli* was found on the exoskeleton from flies.
- Antimicrobial resistance determinants were diverse among *E. coli* clones.
- Flies could facilitate the spreading of antimicrobial resistance genes.

GRAPHICAL ABSTRACT



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ABSTRACT

The life cycle of synanthropic flies and their behavior, allows them to serve as mechanical vectors of several pathogens. Given that flies can carry multidrug-resistant (MDR) bacteria, this study aimed to investigate the spread of genes of antimicrobial resistance in *Escherichia coli* isolated from flies collected in two dairy farms in Brazil. Besides antimicrobial resistance determinants, the presence of virulence genes related to bovine colibacillosis was also assessed. Of 94 flies collected, *Musca domestica* was the most frequently found in the two farms. We isolated 198 *E. coli* strains (farm A = 135 and farm B = 63), and >30% were MDR *E. coli*. We found an association between bla_{TEM} and phenotypical resistance to ampicillin, or chloramphenicol, or tetracycline; and bla_{CTX-M} and resistance to cefoperazone. A high frequency (86%) of phylogenetic group B1 among MDR strains and the lack of association between multidrug resistance and virulence factors suggest that antimicrobial resistance possibly is associated with the commensal bacteria. Clonal relatedness of MDR *E. coli* performed by Pulsed-Field Gel Electrophoresis showed wide genomic diversity. Different flies can carry clones, but with distinct antimicrobial resistance pattern. Sanger sequencing showed that the same class 1 integron arrangement is displayed by apparently unrelated strains, carried by different flies. Our conjugation results indicate class 1 integron transfer associated with tetracycline resistance. We report for the first time, in Brazil, that MDR *E. coli* is carried by flies in the milking environment. Therefore, flies can act as carriers for MDR strains and contribute to dissemination routes of antimicrobial resistance.

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1. Introduction

Rural, as well as urban environments, enable fly development. This proliferation ability within environments modified by humans is defined as synanthropy. Manure and decaying organic matter are the main food sources for maggot and adult synanthropic flies. Due to the contact with these organic substrates, flies are classically known as carriers of various pathogens (Povolný, 1971). The continuous use of antimicrobials in livestock favors the occurrence of antimicrobial resistance in commensal microorganisms, due to selective pressure (Oliver et al., 2011). So, the close contact between manure and flies assign them as a spreading agent for pathogenic and antimicrobial resistant bacteria.

In the last decade or so, flies have been recognized as vectors of multidrug-resistant bacteria. Marshall et al. (1990) were the first to discuss that flies could represent a dissemination route of antimicrobial resistance genes among the animals of a farm when they sought to understand the natural conditions that contributed to bacterial spread and movement of plasmids in the environment. Later, Rybaříková et al. (2010) found overlapping antimicrobial resistance traits and clones among *Escherichia coli* isolated from symbovine flies and cattle feces, suggesting the spread of resistant strains from manure by flies.

After detecting *E. coli* clones from flies and calf feces with the same antimicrobial resistance profile, Usui et al. (2015, 2013) suggested that flies can disseminate antimicrobial resistance genes not only within farm but also between farms. Similarly, Solà-Ginés et al. (2015) found the same Extended-spectrum β-lactamase (ESBL)-producing *E. coli* clone isolated from flies within two broiler farms and suggested it may have happened because flies can travel long distances.

In 2016, Schaumburg et al. (2016) investigated antimicrobial resistance in bacteria from rural and urban areas of Münster city in Germany and compared them to isolates from humans. In view of the high similarity of the isolates, they suggested that there could be a common source of transmission of bacteria from flies to humans.

More recently, it was shown that flies played a role in bla_{NDM} (New Delhi metallo-β-lactamase) and *mcr-1* (plasmid-mediated colistin resistance) gene propagation among chickens and dogs within a farm (Wang et al., 2017).

Here we report that flies can carry multidrug-resistant *Escherichia coli* on dairy farms in Brazil. We speculate that flies play a considerable role in the spread of antimicrobial resistance in the dairy farm and the environment.

2. Material and methods

2.1. Ethical statement

The synanthropic flies used in this study were collected in private farms with the owners' permission. Ethical approval is not required.

2.2. Flies sampling and Escherichia coli isolation

Flies were collected in the area around calf barns using a sweep net in two dairy farms (A and B) 35 km apart (Botucatu, São Paulo, Brazil), where bovine breeding is common. The sweep net was disinfected before the use with 10% bleach for 30 min and washed between the collect on farm A and farm B. We made collections (on one day in June 2015) in the morning for two hours per farm. We transferred the sets of the flies collected from each capture, introducing sterile tubes into sweep net. Flies were frozen to death at −10 °C for 30 min. Thereafter, *E. coli* from the external surface of flies were cultivated adding 2 mL of EC broth medium (selective medium for detection of coliforms and *E. coli*) to tubes containing the flies. The tubes were incubated at 37 °C overnight. Flies were removed and kept in 70% ethanol for species identification using morphology. A loop from EC broth was placed onto MacConkey agar and five colonies with typical *E. coli* morphology were selected from each plate and confirmed by biochemical tests.

2.3. Antimicrobial susceptibility

We tested *Escherichia coli* strains by disc diffusion test in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012a, 2012b) using ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), cephalexin (30 μg), cefoperazone (75 μg), ceftiofur (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), enrofloxacin (5 μg), ciprofloxacin (5 μg) gentamicin (10 μg), sulfamethoxazole/trimethoprim (25 μg) and tetracycline (30 μg). *E. coli* ATCC 25922 was used as antimicrobial susceptibility control. Additionally, third-generation cephalosporin-resistant *E. coli* was analyzed by double disc synergy using aztreonam (30 μg), ceftriaxone (30 μg), cefotaxime (30 μg), ceftazidime (30 μg) to 20 mm apart from amoxicillin/clavulanic acid (30 μg) disc (Jarlier et al., 1988). Multidrug-resistant strains (MDR, resistant to ≥3 antimicrobial classes) (Magiorakos et al., 2012) were selected for *E. coli* phylogeny assay and pulsed-field gel electrophoresis.

2.4. Antimicrobial resistance genes and virulence factors

Antimicrobial resistance and virulence genes were detected by PCR (Table 1). Bacterial DNA was released by boiling and 2 μL of supernatant was added to PCR master mix containing 1.5 U Taq polymerase; 1.5 μL of 10× PCR buffer; dNTP mix to 200 μM; 2 mM $MgCl_2$; 0.5 μL of each primer in appropriated concentrations (Table 1) and ultrapure sterile water to complete a final volume of 15 μL.

2.5. Escherichia coli Phylogeny

Multidrug-resistant strains were assigned to ECOR phylogenetic groups by PCR (Clermont et al., 2013).

2.6. Investigation of cassettes genes within class 1 integron

Sequencing was conducted with 5′CS and 3′CS primers (Table 1) from PCR products purified with AxyPrep PCR Clean Up® Kit (Axygen Biotechnology) using BigDye Terminator v.3.1 Cycle Sequencing Kit and ABI-PRISM 3700 DNA Analyzer (Applied Biosystems, USA). Contigs analyses were done with Sequencher 4.7 software (Gene Codes Corporation) and online tools BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and ORF Finder (National Center for Biotechnology Information - NCBI) (https://www.ncbi.nlm.nih.gov/orffinder/). For the completeness of sequence structure, we performed linkage PCRs using integron structure genes and sequencing, basing primer walking approach (Table 1).

2.7. Conjugation assay

We performed conjugation assays using integron-positive *E. coli* as donors and azide-resistant *E. coli* J53 as recipient strain. Experiments were done as described previously by Soufi et al. (2009) with minor modifications. From *E. coli* growth in Luria-Bertani broth (37 °C for 18 h), donors and recipient strains were mixed in 1:10 (v/v) proportion, respectively, and incubated at 37 °C for 18 h. For transconjugants selection, 100 μL of the mix culture was streaked onto Mueller Hinton agar plates containing sodium azide and tetracycline and incubated at 37 °C. The bacterial growth was verified after 24 h, 48 h, and 72 h. To confirm the conjugation, genes harbored in the donor strains were investigated in transconjugant strains by PCR. Plasmids were detected using alkaline lysis (AccuPrep® Plasmid Mini Extraction Kit).

2.8. PCR-based replicon typing (PBRT)

Inc./replicon from integron-positives *E. coli* were assessed with PCR-based replicon typing (Carattoli et al., 2005).

Table 1
Primers used for detection of antimicrobial resistance and virulence genes and integrons structure, within *Escherichia coli* strains isolated from external surface of flies collected in two dairy farms in Brazil.

Primer	Product	Sequence (5′-3′)	Tm*	bp**	Conc.	Ref.
Virulence factors						
eae	intimin	GACCCGGCACAAGCATAAGG CCACCTGCAGCAACAAGAGC	63 °C	384	60	(Yu and Kaper, 1992)
stx1	Shiga toxin	AAGTTGCAGCTCTCTTTGAATA TGCAAACAAATTATCCCCTGAG	50 °C	364	90	(Ojeniyi et al., 1994)
stx2	Shiga toxin	GGGCAGTTATTTTGCTGTGGA GTATCTGCCTGAAGCGTAA	50 °C	386	90	
hlyA	hemolysin	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATCCCGTCA	63 °C	1177	150	(Yamamoto et al., 1995)
K99	colonization factor	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	50 °C	314	60	(Franck et al., 1998)
LTII	heat-labile enterotoxin	AGATATAATGATGGATATGTATC TAACCCTCGAAATAAATCTC	48 °C	300	90	(Schultsz et al., 1994)
STa	heat-stable enterotoxin	TCCGTGAAACAACATGACGG ATAACATCCAGCACAGGCAG	48 °C	244	60	(So and McCarty, 1980)
β-lactams						
ampC	ampC β-lactamases	CCCCGCTTATAGAGCAACAA TCAATGGTCGACTTCACACC	61 °C	634	30	(Féria et al., 2002)
bla_{TEM}	ESBL	TCGGGGAAATGTCGCG TGCTTAATCAGTGAGGCACC	61 °C	972	30	(Cao et al., 2002)
bla_{SHV}	ESBL	TTATCTCCCTGTTTAGCCACC GATTTGCTGATTTCGCTCGG	61 °C	795	60	
bla_{CTX-M}	ESBL	CGATGTGCAGTACCAGTAA TTAGTGACCAGAATCAGCGG	60 °C	585	60	(Batchelor et al., 2005)
Tetracycline						
tetA	efflux pump system	GTAATTCTGAGCACTGTCGC CTGTCCTGGACAACATTGCTT	62 °C	937	150	(Guardabassi et al., 2000)
Integrons						
intI1	class 1 integrase	GGGTCAAGGATCTGGATTTCG ACATGGGTGTAAATCATCGTC	62 °C	483	60	(Mazel et al., 2000)
5′CS 3′CS	inserted cassette region	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	60 °C	V§	60	(Lévesque et al., 1995)
intI2	class 2 integrase	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	62 °C	788	60	(Mazel et al., 2000)
attI2-F *orfX*-R	inserted cassette region	GACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	58 °C	V§	60	(Machado et al., 2005)
dfrA7/dfrA17	trimethoprim resistance	CAGAAAATGGCGTAATCG TCACCTTCAACCTCAACG	55 °C	345	60	(Frech et al., 2003)
qac	ammonium quaternary resistance	GGCTGGCTTTTTCTTGTTATCG TGAGCCCCATACCTACAAAGC	62 °C	287	60	(Mazel et al., 2000)

Tm*: Melting temperature; bp**: product length; Conc.: primers concentrations (ng/μL); Ref.: Reference; V§: variable; ESBL: Extended-spectrum β-lactamase.

2.9. Statistical analysis

Presence or absence of multidrug resistance versus integrons and multidrug resistance versus virulence factors were compared with Fisher's exact test ($p = 0.05$). Resistance phenotypes and resistance genes were analyzed with logistic regression ($p = 0.05$).

3. Results

We isolated 198 *E. coli* strains from the external surface of 94 flies (farm A: 135 strains from 57 flies; farm B: 63 strains from 37 flies). Flies most often found on farm A were *Musca domestica* (Linnaeus, 1758) ($n = 20$), *Fannia* spp. ($n = 14$) and *Stomoxys calcitrans* (Linnaeus, 1758) ($n = 10$). On farm B the most frequently found were *M. domestica* ($n = 10$), *Hippelates* spp. ($n = 7$), and Acalyptratae ($n = 7$) and *Fannia* spp. ($n = 5$).

On farm A, antimicrobial resistance genes detected in *E. coli* strains included bla_{TEM} (36.3%; 49/135), *tetA* (14.8%; 20/135), bla_{CTX-M} (11.1%; 15/135) and *ampC* (4.4%; 6/135). Virulence genes found were *eae* (2.9%; 4/135), *stx1* (5.2%; 7/135), *hlyA* (8.1%; 11/135), K99 (3.7%; 5/135), LTII (9.6%; 13/135), and STa (3.7%; 5/135). The seven *stx1*-positive *E. coli* were isolated from *M. domestica*. On farm B, only five strains harbored *ampC*, and no strain showed any of the investigated virulence factors.

We found 60 multidrug-resistant *E. coli* from farm A and only two from farm B. Of these MDR *E. coli*, 52 were assigned to phylogenetic group B1 and eight to group E. The two MDR *E. coli* from farm B were assigned to the B1 phylogenetic group. Strains from farm A were primarily resistant to ampicillin, amoxicillin/clavulanic acid, tetracycline, sulfamethoxazole/trimethoprim, enrofloxacin, chloramphenicol and ciprofloxacin. On farm B, they are resistant to ampicillin and amoxicillin/clavulanic acid. Phenotypical antimicrobial resistance frequencies are shown in Fig. 1.

Multidrug resistance was associated with the presence of integron ($p = 0.0012$). There was no association between multidrug resistance and *eae* or *hlyA*, or LTII or STa ($p > 0.05$). In contrast, K99 was significantly associated with multidrug resistance ($p = 0.0158$), and *stx1* gene was associated with multidrug resistance default ($p = 0.0171$). Also, there was association between bla_{TEM} and phenotypic resistance to ampicillin ($p = 0.0242$), chloramphenicol ($p = 0.0179$) or tetracycline ($p = 0.0056$). The bla_{CTX-M} was associated with phenotypical resistance to cefoperazone ($p = 0.0458$). The *ampC* and *tetA* genes were not associated with phenotypical resistance to any of the tested antimicrobials.

Class 1 integron structure from eight *E. coli* positives (*int11* + 5′CS-3′ CS) was investigated by PCR and sequenced by primer walking. All strains showed the same integron array (*int1-dfrA7-attC-qacEΔ1*), with only one *dfrA7* cassette gene (GenBank accession no. MF445023,

MF445024, MF445025, MF445026, MF445027, MF46502, MF465027, MF465028). No *E. coli* had class 2 integron.

Forty-three MDR *E. coli* were selected for PFGE typing in accordance with antimicrobial resistance patterns of integron-positive strains and MDR from farm B (Table 2). Thirty-six pulsotypes were generated (Fig. 2), demonstrating wide genomic diversity. Four *E. coli* (three from farm A and one from farm B) showed 100% similar fingerprints. These *E. coli* clones were isolated from different sets of flies (MA39, MA9e, and MB2). Moreover, two isolates from Farm A and one from Farm B displayed >90% similarity (MA6a, MA2d, and MB1d). We considered clones as the strains with >90% of genomic similarity (Fig. 2).

Conjugation experiments conducted with integron-positive *E. coli* using tetracycline selection showed the transfer of integron genes, bla_{CTX-M} and *tetA*. PCR results for the transconjugant strains showed that three donor strains transferred all genes. Of these seven bla_{TEM}-positive strains, four strains did no transfer bla_{TEM} by tetracycline-associated conjugation. All transconjugants received plasmids.

Additionally, PCR-based replicon typing (PBRT) was performed on all eight integron-positive *E. coli*. Four strains (MA17a, MA17c, MA17d, MA17e) displayed Inc./replicon groups I, HI1, HI2, FIA, FIB, P, K, F; one strain (MA17b) harbored I, N, FIA, FIB, P, K, F groups; one (MA26a) harbored I, N, HI1, HI2, FIB, P, K, F; one (MA26b) N, HI1, HI2, P, K, and one strain (MA26d) harbored I, N, HI1, HI2, P, K groups.

4. Discussion

Our results show different antimicrobial resistance profiles among commensal *E. coli* clones, carried by different flies (isolates from *Chrysomya megacephala*, Muscidae, *Fannia* sp. and, *Hippelate*s sp.), suggest that resistance gene dissemination can be independent of multidrug-resistant clone spread. In contrast, either different individuals of flies or the same individual can carry unrelated strains, but with the same genotype. This is evident in integron-positive *E. coli* since isolates from different flies showed the same gene array. Furthermore, we find flies from different farms (A or B), carrying MDR *E. coli* clone (genomic similarity ≥90%), and harboring distinct antimicrobial resistance markers.

Diversity and similarity of antimicrobial resistance determinants in bacterial clones and unrelated strains can be due to horizontal gene transfer, which is a known process related to antimicrobial genes dissemination between strains (Stokes and Gillings, 2011). Besides, we believe that flies movement can contribute to the spread of a wide range of strains in the environment and offer an advantage in genes transposition.

Previous studies have discussed the role of flies as vectors of antimicrobial resistance bacteria (Blaak et al., 2014; Davari et al., 2010; Literak et al., 2009; Liu et al., 2013; Macovei and Zurek, 2006; Marshall et al., 1990; Rahuma et al., 2005; Rybaříková et al., 2010; Schaumburg et al., 2016; Solà-Ginés et al., 2015; Usui et al., 2015, 2013; Von Salviati et al., 2015; Wang, 2013; Wang et al., 2017; Zurek and Ghosh, 2014). In Brazil, Almeida et al. (2014) isolated *E. coli*, *Salmonella* spp. and, *Staphylococcus* spp. from the internal and external surfaces of the flies collected in 30 dairy farms, but they did not find antimicrobial resistant strains. Therefore, our results constitute the first report, from Brazil, of MDR *E. coli* isolated from flies.

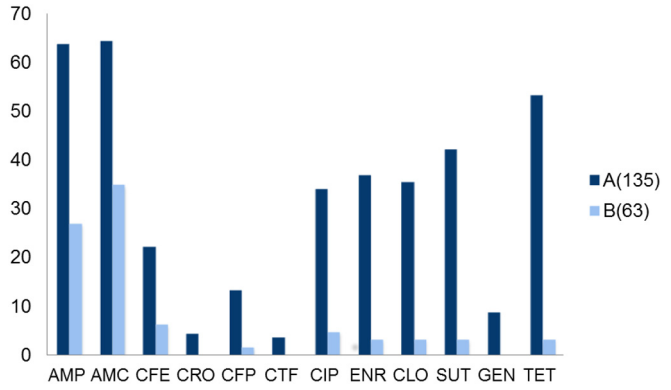


Fig. 1. Antimicrobial resistance frequencies of *E. coli* from flies collected on two dairy farms (A and B), located in Botucatu, SP, Brazil. Farm A with 135 *E. coli* strains; Farm B with 63 *E. coli* strains. AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CFE, cefalexin; CRO, ceftriaxone; CFP, cefoperazone; CTF, ceftiofur; CIP, ciprofloxacin; ENR, enrofloxacin; CLO, chloramphenicol; SUT, sulfamethoxazole/trimethoprim; GEN, gentamicin; TET, tetracycline.

Table 2
Multidrug-resistant *E. coli* groups selected for PFGE assay.

	Antimicrobial resistance
Group I	AMP/AMC/CFE/CFP/CIP/ENR/CLO/SUT/TET
Group II	AMP/AMC/CFE/CRO/CFP/CTF/CIP/ENR/SUT/GEN/TET
Group III	AMP/AMC/CFE/CIP/ENR/CLO/SUT/TET
Group IV	AMP/AMC/CIP/ENR/CLO/SUT/TET
Group V	AMP/AMC/CFP/CIP/ENR/CLO/SUT/TET
Group VI	AMP/AMC/CFE/CIP/ENR/CLO/SUT/GEN/TET

AMP, ampicillin; AMC, amoxicillin/clavulanis acid; CFE, cefalexin; CRO, ceftriaxone; CFP, cefoperazone; CTF, ceftiofur; CIP, ciprofloxacin; ENR, enrofloxacin; CLO, chloramphenicol; SUT, sulfamethoxazole/trimethoprim; GEN, gentamicin; TET, tetraciclyne.

Flies can disseminate bacteria through exoskeletons, regurgitation and excretes (Greenberg, 1973), acting as bioenhanced vector (Kobayashi et al., 1999). Some flies structures facilitate microorganisms carrier (Sarwar, 2015). In this context, Junqueira et al. (2017) said that the microbiome from the external surface of the blowflies and houseflies highlighted their role as mechanical vectors of pathogens due to physical contact. Here, we investigated the role of them as mechanical vectors of MDR *E. coli*, because external parts of the flies are those in first contact with the contaminated (feces and garbage) or uncontaminated environment (food).

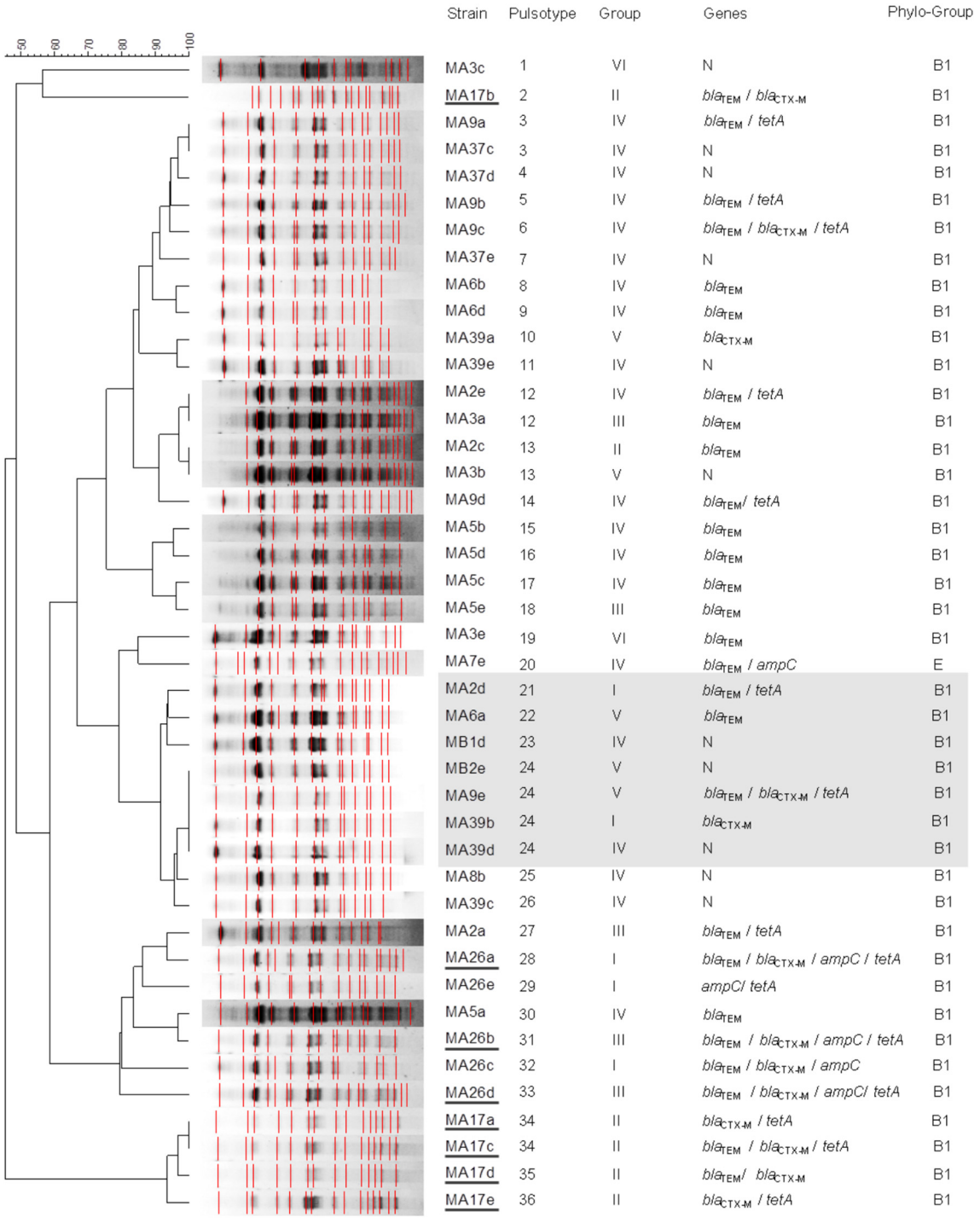
The same MDR *E. coli* clones isolated from two farms located a long distance from each other indicates regional spreading of clones. This spread may have occurred through several possibilities such as water sources, human contact, fomites, wild animals, and/or flies.

Entomology studies are unclear as to the flight distance of flies, which diverge according to species and stimuli (Jones et al., 1999). Some studies have reported flight distances around 6–7 km (Nazni et al., 2005; Winpisinger et al., 2005). However, it seems possible that longer distances are reached since there are estimates that dispersal of the flies can reach 22 km or 30 km (Jones et al., 1999; Nazni et al., 2005), in accordance with data discusses here. Also, we cannot rule out the flies' movement by modes of transport such as cars, buses, milk trucks, delivery trucks, and others.

On examination of the data from farm A, there was not an association between multidrug resistance and virulence presence, except for K99 gene. >86% of strains were placed into phylogenetic group B1, known as commensals and most of the *E. coli* isolated from animals belongs to this group (Tenaillon et al., 2010). These results suggest that multidrug resistance pressure has emerged in commensal strains.

In addition, we found low frequencies of virulence genes in strains from flies on farm A and none from flies on farm B. Moreover, all seven *E. coli* positive for Shiga toxin gene (*stx1*) were isolated from *M. domestica*, indicating a potential association between Shiga toxin-producing *E. coli* (STEC) and *M. domestica*. In this regard, Moriya et al. (1999) were the first to showed STEC transmission by *M. domestica*, when they investigated an outbreak of the enterohemorrhagic colitis in children in Japan. At that time the authors concluded that flies had served as mechanical vectors.

Escherichia coli from farm A showed more phenotypic and genotypic traits of antimicrobial resistance when compared with strains from farm B. These differences between the two farms can be due to distinct cattle management in each farm. Animals' agglomeration in farm A would possibly facilitate the circulation and maintenance of multidrug-resistant and/or pathogenic strains among animals, implying more



Strain	Pulsotype	Group	Genes	Phylo-Group
MA3c	1	VI	N	B1
MA17b	2	II	bla_{TEM} / bla_{CTX-M}	B1
MA9a	3	IV	bla_{TEM} / *tetA*	B1
MA37c	3	IV	N	B1
MA37d	4	IV	N	B1
MA9b	5	IV	bla_{TEM} / *tetA*	B1
MA9c	6	IV	bla_{TEM} / bla_{CTX-M} / *tetA*	B1
MA37e	7	IV	N	B1
MA6b	8	IV	bla_{TEM}	B1
MA6d	9	IV	bla_{TEM}	B1
MA39a	10	V	bla_{CTX-M}	B1
MA39e	11	IV	N	B1
MA2e	12	IV	bla_{TEM} / *tetA*	B1
MA3a	12	III	bla_{TEM}	B1
MA2c	13	II	bla_{TEM}	B1
MA3b	13	V	N	B1
MA9d	14	IV	bla_{TEM}/ *tetA*	B1
MA5b	15	IV	bla_{TEM}	B1
MA5d	16	IV	bla_{TEM}	B1
MA5c	17	IV	bla_{TEM}	B1
MA5e	18	III	bla_{TEM}	B1
MA3e	19	VI	bla_{TEM}	B1
MA7e	20	IV	bla_{TEM} / *ampC*	E
MA2d	21	I	bla_{TEM} / *tetA*	B1
MA6a	22	V	bla_{TEM}	B1
MB1d	23	IV	N	B1
MB2e	24	V	N	B1
MA9e	24	V	bla_{TEM} / bla_{CTX-M} / *tetA*	B1
MA39b	24	I	bla_{CTX-M}	B1
MA39d	24	IV	N	B1
MA8b	25	IV	N	B1
MA39c	26	IV	N	B1
MA2a	27	III	bla_{TEM} / *tetA*	B1
MA26a	28	I	bla_{TEM} / bla_{CTX-M} / *ampC* / *tetA*	B1
MA26e	29	I	*ampC*/ *tetA*	B1
MA5a	30	IV	bla_{TEM}	B1
MA26b	31	III	bla_{TEM} / bla_{CTX-M} / *ampC* / *tetA*	B1
MA26c	32	I	bla_{TEM} / bla_{CTX-M} / *ampC*	B1
MA26d	33	III	bla_{TEM} / bla_{CTX-M} / *ampC*/ *tetA*	B1
MA17a	34	II	bla_{CTX-M} / *tetA*	B1
MA17c	34	II	bla_{TEM} / bla_{CTX-M} / *tetA*	B1
MA17d	35	II	bla_{TEM}/ bla_{CTX-M}	B1
MA17e	36	II	bla_{CTX-M} / *tetA*	B1

Fig. 2. Pulsed-field gel electrophoresis (PFGE) dendrogram using *Xba*I-macrorestriction, antimicrobial resistance genes, and phylo-groups found in *E. coli* isolated from flies collected from two dairy farms in Brazil. Patterns generated by UPGMA, using Dice coefficient, tolerance and optimization 1% (GelCompar, Applied Maths, Belgium). Clones are highlighted. Numbers 1–36 perform pulsotypes. I to VI perform antimicrobial susceptibility groups. Class 1 integron-positive *E. coli* are underlined. N: negative. NT: no type.

frequent outbreaks and antibiotic intervention at higher doses. Despite antimicrobial resistance in strains from flies (Fig. 1) has not followed the regular antimicrobial use in calves (ceftiofur, enrofloxacin and gentamicin), these findings suggest that: i – flies could play an important role in the dissemination of MDR *E. coli* and pathogenic strains in dairy farms, including the potential introduction of antimicrobial resistant determinants from outside farms; ii – antimicrobial resistance genes could improve fitness advantages. The next step would be to assess antimicrobial resistance and clonal relatedness of *E. coli* isolated from cattle feces and flies.

Integron-positive *E. coli* contained a single gene cassette *dfrA7* were isolated from different flies (MA17, *M. domestica*, and MA26 *Fannia* sp.). They exhibited different pulsotypes, suggesting that *dfrA7* may have regional dissemination. In this regard, Labar et al. (2012) discussed the infrequent arrangement of class 1 integron containing one *dfrA7* copy. They found high frequencies of class 1 integrons with single copies of *dfrA7* in *E. coli* from healthy humans in Nigeria and Ghana. To our knowledge, there are no reports of *dfrA7* gene cassettes in integrons in Brazil from human or animal isolates.

The results of conjugation assays indicate that antimicrobial selective pressure targeted to commensal *E. coli* and horizontal gene transfer of class 1 integron-associated with tetracycline resistance in strains harboring plasmids known to spread these genes, since replicon groups I, N e K are associated with the spread of bla_{CTX-M} (Carattoli, 2009) and all integron-positive *E. coli* harbored IncP related to class 1 integrons and spread of tetracycline resistance genes (Popowska and Krawczyk-Balska, 2013).

In this context, detection of gene cassettes within integrons, the PFGE fingerprints, and the antimicrobial resistance profile support the dissemination not only of the MDR clones, but also antimicrobial resistance genes, so that gene transfer possibly took place in a horizontal manner and local dissemination of the strains could have been facilitated by flies.

In this study, we provide evidence from Brazil for the inclusion of flies in the dissemination routes of environmental antimicrobial resistance, which is shown to be favored through the flies' synanthropic and communicative behavior.

5. Conclusions

Flies seem to play an important role in antimicrobial resistance spread in the dairy farm environment. These vectors can carry antimicrobial resistant strains in the environment acting as MDR bacteria carriers. They could also either insert, take out or maintain antimicrobial resistance in any environment. Importance strains recovered from the external surface of flies should be investigated due to their potential for contact between many surfaces. This intimate contact contributes to strain dissemination and raises a public health concerns.

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