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Potential of saprophage Diptera to acquire culturable livestock-associated antibiotic-resistant bacteria

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Summary

The increasing prevalence of antibiotic resistance among bacteria is one of the most intractable challenges in 21st-century public health. Dipterans that associate with livestock, livestock waste products and cadavers have the potential to acquire livestock-associated antibiotic-resistant bacteria (LA-ARB) and transmit them to humans. In this study, piglet cadavers were used to attract saprophage dipterans from the environment and those dipterans were sampled for the presence of LA-ARB. In the first trial, culturable microbes resistant to both aminoglycoside and β-lactam antibiotics were found in all cadavers and masses of dipteran larvae, and in three-quarters of adult dipterans. In the second trial, over 130 culturable bacterial colonies resistant to β-lactams were isolated from the cadavers, larval and adult dipterans. Over 100 of those colonies were coliform or metabolically similar bacteria. Adult dipterans carried β-lactam resistant staphylococci, whereas those bacterial types were absent from larval dipterans and cadavers, suggesting they were picked up from elsewhere in the environment. This research indicates that LA-ARB are ubiquitous in pig farms, and dipterans have the potential to carry medically important microbes. Further research is encouraged to determine the extent to which dipterans acquire microbes from animal agriculture relative to other environments.

KEYWORDS

antibiotic-resistant bacteria, Calliphoridae, coliform, mechanical vector, saprophage, *Staphylococcus*

1 | INTRODUCTION

Antibiotic-resistant bacteria (ARB), those that have developed resistance to one or more modern antibiotics, present a major health crisis. Approximately 2 million people in the USA get infected with ARB each year, with 23,000 dying (CDC, 2014). Exposure to ARB can occur in many ways. Hospital-acquired ARB have received much attention (Struelens, 1998); however, ARB are also notably transmitted to humans in other environments. Animal feeding operations (AFOs) are one such environment. The treatment of animals with antibiotics (some of which are the same as those used in humans) to promote growth or prevent bacterial diseases is estimated to account for over half (up to 70%) of the total antibiotics used in the USA (Hribar, 2010). Inappropriate use of antibiotics has been reported to select for resistance traits in microbes (Hildreth, Burke, & Glass, 2009; Mellon, Benbrook, & Benbrook, 2001). Most antibiotics used for preventing or treating infections in humans or animals as well as for promoting faster growth of livestock are only partially metabolized and are then discharged along the excreta, either to sewage treatment plants or straightforward in waters or soils (Dolliver & Gupta, 2008; Martinez, 2009). Thus, the presence of these compounds in animals, their waste and the environment may be a strong selective agent for the evolution of ARB, also called livestock-associated antibiotic-resistant bacteria (LA-ARB).

Several different LA-ARB are of particular concern in AFOs. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been identified in European pig farms since the early 2000s and has been found to be

transmitted to farm workers and other local populations (Verhegghe et al., 2013; Voss, Loeffen, Bakker, Klaassen, & Wulf, 2005). These MRSA strains have the ability to cause disease outbreak in humans. Antibiotic-resistant *Escherichia coli* has also been linked to food animals (Friese et al., 2013), which are known to acquire the highly pathogenic strain O157:H7 (Keen, Wittum, Dunn, Bono, & Durso, 2006). These and other bacteria easily spread through animal populations due to the high densities and enclosed rearing conditions. Transmission of LA-ARB to humans can occur in several ways, including poor waste management and food handling, manure-based fertilizers and by mechanical transmission by waste-associated animals (Wei, Ishida, Miyanaga, & Tanji, 2014; Yang, Zhang, Guo, & Tian, 2016).

Release of antibiotics, resistance genes and ARB into the environment and potentially allows other organisms such as insects to acquire LA-ARB. Many insects, such as dipterans, are strongly associated with animal agriculture, particularly the animals themselves and their waste streams (Blaak et al., 2014; Pace, Talley, Crippen, & Wayadande, 2017). The potential of these insects to transmit LA-ARB to humans is high. For instance, several species of dipterans are known to transmit pathogens to humans, including important intestinal microbes that may be ARB such as *Clostridium difficile*, *Salmonella* spp. and *E. coli* (Blazar, Lienau, & Allard, 2011; Burt, Siemeling, Kuijper, & Lipman, 2012; Ranjbar, Izadi, Hafshejani, & Khamesipour, 2015; Wales et al., 2010). Dipterans are uniquely important as possible vectors of LA-ARB. These species, mostly muscomorphs, frequently encounter both humans and animals, possess sticky footpads (pulvilli) and salivate and/or regurgitate before feeding (Pace et al., 2017). Although dipterans associated with faecal waste are known for their ability to pick up LA-ARB (Wales et al., 2010), less is known about the potential of other dipterans, including carrion-feeding (saprophage) species like calliphorids and sarcophagids, to acquire and potentially transmit LA-ARB (Tomberlin et al., 2017).

In this study, we collected saprophage dipterans from livestock cadavers and sampled them for the presence of staphylococci and faecal coliforms. We selected saprophage dipterans because of their ubiquity in the environment and association with animals (Tomberlin et al., 2017). Because antibiotic resistance is ubiquitous in AFOs, even to antibiotics not actually used by the AFO (Wichmann, Udikovic-Kolic, Andrew, & Handelsman, 2014), we screened for resistance, not only to aminoglycosides (the only antibiotic used at the source farm), but also to β-lactams, compounds for which resistance is well characterized. The objective of this study was to assess the potential of saprophage dipterans to link LA-ARB in AFOs to human populations. Specifically, we investigated the capacity of immature and adult dipterans attracted to and feeding on animal cadavers to accumulate and vector culturable ARB to human populations.

Impacts

- Saprophage dipterans have the potential to transmit livestock-associated antibiotic-resistant bacteria (ARB) to humans.
- Culturable coliform bacteria resistant to antibiotics were found in all piglet cadavers tested in this study, and all saprophage dipteran larval masses carried those same bacterial taxa according to selective and differential media.
- Adult saprophage dipterans also carried culturable ARB, but most were different bacterial taxa, suggesting that those dipterans have the ability to acquire ARB from the environment as well as dead livestock.

2 | METHODS

2.1 | Background

We performed two trials, one in September 2015 and the other in May 2016. For both trials, stillborn piglets that were found in and removed from farrowing pens at that time were obtained from a Pilgrim's Pride commercial farm in Stephens County, Georgia and refrigerated in sealed sterile plastic bags until use (<24 hr). Live-birthed piglets on that farm are normally treated with gentamicin as part of neonate processing; the stillborn piglets obtained for this study were not treated. The piglets were placed into three 90 × 45 × 45 cm wooden cages framed with hardware cloth to prevent scavenging. These cages were placed at the edge of a 10-m-wide mowed path through a 31 ha mixed deciduous and pine forest in Hall County, GA (34°14′29.59N, 83°51′58.11W). The forest primarily consists of *Pinus taeda*, *Quercus nigra*, *Quercus phellos*, *Carya* spp. and invasive *Ligustrum sinense*. Previous research has shown that the dominant local saprophage dipterans are *Lucilia coeruleiviridis* (Calliphoridae) and *Phormia regina* (Calliphoridae) (Munro, H. L., Lampert, E.C., unpublished data).

FIGURE 1 Piglet cadavers at 24 hr in the environment. An adult *Lucilia* sp. (Diptera: Calliphoridae) is indicated in the circle. Photograph by E. Lampert

2.2 | Occurrence of resistant bacteria

Six cadavers were laid on the soil, two per cage, at 11:00 a.m. on 15 September 2015 (Figure 1). Each cadaver was sampled for microbes 48 hr later. Cadavers were sampled in the cages by swabbing the entire surface with sterile cotton swabs moistened with sterile 0.85% NaCl (normal saline), and masses of dipteran larvae were sampled in the same manner. All masses of dipteran larvae (1–4 per pig) (Figure 2) were sampled together. Adult dipterans (21) were collected individually using a bleach-sanitized aerial net. Adult dipterans were placed individually into small clean plastic ziplock bags and refrigerated until they were returned to the laboratory (all collecting took ~1 hr). Upon return to the laboratory, adult dipterans were placed individually using flame-sanitized forceps into glass vials containing 3 ml chilled sterile saline solution and immediately crushed using sterile glass rods. Remains were vortexed for 1 min to separate any internal or external microorganisms from dipteran issues and suspended in saline, and sterile cotton swabs were used to inoculate the suspension onto nutrient agar plates.

Microbes were cultured by swabbing the entire surface of nutrient agar (BD Difco, Franklin Lakes, New Jersey, USA, #213000) Petri plates. Each sample was swabbed onto four plates, one plate containing no antibiotic (control plate) and the others containing 10 μg/ml gentamicin, 100 μg/ml streptomycin and 100 μg/ml ampicillin. All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA), and concentrations were recommended by the manufacturers. These antibiotics were selected so that the (i) antibiotic used at the farm, gentamicin, (ii) a different aminoglycoside, streptomycin, and (iii) ampicillin, a semi-synthetic β-lactam, were all included. Plates were sealed and cultured at 37°C for 48 hr, with an initial observation at 24 hr. The presence or absence of bacterial colonies on each plate was recorded, and a 2 × 4 × 3 contingency table analysis was performed, in which the two outcomes (growth or lack of growth of culturable microorganisms) were compared among the four antibiotic media (control, gentamicin, streptomycin and ampicillin) and three sample sources (cadavers, larval dipteran masses and adult dipterans).

2.3 | Coliform-*Staphylococcus* screening

Seven piglet cadavers were obtained, handled and placed in the same cages as described previously, two per cage (with a third in one cage), at 11:00 a.m. on 09 May 2016. Cadavers, larval dipteran masses and adult dipterans were sampled 48 hr later. Sampling methods were identical to the first trial, except 30 adult dipterans, 10 from each cage, were collected. Each sample was swabbed onto two nutrient agar plates, one with and one without 100 iu/ml penicillin. After 48 hr at 37°C, individual bacterial colonies were isolated using quadrant streaking on sterile nutrient agar plates. All single pure colonies were cultured for 24 hr at 37°C on both mannitol salt agar (MSA) plates (BD Difco # B11407) to screen for staphylococci and MacConkey agar (BD Difco # DF0075-17-1) plates to screen for coliforms.

A 2 × 2 × 3 contingency table analysis was performed, in which the two outcomes were compared among two types of antibiotic media (control, penicillin) and three sample sources (as above). A one-way analysis of variance (ANOVA) was also used to compare the mean numbers of different morphologically distinguishable colonies cultured from the penicillin-containing plates among the three types of samples. All analyses were performed with SPSS v.23.0 (Microsoft, 2015).

3 | RESULTS

3.1 | Occurrence of resistant bacteria

All adult dipterans that were sampled were members of Calliphoridae; however, they could not be identified to species because they were destructively sampled. Eighteen of the 21 collected

FIGURE 2 Piglet cadaver after 48 hr in the environment, showing large masses of dipteran larvae feeding on the head and umbilical region. Bacteria were sampled at this time. Photograph by E. Lampert

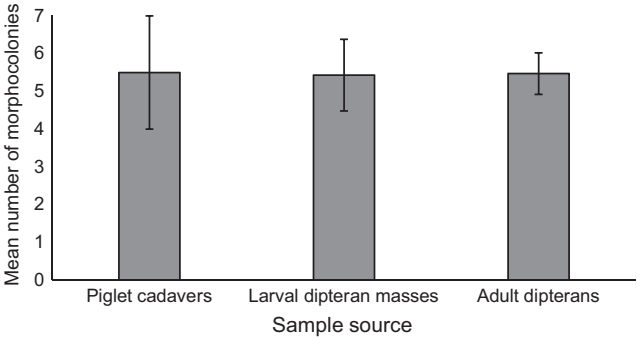

FIGURE 3 Mean (±*SE*) number of different culturable penicillin-resistant bacteria colonies cultured from piglets (*n* = 4 plates), larval dipterans feeding on those piglets (*n* = 8) and adult dipterans (*n* = 17) visiting those piglets

TABLE 1 Bacterial growth on nutrient agar plates and plates with three different antibiotics added in the 2015 trial. Fractions indicate the number of samples out of the total samples taken that yielded at least one type of bacterium

Sample	Nutrient Agar	10 μg/ml Gentamicin	100 μg/ml Streptomycin	100 μg/ml Ampicillin
Piglets	6/6	6/6	5/6	6/6
Larval masses	6/6	6/6	6/6	6/6
Adult dipterans	18/21	5/21	4/21	6/21
Total	30/33	17/33	15/33	18/33

swabs from the adult dipterans yielded significant growth of culturable bacteria colonies, indicating that colony-forming units (CFUs) were present in all but three swabbed samples; those three were discarded. All but one of the plates swabbed with samples taken from cadavers and larval masses showed bacterial growth, regardless of the type of antibiotic present (Table 1). Eighteen of the 21 adult dipterans yielded culturable bacteria. Of those 18, nine carried bacteria that were resistant to at least one antibiotic (Table 1); four adult dipterans carried culturable bacteria resistant to at least two antibiotics, two of those four carried culturable resistant to all three antibiotics (Table 2). The two adult dipterans carrying culturable bacteria resistant to all three antibiotics were collected from the same cage containing two cadavers, and four of the five adult dipterans carrying culturable bacteria resistant to gentamicin were also collected from that same cage. Adult dipterans carrying culturable bacteria resistant to ampicillin and streptomycin were collected from all three cages. Culturable bacteria sampled from cadavers and larval masses were several times more likely to grow on each of the three types of antibiotic-treated plates compared to those sampled from adult dipterans (ampicillin, $\chi^2_2 = 15.71$, $p < .0001$; streptomycin, $\chi^2_2 = 16.58$, $p < .0001$, gentamicin, $\chi^2_2 = 17.75$, $p < .0001$). Of the three sample types, the likelihood of culturable bacteria growing on different types of antibiotic-treated media only varied among adult dipteran samples ($\chi^2_3 = 25.70$, $p < .0001$, $p > .05$ for both cadaver and larval mass samples) (Tables 1 and 2).

TABLE 2 Presence (1) and absence (0) of bacteria resistant to ampicillin, streptomycin and gentamicin on nine of 21 adult calliphorids collected in 2015

	Antibiotic-resistant bacteria		
Dipteran	10 μg/ml gentamicin	100 μg/ml streptomycin	100 μg/ml ampicillin
1	1	0	0
2	0	0	1
4	1	1	1
8	0	0	1
9	0	0	1
11	0	1	1
14	1	1	1
15	1	1	0
16	1	0	0
Number of flies	5	4	6

3.2 | Coliform—*Staphylococcus* trial

All 44 nutrient agar plates yielded multiple types of culturable bacterial colonies, indicating that live culturable bacteria were present on all swabbed samples. All of the samples taken from larva masses and cadavers yielded culturable bacteria resistant to penicillin, while 17 of 30 sampled adult dipterans (57%) yielded culturable antibiotic-resistant culturable bacteria (Table 3). While a trend was found that the frequency of penicillin-treated plates showing microbial growth varied among the three sample sources ($\chi^2_2 = 4.76$, $p = .09$), there was no evidence that the presence of cultured bacteria on the plate was dependent on either sample type or presence/absence of antibiotic ($\chi^2_2 = 3.70$, $p = .157$). Moreover, the number of distinguishable culturable bacteria morphotypes growing upon penicillin-treated plates did not differ among the three sample sources (adult dipteran, cadaver and larval mass), even when plates with counts of "0" were included in the analysis ($F_{2,41} = 1.53$, $p = .23$) (Figure 3).

We were able to culture on antibiotic-treated media 134 distinct morphospecies from 16 adult dipterans, seven cadavers and seven larval masses. The majority (120) of these culturable bacteria were Gram-negative, with 105 of those potentially coliforms based on their ability to ferment lactose (Table 4). The other 15 Gram-negative CFUs that were cultured on MacConkey agar showed no lactose-fermentation ability. Tentatively, 14 CFUs that cultured in the high-salt environment of the MSA plates were identified as *Staphylococcus* species (eight of which fermented mannitol [possibly *S. aureus*] and six of which were non-fermenters of mannitol [possibly *S. epidermidis*]) (Table 4). Interestingly, of the four types of penicillin-resistant Gram-positive CFUs grown on MSA, potential pathogens (mannitol fermenters) were found only on adult dipterans and not on the piglets or larval masses. Similarly, potential

TABLE 3 Bacterial growth on nutrient agar plates and plates with 100 iu/ml penicillin added. Fractions indicate the number of samples out of the total samples taken that yielded at least one type of bacterium

Sample	Nutrient agar	100 iu/ml penicillin
Piglets	7/7	7/7
Larval masses	7/7	7/7
Adult dipterans	30/30	17/30
Total	44/44	31/44

TABLE 4 Number of colonies of each of four types of culturable penicillin-resistant bacteria found on piglets, dipteran larvae feeding on those piglets and adult dipterans visiting those piglets

	Gram-positive		Gram-negative	
Sample	Mannitol fermenters	Mannitol non-fermenters	Lactose fermenters	Lactose non-fermenters
Piglet	0	1	13	0
Larval masses	0	5	26	0
Dipteran adults	8	0	66	15
Total	8	6	105	15

Gram-negative pathogens (non-lactose fermenters) were found only on the adult dipterans.

4 | DISCUSSION

The level of antibiotic resistance we encountered was astounding both in frequency and in scope. There was a high prevalence of antibiotic resistance to four antibiotics from two groups of antibiotics; aminoglycosides (gentamicin and streptomycin) and β-lactams (penicillin and ampicillin). The two groups have different modes of action. Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life threatening infections (Gilbert, 1995). Gentamicin is a good example of aminoglycosides, and they are useful in the treatment of Gram-negative bacillary infections (Mingeot-Leclerq, Glupczynski, & Tulkens, 1999). Only gentamicin is routinely used on the farm under consideration and on live-birthed piglets as part of neonatal care. It was, therefore, not surprising that the resistance profile of the culturable bacteria to gentamicin and streptomycin was very similar (Table 1). However, resistance to ampicillin and penicillin (which are not in use at the farm) was even broader than that of the aminoglycosides. Resistance to penicillin is to be expected in Gram-negative bacteria, which are by nature penicillin-resistant but susceptible to semi-synthetic ampicillin (Acred, Brown, Turner, & Wilson, 1962; Petri 2011). Culturable ARB were found on all three samples (stillborn piglets, dipteran larvae masses and adult dipterans). This level of resistance to broad-spectrum antibiotics by so many isolates, both Gram-positive and Gram-negative, is of great concern, especially when the bacteria involved belong to potentially pathogenic taxa.

Overall, we found more evidence that ARB were ubiquitous in the sampled animals, even those that had not been treated with antibiotics, and that the exteriors of dipteran larvae feeding on ARB-containing carrion could pick up ARB. We also found that adult dipterans rarely do, but are able to pick up LA-ARB from piglet cadavers. Transfer of microbes from livestock to dipterans and vice versa has been documented by other studies. Houseflies can also disseminate bacteria by harbouring them on their external body parts, although microbe survival may be affected by drying during flight (Mian, Maag, & Tacal, 2002; Yap, Kalpana, & Lee, 2008). Other studies have also documented dipterans (blowflies and houseflies) as efficient mechanical vectors of pathogenic bacteria and carriers of Gram-negative bacteria (genera *Salmonella* and *Escherichia*) in their guts (Pace et al., 2017).

One interesting and intriguing outcome of this study was the presence of potentially pathogenic Gram-negative bacteria possibly *Salmonella* species, and Gram-positive bacteria, which include *S. aureus*, in adult dipterans only and not on the cadavers or larvae. Although the adult dipteran population was not sampled for ARB prior to leaving piglets outside in cages (due to the fact that they were not reared by the researchers, but collected on site as a product of the environment), we found possible evidence that adult dipterans can rapidly pick up culturable ARB from the environment. It is therefore likely that the adult dipterans obtained pathogens from both the cadavers and the environment. *E. coli* O157:7 and *Salmonella enterica* have been shown to be transmitted from inoculated manure to lettuce mechanically (Pace et al., 2017). We did not, however, serotype the cultured CFUs for this study.

The findings indicate the need for further research in the natural incidence of ARB in dipteran populations to help clarify the extent to which adult dipterans can pick up and transmit ARBs. In addition, further screening for pathogenic microbe taxa can inform researchers about the potential dangers of fly transmission of ARB. Because of dipteran life cycles, and the presence of larvae on carrion, further research should look into the possibility of ARB that could be carried over from immature to adult life stages.

This study provides evidence that dipteran populations in animal rearing operations are potential vectors of ARB that include potentially pathogenic strains. We suggest that exposure to those populations should be monitored and minimized to limit the possible transmission of pathogenic ARB to the human population. Further work could more specifically identify the ARB to species and strain level through pulse field gel electrophoresis, and sequence-based techniques can both identify the antibiotic resistance genes in microbial populations associated with dipterans as well as identify non-culturable microorganisms of potential public health importance. Standard antibiotic sensitivity testing using dilution methods may further characterize the level of resistance. It would also be informative to continue the sampling the decomposing cadavers for longer periods of time to determine any microbiome profile changes with further decomposition of carcasses as was performed by other researchers (Weatherbee, Pechal, & Benbow, 2017).

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CONFLICT OF INTEREST

We declare no conflict of interest.

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