

Antimicrobial-resistant bacteria and microbiota identified among flies and their sympatric animals

by

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Abstract

Antimicrobial resistance is rising globally at an alarming rate as one of the most serious public health challenges. There is increasing evidence that the environment plays a major role in development and transmission of antimicrobial resistance genes. However, studies on the environmental connection to the spread of these important genes are scarce. Filth flies have well adapted to live in close association with humans and animals. They are attracted to microbe-rich organic materials for reproductive purposes. As a result, they can acquire bacteria including antimicrobial-resistant bacteria from animal wastes and act as a potential vector of bacterial infections. The research conducted for this dissertation aims to investigate the role of flies as a sentinel candidate for surveillance of antimicrobial resistance by isolating antimicrobial-resistant bacteria from flies and analyzing the relationship between flies, their environment, and the antimicrobial resistance shared between them. Flies were collected from six locations in Auburn, Alabama and split into three sets for analysis. Additionally, fecal samples were collected from sympatric animals in four animals' husbandries at the same time flies were trapped. *Escherichia coli* and *Klebsiella pneumoniae* were isolated from 38.0% and 16.7%, respectively, of flies. These bacteria included multidrug-resistant and extended-spectrum beta-lactamase (ESBL)-producing strains. Notably, two flies at a dog kennel were found to have ESBL-producing *E. coli* isolates that belong to the same sequence type (ST68) and serotype (O25:H6). Moreover, those isolates were found to harbor the same virulence and antimicrobial resistance genes identified by whole genome sequencing, indicating that the isolates are closely related, and flies might have acquired them from the same source. Microbiota and antimicrobial resistance genes as well as mobile genetic elements (ARG/MGE) detected in flies from the second set were compared to those present in fecal samples of sympatric animals. Microbiota of the flies were found to be

more similar to the microbiota of the feces of their sympatric animals than to microbiota of feces of other animals. Similarly, resistome analysis of ARG/MGE showed that 91.7% of ARG/MGE detected in flies were also found in feces. Flies in the third set were used for isolation of a total of 485 colistin-resistant bacteria, the majority of which were naturally resistant bacteria. A colistin-resistant *K. pneumoniae* strain was analyzed by whole genome sequencing. The genome does not harbor any known genes or mutations conferring resistance to colistin. However, the genome contains various virulence determinants involved in adhesion, iron acquisition and, most importantly, the regulator gene *magA*, which contributes to *K. pneumoniae* virulence by hypermucoviscosity phenotype. In total, the analysis of these flies and feces of sympatric animals shows that flies might mirror bacterial communities from the environment, including those with clinical importance and antimicrobial resistance phenotype. However, further investigation with long-term studies on more geospatially distinct populations is needed to determine the suitability of flies as sentinels for antimicrobial resistance surveillance programs.

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List of Abbreviations

AMR	antimicrobial resistance
ARG	antimicrobial resistance gene
bp	base pair
CARD	Comprehensive Antibiotics Resistance Database
cgMLST	core-genome multilocus sequence typing
cgST	core-genome sequence type
COG	Cluster of orthologous groups
CRE	carbapenem resistant <i>Enterobacteriaceae</i>
ESBL	extended-spectrum β -lactamase
Gb	gigabase ($*10^9$ bases)
HGT	horizontal gene transfer
IMViC	indole, methyl red, Voges-Proskauer, and citrate tests
KEGG	Kyoto Encyclopedia of Genes and Genomes
$_L$ -Ara4N	4-amino-4-deoxy- $_L$ -arabinose
LPS	lipopolysaccharide
Mb	megabase ($*10^6$ bases)
MDR	multidrug-resistant
MFS	major facilitator superfamily
MGE	mobile genetic element
MIC	minimal inhibitory concentration
MLS _B	macrolides, lincosamide and streptogramins B
MLST	multilocus sequence typing

MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
OTU	Operational Taxonomic Unit
PEtN	phosphoethanolamine
RND	Resistance-Nodulation-Division superfamily
spp.	species
T6SS	type VI secretion system
VFDB	Virulence Factors Database
WGS	whole genome sequencing

Chapter 1

Literature Review

Abstract

The successes in antibiotic discoveries that occurred in the 20th century during the “antibiotics golden era” have helped combat bacterial infections. However, bacteria have evolved antimicrobial resistance (AMR) mechanisms. AMR is a serious public health threat that is rising globally at an alarming rate. Understanding the emergence and mode of transmission of AMR helps to prevent and control that global crisis. While multiple active surveillance programs have been established to monitor AMR, studies on the environmental link to antimicrobial spread are lacking. Filth flies are well-known mechanical and biological vectors of bacterial pathogens including antimicrobial-resistant bacteria but there is little data on their role in spreading microbial community and antimicrobial resistance. In this review, emergence, dissemination, and mechanisms of antimicrobial resistance with highlights on extended-spectrum beta lactamases and colistin resistance will be discussed. Also, “filth” fly’s role as mechanical and biological vectors of bacteria, as well as aspects of fly life cycles that result in their interaction with microbes, will be reviewed. Finally, current surveillance methods will be discussed.

1.1 Antimicrobial Resistance

1.1.1 Causes and emergence of the crisis

Most current antibiotics were discovered during the “Golden Age” of antibiotics from 1940 to 1962. Penicillin was the first antimicrobial agent discovered, in 1928 by Sir Alexander Fleming (Masuda and Ohya, 1992). It was released for clinical use in 1943 to treat bacterial infections. However, penicillin-resistant strains were identified at the same time among the soldiers during the World War II, and penicillin resistance became a substantial health problem in the 1950’s. Antimicrobial resistance (AMR) is defined as the presence of a genetically intrinsic or acquired resistance mechanism in a bacterium that is otherwise naturally susceptible to a particular antibiotic via a mutation or acquisition of resistance genes. AMR is one of the major threats to public health, causing at least 2.8 million infections and resulting in approximately 35,000 deaths every year in the United States (Hoelzer *et al.*, 2017; U.S. Centers for Disease Control and Prevention, 2019). Mortality due to AMR infections has been estimated to contribute to 10 million deaths annually worldwide by 2050 (O’Neill, 2016). Therefore, understanding the emergence and the mode of transmission of AMR will help to prevent and control the global crisis.

As early as 1945, Sir Alexander Fleming warned about the emergence of AMR when he said, “the public will demand [the drug and] ... then will begin an era ... of abuses. The microbes are educated to resist penicillin....” (Ventola, 2015). Although it is difficult to directly link the use of antibiotics to the emergence of AMR, there is a strong evidence showing that abuse of broad-spectrum antibiotics has selected and favored the survival of resistant strains by eliminating other susceptible competitive bacteria, and thus increasing the accessibility to available nutrients (de Man *et al.*, 2000). Furthermore, horizontal gene transfer (HGT) that

occurs among bacteria allows the transfer of antimicrobial resistance genes (ARG) among bacteria within the same or different species (Read & Woods, 2014). A study performed by the Information Medical Statistics Company estimated that 22 antibiotic doses were prescribed per person every year in the United States (Van Boeckel *et al.*, 2014). Unfortunately, up to 50% of prescribed antibiotics are inappropriately indicated for the treatment, choice of drug, or duration (U.S. Centers for Disease Control and Prevention, 2013). For example, more than 90% of patients with community-acquired pneumonia receive antibiotics without the pathogen being identified (Bartlett, 2011); and up to 60% of antibiotics prescribed in intensive care units are prophylactic and/or unnecessary (Roberts *et al.*, 2014).

In some developing countries, antibiotics are available without a prescription, and their use is unregulated. In a study conducted by the Netherlands Institute for Health Services Research (Nivel), 7% of antibiotics used in human medicine in Europe are taken without prescriptions because antibiotics are available without a prescription in some southern European countries such as Romania and Greece. Unfortunately, use of antibiotics without prescription is not well documented in developing countries, and most surveys rely on patient reporting of antimicrobial use (Morgan *et al.*, 2011).

The overconsumption of antimicrobials extends beyond human use to animals and livestock. Antibiotics are extensively used as growth promoters in livestock especially in developing countries, to improve the livestock production (Van Boeckel *et al.*, 2015). The mechanism by which antimicrobial growth promoters work is not clear. Four hypotheses have been proposed to explain antimicrobial growth promoters' action: 1) protection of nutrients against bacterial destruction; 2) improvement of nutrient absorption through intestinal barriers; 3) reduction of intestinal bacterial toxins; and 4) reduction in the incidence of intestinal

infections (Feighner and Dashkevicz, 1987). China is the world's largest consumer of antibiotics, five times greater than the U.S. and Europe combined. A total of 97,000 tons of antimicrobials are used in Chinese farms, with 30% used as growth promoters. This extensive use of antimicrobials has resulted in the presence of antimicrobial residues as well as resistant bacteria in livestock products, waste, and environmental water (Tang *et al.*, 2016). Prior to its ban in 2017 in the United States (FDA, 2016), inclusion of antimicrobials in animal feed was extensively practiced. In 2011, a total of 13,542 tons of antibiotics were purchased as animal growth promoters (FDA, 2011), while only 3,290 tons were used in human medicine (FDA, 2012). Although controversial, using antimicrobials as growth promoters can contribute to selection of resistant bacteria in the animals' microbiota which can be transmitted to consumers, other animals, or to the environment through animal waste (Xi *et al.*, 2009).

The environment plays a major role in development and transmission of AMR. Soil and environmental water are considered vital reservoirs for antimicrobial resistance genes (D'Costa *et al.*, 2006). The release of antimicrobial residues into the environment and consequent bacterial exposure to subinhibitory concentrations increase the rate of spontaneous and random mutations in the bacterial genome (Morero *et al.*, 2011), plasmid transfer via conjugation (Johnson *et al.*, 2015), and transposon activity (Hocquet *et al.*, 2012). There is a strong evidence that ARG in pathogenic bacteria have been originally acquired from environmental sources (Rhodes *et al.*, 2000; Forsberg *et al.*, 2012). Sewage is widely recognized as a "hotspot" for the development and a point source for spread of resistant bacteria even in the absence of antimicrobial residues (Bengtsson-Palme *et al.*, 2016). It is important to recognize that ARG harbored by bacteria in the environment can mediate essential functions in the bacterial cell other than AMR. For example, the enzyme encoded by chromosomal gentamicin-acetylating gene, *aac(2')-Ia*, has been shown

to be involved cell wall metabolism and responsible for acetylation of peptidoglycan in *Providencia stuartii* (Payie *et al.*, 1995; Macinga and Rather, 1999).

HGT is crucial in spread of ARG between bacteria, often under selection pressure from antimicrobial agents or biocides such as disinfectants (Jutkina *et al.*, 2016; Zhang *et al.*, 2017). Although sewage and wastewater are hotspots for HGT among bacteria as they provide sufficient nutrients and contain antibiotic residues and diverse species of bacteria from different sources (Schluter *et al.*, 2008), the transfer of ARG in sewage plants remains difficult to demonstrate due to the lack of technologies that are able to determine such transfers. Furthermore, detection of the genes and/or resistant bacteria are not sufficient to demonstrate the occurrence of the HGT.

1.1.2 Extended-spectrum beta lactamases (ESBL)

Beta-lactams are the most frequently prescribed antibiotics in the United States (Hick *et al.*, 2015). Based on chemical structures, the large number of available β -lactams can be subdivided into 5 groups: penicillins, cephalosporins, cephamycins, carbapenems, and monobactams. (Finch *et al.*, 2012). Bacterial resistance to β -lactams can be caused by modification of target sites, reduced permeability, efflux, or drug degradation by β -lactamases (Wilke *et al.*, 2005). β -Lactamases are the primary resistance mechanism in Gram-negative bacteria. The enzymes hydrolyze the amide bond of the carbonyl moiety of the β -lactam ring (Fisher *et al.*, 2005). Penicillin-resistant strains were first identified in the 1940's. That accelerated the need for next-generation antibiotics such as cephalosporins to overcome the resistance. Currently, there are 5 generations of cephalosporins: first-generation (cefazolin, cephalexin), second-generation (cefoxitin, cefotetan, cefmetazole, cefaclor, cefpodoxime, and cefuroxime), third-generation (cefixime, cefodizime, cefotaxime, cefteteram, and ceftizoxime),

fourth-generation (cefepime, ceftuprenam, cefoselis, ceftazopran, ceftiprome, and ceftquinome), and fifth-generation (ceftaroline). The discovery of cefotaxime (the third-generation cephalosporin) in 1976 was a groundbreaking discovery in antimicrobial therapy because of its extended-spectrum and resistance to penicillinases. However, extended-spectrum β -lactamase (SHV-2) was identified in 1983 with a point mutation in *bla_{SHV-1}* encoding penicillinase, which resulted in glycine replacement with serine at position 238 in SHV-1 β -lactamase (Knothe *et al.*, 1983). Since then, 36 SHV allelic variants have been described in NCBI Beta-Lactamase Data Resources (Lahey list of beta-lactamases) in a wide range of *Enterobacteriaceae* members, *Pseudomonas aeruginosa*, and *Acinetobacter* species to mediate resistance to extended-spectrum β -lactams (Poirel *et al.*, 2004; Liakopoulos *et al.*, 2016). Along with the SHV family, TEM and CTX-M β -lactamases are the most identified ESBL families. The first two reported TEM enzymes were TEM-1 and TEM-2 from an *E. coli* isolate in 1965 (Datta and Kontomichalou, 1965). Both enzymes do not have degradation activity on extended-spectrum cephalosporins. However, two amino acid substitutions in TEM-2 result in a new allelic variant (TEM-3) that has extended activity against cefotaxime (Sougakoff *et al.*, 1988). As of today, there are total of 142 TEM allelic variants in NCBI Beta-Lactamase Data Resources, among which there are 85 ESBL enzymes. CTX-M enzymes are a group of 140 ESBL enzymes reported to date that confer resistance to third-generation and fourth-generation cephalosporins (Poirel *et al.*, 2002; Bonnet, 2004; Sturenburg, 2004). Other ESBL families include allelic variants of AmpC, BEL, CARB, CMY, OXA, and PER β -lactamases.

1.1.3 Colistin resistance

Colistin, polymyxin E, is a member of cationic decapeptide polymyxin antimicrobial agents that are isolated from *Paenibacillus polymyxa* (Storm *et al.*, 1977). Polymyxins were discovered in the 1940's and were in use until the 1970's when their use waned due to their nephrotoxicity and neurotoxicity. Colistin has been extensively used in veterinary medicine and as a growth promoter in livestock. The emergence of carbapenem resistant *Enterobacteriaceae* (CRE), *P. aeruginosa*, and *A. baumannii* has necessitated resurgence of polymyxins in human medicine as the last-resort antimicrobial agent with a narrow-spectrum activity. There are 5 groups in polymyxins (A-E), with only polymyxin B and colistin approved for clinical use as the last-resort for multidrug-resistant Gram-negative bacteria (Li *et al.*, 2006). The consensus mechanism of action is that polymyxins disturb the outer membrane via binding to the lipid A component of lipopolysaccharides (LPS), as diaminobutyric acid residues in polymyxins provide electrostatic attractions to anionic phosphate groups of lipid A, displacing divalent cations that stabilize the LPS (Figure 1.1). A secondary mode of action of polymyxins involves cell membrane penetration and inhibition of type II-quinone oxidoreductases.

Colistin resistance has led to fear of pan-drug resistance. Bacteria survive colistin along with cationic peptides by remodeling the LPS to reduce the net negative charges. Such modifications occur as a result of substituting the 4' phosphate group with cationic 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or substituting the 1' phosphate group with phosphoethanolamine (PEtN) in order to neutralize the net charge of LPS and therefore having lower affinity to cationic peptides. The strategies and enzymes involved in LPS modifications differ between genera. In some *Enterobacteriaceae* such as *Salmonella enterica*, *K. pneumoniae*, and *E. coli*, polymyxin resistance requires activation of PmrA-PmrB, PhoP-PhoQ, and/or CrrA-CrrB two component regulatory systems (Figure 1.2). These two-component regulatory systems

are also produced by colistin susceptible Gram-negative bacteria, but some mutations result in consecutive expression of the two-component regulatory systems (Jayol *et al.*, 2014; Jayol *et al.*, 2015; Poirel *et al.*, 2015). Tables 1.1, 1.2, and 1.3 summarize known mutations reported in NCBI's Pathogen Detection Reference Gene Catalog.

PmrB is the sensor histidine kinase of PmrA-PmrB two component regulatory system. It is activated by autophosphorylation and phosphorelay to the response regulator PmrA, which in turn upregulates expression of various LPS modification genes including *arnBCADTEF* operon (also known as *pmrHFIJKLM*) and *pmrC* gene resulting in addition of L -Ara4N and PEtN, respectively (Figure 1.2) (Gunn *et al.*, 1998; Gatzeva-Topalova *et al.*, 2005; Yan *et al.*, 2007). In some *K. pneumoniae* strains, CrrA-CrrB two component regulatory system is involved in colistin resistance by CrrA-mediated activation of expression of *crrC*, which phosphorylates PmrA and consequently upregulates *arnBCADTEF* operon (Cheng *et al.*, 2016). PmrA can also upregulate expression of *pmrF*, encoding a regulator that represses expression of *lpxT* responsible for phosphate addition into lipid A. Subsequently, less phosphate groups are added to LPS results in less negative charge of LPS (Touzé *et al.*, 2008).

Indirect activation of PmrB can be triggered by PhoP-PhoQ through activation of the linker protein PmrD. The regulatory protein, PhoP, activates PmrD which then binds to the phosphorylated PmrA to inhibit PmrB-mediated dephosphorylation (McPhee *et al.*, 2006; Cheng *et al.*, 2010). Alternatively, PhoP can upregulate the expression of *arnBCADTEF* operon independently of PmrA (Mitrophanov *et al.*, 2008). MgrB is a small transmembrane peptide that works as a negative feedback to inhibit PhoQ phosphorelay (Lippa and Goulian, 2009). Some Gram-negative bacteria such as *Proteus* spp., *Providencia* spp., *Morganella morganii*,

Burkholderia spp., and *Serratia marcescens* are naturally resistant to colistin with their LPS containing L-Ara4N (Sidorczyk *et al.*, 1983; Falagas and Kasiakou, 2005).

The global prevalence of colistin resistance has become a significantly major concern since the emergence of plasmid-mediated mobile colistin resistance (*mcr*) genes. *mcr-1* was first isolated from *E. coli* strains in China (Liu *et al.*, 2016). Since then, 9 more variants of *mcr* genes (*mcr-2* to *mcr-10*) have been reported (Table 1.4) (Xavier *et al.*, 2016; Yin, *et al.*, 2017; Carattoli *et al.*, 2017; Borowiak *et al.*, 2017; AbuOun *et al.*, 2018; Yang *et al.*, 2018; Wang *et al.*, 2018; Carroll *et al.*, 2019; Wang *et al.*, 2020). MCR proteins are PEtN transferase metalloenzymes containing four zinc ions (Ma *et al.*, 2016; Coates *et al.*, 2017). They add PEtN to 1(4')-phosphate moieties to lipid A thus reducing the negative charge of LPS, resulting in less affinity of LPS to colistin (Liu *et al.*, 2016). The emergence of *mcr* genes poses a major health threat because they can rapidly spread via horizontal gene transfer. Except the allelic variant of *mcr-1* (*mcr-1.10*) in *Moraxella poric* and *mcr-6* in *Moraxella pluranimalium*, dissemination of plasmid-containing *mcr* genes to non-fermentative organisms such as *P. aeruginosa* and *A. baumannii* has not been reported yet. However, the plasmids can be transferred to *P. aeruginosa* and *A. baumannii* during conjugation experiments in the laboratory (Liu *et al.*, 2016).

1.2 “Filth” flies

Filth flies are the flies that feed and breed on decaying organic material such as human feces and animal manure. There are two main characteristics that are shared by filth flies: they are coprophagic (feed on feces and manure) and synanthropic (live in a close association with human and domestic animals). Among over 125,000 different species of flies, the filth flies that are mostly involved in bacterial dissemination are houseflies and blowflies. They are often found

in abundance in areas of human and animal activities such as food facilities, hospitals, markets, and poultry and livestock facilities. They act as transmission vectors for bacterial organisms due to their unrestricted movement even for long distances (5-7 km) (Greenberg, 1965).

1.2.1 Fly-microbe interactions

Female houseflies lay about 100-150 eggs at a time in microbe-rich masses such as decaying organic material, the substrate characteristics of oviposition sites. Eggs hatch within 8-24 hours (8-12 hours under optimal temperature 25-35°C) into first-instar larvae, which utilize bacteria in the masses as a source of food and develop into two additional instars. The third-instar larvae migrate to a dry and cool place for metamorphosis (elimination of larval organs and development of pupation) to form pupae (Čičková *et al.*, 2012).

Numerous studies have demonstrated the importance of bacteria as a food source to larval development as larvae fail to survive and develop in axenic environments (Zurek *et al.*, 2000). Within organic materials, bacterial communities change dramatically due to changes in pH, water content, and availability of oxygen. Some bacterial species are known to attract larvae and required for larval development. For example, *Proteus mirabilis* is known to produce volatile swarming signals in decaying organic material that might attract filth flies for breeding (Ma *et al.*, 2012). Some organisms such as *Klebsiella oxytoca* show a reverse effect on female houseflies' attraction from induction to inhibition (Lam *et al.*, 2007). In addition, some bacterial species are utilized more effectively by flies than others. Rochon *et al.* (2004) demonstrated that larvae of houseflies reared on *E. coli* had better survival than larvae of stable flies growing on *E. coli*. Most bacterial taxa in feces and manure are digested by larvae, yet some survive and remain in the larval alimentary tract. Bacterial consumption diminishes during development of the third-

instar larvae before pupation and returns during metamorphosis. In pupae, ingested bacteria inside the lining of larval gut are shed which results in loss of most bacteria (Zurek and Nayduch, 2016). Remaining bacteria are digested in the alimentary canal of an adult housefly, which is protected with two layers of a non-cellular peritrophic matrix, which functions as a barrier containing antimicrobial peptides and digestive enzymes (Nayduch *et al.*, 2005). In conclusion, the carriage of bacteria from early stages to adults requires bacterial resistance against proteolytic and expulsive processes during development. Therefore, newly emerged adult flies carry relatively small concentrations of bacteria from pupae; and survival of bacteria during metamorphosis is solely dependent on bacterial species, not bacterial abundance, in rearing media (Su *et al.*, 2010; Bahrndorff *et al.*, 2014).

Although bacteria are not the main nutritional source for adult flies, all life stages of flies have adapted to live in a close association with human feces and animal manure, which are microbe-rich environments attracting adult female houseflies for oviposition and thus facilitating microbial acquisition by adult houseflies. Ingested bacteria are initially harbored in the crop where they are either predigested by salivary digestive enzymes or regurgitated as vomit droplets (Stoffolano and Haselton, 2013). Ingested bacteria are then transferred to the alimentary canal through the proventriculus. There are three regions of the alimentary canal: midgut, hindgut, and rectum. The midgut is covered with the peritrophic matrix, whereas other regions are protected with cuticle to prevent bacterial access to epithelial cells (Steinhaus, 1952). In the midgut, ingested bacteria are captured in the peritrophic matrix, where they are sensed by pattern-recognition receptors that elicit innate immune responses such as production of antimicrobial peptides. Studies of temporospatial fate of bacteria inside the midgut have shown that all bacterial species were consistently contained in the peritrophic matrix. In such cases, flies serve as

environmental reservoirs for bacteria, especially the ones that are highly resistant to antimicrobial peptides such as *Salmonella* Typhimurium and *P. aeruginosa*, which not only persist but also proliferate in the peritrophic matrix in high numbers (Joyner *et al.*, 2013; Chifanzwa and Nayduch, 2017). *E. coli* O157:H7, on the other hand, was found to be able to survive and proliferate in the crop for 3 days (Wasala *et al.*, 2013). Another example is *Aeromonas caviae*, which was shown to proliferate inside experimentally-fed houseflies for 3 days and remain viable for 8 days (Nayduch *et al.*, 2002).

Upon exposure of flies to microbe-rich environments, bacterial organisms can easily stick to the exoskeleton and be transmitted to humans and animals through translocation (Figure 1.3). Enteric bacteria are the most abundant organisms in flies due to their abundance in feces and manure, and these isolated bacteria include potential pathogens, with some having antimicrobial resistance phenotypes. The bacteria include but not limited to enterohemorrhagic *E. coli* (EHEC) (Iwasa *et al.*, 1999), enteroaggregative *E. coli* (EAEC) , enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) (Khin Nwe *et al.*, 1989), *Shigella* spp. (Butler *et al.*, 2010), *Salmonella* spp. (Hemmatinezhad *et al.*, 2015), *Campylobacter jejuni* (Szalanski *et al.*, 2004), *Vibrio cholerae* (Khin Nwe *et al.*, 1989), *Klebsiella* spp. (Davari *et al.*, 2010), *Pseudomonas* spp. (Hemmatinezhad *et al.*, 2015), *Helicobacter pylori* (Allen *et al.*, 2004), and *Clostridium* spp. (Bahrndorff *et al.*, 2017).

The microbiome of filth flies is dynamic, with presence of host-specific bacterial communities due to acquisition of bacteria from similar habitats and large variations affected by the lifestyle of flies (Bahrndorff *et al.*, 2017; Junqueira *et al.*, 2017).

1.3 Surveillance for AMR

Antimicrobial resistance has become a global serious threat to public health. Early detection of antimicrobial resistance is critical to understand the origin and to establish and implement prevention strategies. More importantly, surveillance of antimicrobial resistance is a vital element to provide information about changes in susceptibility and prevalence of resistance, to establish trends in pathogen antimicrobial resistance, to identify emergence of new resistance mechanisms, and to help in the control of resistance. Thus, a well-planned surveillance study should provide data to determine changes of antimicrobial susceptibility, to assess the progress of resistance, and to determine whether resistance is associated with an outbreak or an infection. The United States National Antimicrobial Resistance Monitoring System (NARMS) is a national surveillance program established in 1996 to monitor resistance trends by providing data on the extent and temporal trends of resistance in enteric organisms such as *Salmonella* spp. and *Campylobacter*. NARMS is a collaborative program by the Department of Agriculture (USDA), the Centers for Disease control and Prevention (CDC), and the Food and Drug Administration (FDA) to connect human, animal, and environment health through passive and active sampling methods (Ginevan, 2002).

Passive surveillance is defined as continuing monitoring of infections based on investigating bacteria isolated from clinically diseased groups. It is considered passive since the decision of inclusion or exclusion of data collection is made by clinicians not the investigators or authorities, and such practices are questionable because of inter-laboratory variations (Masterton, 2000). Passive surveillance is usually inexpensive and continuous. It also provides an early warning for emerging resistance. On the other hand, limitations of passive surveillance are substantial, as the data may not represent the bacterial population since only disease-related isolates are being investigated (Mather *et al.*, 2016). Data collection in this manner is of little

value in itself because these data can be biased and frequently collected after failure of initial treatments, with potentially missed cases due to a lack of urgency sensed by healthcare providers for the collection of passive data.

Active surveillance is defined as a planned periodic collection of targeted and representative samples. Consequently, data collected through active surveillance better reflect the characteristics of the general population. In contrast to passive surveillance, samples to be included in active surveillance are selected through a formal sampling process initiated by the investigator. In other words, appropriate target populations are defined first, before sampling. For example, in active surveillance of antimicrobial resistance in the food chain, samples that are collected from live healthy animals, carcasses, and retail meat are used to estimate trends of resistance over time (Mather *et al.*, 2016). This targeted surveillance method is often done in the form of single or repeated surveys to provide trends in a longer duration. Contrary to passive surveillance, active surveillance can be costly since it targets putatively healthy subjects that may not carry antimicrobial-resistant organisms. Active surveillance overcomes the two general sources of bias inherent in passive surveillance: 1) passive surveillance targets bacterial isolates from disease-related individuals, and 2) it solely depends on clinicians' submission. On the other hand, active surveillance screens putatively healthy individuals and meat in a population-based sampling method.

To overcome limitations of passive and active sampling methods, sentinel surveillance can be implemented to monitor the rate of occurrence of antimicrobial resistance in a sub-population to estimate trends of resistance in the environment. The primary objective of sentinel surveillance is to provide an accurate estimate of prevalence and early detection of occurrence (Schrag *et al.*, 2002). Some U.S. states have adopted and implemented sentinel surveillance as an

alternative surveillance method to estimate prevalence and to monitor trends of antimicrobial resistance in a population based on data collected from sub-populations such as hospitals (Breiman *et al.*, 1994; Richards *et al.*, 1999; Jernigan *et al.*, 2001; Rodríguez-Prieto *et al.*, 2015). However, such surveillance methods have not shown a high degree of accuracy in their estimation (Schrag *et al.*, 2002). In contrast, sentinel samples have been used as feasible indicators to estimate prevalence of pathogens such as sentinel chickens for West Nile virus (Scott *et al.*, 2001), feral pigs for *Mycobacterium bovis* (Nugent *et al.*, 2002), possum for *Mycobacterium ulcerans* (Carson *et al.*, 2014), and white-tailed deer for *Anaplasma phagocytophilum* (Dugan *et al.*, 2006).

References

- AbuOun, M., Stubberfield, E., Duggett, N., Kirchner, M., Dormer, L., Nunez-Garcia, J., et al. (2018) *mcr-1* and *mcr-2* (*mcr-6.1*) variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to 2015. *J Antimicrob Chemother* **73**: 2904–2904.
- Aghapour Z., Gholizadeh P., Ganbarov K., Bialvaei A., Mahmood S., Tanomand A., et al. (2019) Molecular mechanisms related to colistin resistance in *Enterobacteriaceae*. *Infect Drug Resist* **12**: 965–975.
- Allen, S., Thomas, J., Alexander, N., Bailey, R., and Emerson, P. (2004) Flies and *Helicobacter pylori* infection. *Arch Dis Child* **89**: 1037–1038.
- Bahrndorff, S., de Jonge, N., Skovgård, H., and Nielsen, J. (2017) Bacterial communities associated with houseflies (*Musca domestica*) sampled within and between farms. *PLOS ONE* **12**: e0169753.
- Bahrndorff, S., Gill, C., Lowenberger, C., Skovgård, H., and Hald, B. (2014) The effects of temperature and innate immunity on transmission of *Campylobacter jejuni* (Campylobacterales: *Campylobacteraceae*) between life stages of *Musca domestica* (Diptera: *Muscidae*). *J Med Entomol* **51**: 670–677.
- Bartlett, JG. (2011) Diagnostic tests for agents of community-acquired pneumonia. *Clin Infect Dis* **52**: S296–S304.
- Bengtsson-Palme, J., Hammaren, R., Pal, C., Ostman, M., Bjorlenius, B., Flach, CF., et al. (2016) Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci Total Environ* **572**: 697–712.
- Bonnet, R. (2004) Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother* **48**: 1–14.

- Borowiak, M., Fischer, J., Hammerl, J., Hendriksen, R., Szabo, I., and Malorny, B. (2017) Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in D-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. *J Antimicrob Chemother* **72**: 3317–3324.
- Bourrel, A., Poirel, L., Royer, G., Darty, M., Vuillemin, X., Kieffer, N., et al. (2019) Colistin resistance in Parisian inpatient faecal *Escherichia coli* as the result of two distinct evolutionary pathways, *Journal of Antimicrobial Chemotherapy* **74**: 1521–1530.
- Breiman, R., Butler, J., Tenover, F., Elliott, J., and Facklam, R. (1994) Emergence of drug-resistant pneumococcal infections in the United States. *JAMA* **271**: 1831–1835.
- Butler, J., Garcia-Maruniak, A., Meek, F., and Maruniak, J. (2010) Wild Florida house flies (*Musca domestica*) as carriers of pathogenic bacteria. *Florida Entomologist* **93**: 218–223, 216.
- Cannatelli, A., Di Pilato, V., Giani, T., Arena, F., Ambretti, S., Gaibani, P., et al. (2014a) In vivo evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing *Klebsiella pneumoniae* is associated with low-dosage colistin treatment. *Antimicrob Agents Chemother* **58**: 4399–4403.
- Cannatelli, A., Giani, T., D'Andrea, M., Di Pilato, V., Arena, F., Conte, V., et al (2014b) MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* **58**: 5696–5703.
- Cannatelli, A., Giani, T., Aiezza, N., Di Pilato, V., Principe, L., Luzzaro, F., et al (2017) An allelic variant of the PmrB sensor kinase responsible for colistin resistance in an *Escherichia coli* strain of clinical origin. *Scientific reports* **7**: 5071.

- Carattoli, A., Villa, L., Feudi, C., Curcio, L., Orsini, S., Luppi, A., et al. (2017) Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill* **22**: 30589.
- Carroll, LM., Gaballa, A., Guldemann, C., Sullivan, G., Henderson, LO., and Wiedmann, M. (2019) Identification of novel mobilized colistin resistance gene *mcr-9* in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype Typhimurium isolate. *MBio* **10**: e00853–e00819.
- Carson, C., Lavender, C., Handasyde, K., O'Brien, C., Hewitt, N., Johnson, P., and Fyfe, J. (2014) Potential wildlife sentinels for monitoring the endemic spread of human buruli ulcer in South-East Australia. *PLoS Negl Trop Dis* **8**: e2668.
- CDC. (2013) Antibiotic Resistance Threats in the United States, 2013. Atlanta, GA, USA.
- Cheng, H., Chen, Y., and Peng, H. (2010) Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *J Biomed Sci* **17**:60.
- Cheng, Y., Lin, T., Pan, Y., Wang, Y., Lin, Y., and Wang, J. (2015) Colistin resistance mechanisms in *Klebsiella pneumoniae* strains from Taiwan. *Antimicrob Agents Chemother* **59**: 2909–2913.
- Cheng, Y., Lin, T., Lin, Y., and Wang, J. (2016) Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **60**: 3709–3716.
- Chifanzwa, R. and Nayduch, D. (2017) Dose-dependent effects on replication and persistence of *Salmonella enterica* serovar Typhimurium in house flies (Diptera: *Muscidae*). *J Med Entomol* **55**: 225–229.

- Choi, M. and Ko, K. (2014) Mutant prevention concentrations of colistin for *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* clinical isolates. *J Antimicrob Chemother* **69**: 275–277.
- Čičková, H., Pastor, B., Kozánek, M., Martínez-Sánchez, A., Rojo, S. and Takáč, P. (2012) Biodegradation of pig manure by the housefly, *Musca domestica*: a viable ecological strategy for pig manure management. *PloS one* **7**: e32798–e32798.
- Coates, K., Walsh, T., Spencer, J., and Hinchliffe, P. (2017) 1.12 Å resolution crystal structure of the catalytic domain of the plasmid-mediated colistin resistance determinant MCR-2. *Acta Cryst.* **F73** (2017) 443–449.
- Datta, N. and Kontomichalou (1965) Penicillinase synthesis controlled by infectious *R* factors in *Enterobacteriaceae*. *Nature* **208**: 239–241.
- D'Costa, V., McGrain, K., Hughes, D., and Write, G. (2006) Sampling the antibiotic resistome. *Science* **311**: 374–377.
- de Man, P., Verhoeven, B., Verbrugh, H., Vos, M., and van den Anker, J. (2000) An antibiotic policy to prevent emergence of resistant bacilli. *Lancet* **355**: 973–978.
- Delannoy, S., Le Devendec, L., Jouy, E., Fach, P., Drider, D., and Kempf, I (2017) Characterization of colistin-resistant *Escherichia coli* isolated from diseased pigs in France. *Front microbiol* **8**: 2278.
- Dugan, V., Yabsley, M., Tate, C., Mead, D., Munderloh, U., Herron, M., et al. (2006) Evaluation of white-tailed deer (*Odocoileus virginianus*) as natural sentinels for *Anaplasma phagocytophilum*. *Vector Borne Zoonotic Dis* **6**: 192–207.
- Falagas, ME., Kasiakou, SK. (2005) Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. *Clin Infect Dis* **40**:1333–1341.

- FDA. (2011) Summary report on antimicrobials sold or distributed for use in food-producing animals. (Medicine FaDA-CfV, ed.).
- FDA. (2012) Drug use review. (Services F-DoHaH, ed.).
- FDA, Center for Veterinary Medicine. (2016) FDA reminds retail establishments of upcoming changes to the use of antibiotics in food animals. (Veterinary Feed Additive).
- Feighner S. and Dashkevicz M. (1987) Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. *Appl Environ Microbiol* **53**: 331–336.
- Finch, R., Davey, P., Wilcox, M., and Irving, W. (2012) Antimicrobial Chemotherapy, Oxford University Press, 2012.
- Fisher, J., Meroueh, S., and Mobashery, S. (2005) Bacterial resistance to beta-lactam antibiotics: compelling opportunism, compelling opportunity. *Chem Rev* **105**: 395–424.
- Forsberg, K., Reyes, A., Wang, B., Selleck, E., Sommer, M., and Dantas, G. (2012) The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337**: 1107–1111.
- Gatzeva-Topalova, P., May, A., and Sousa, M. (2005) Structure and mechanism of ArnA: conformational change implies ordered dehydrogenase mechanism in key enzyme for polymyxin resistance. *Structure* **13**: 929–942.
- Ginevan, M. (2002) Assessment of the national antimicrobial resistance monitoring system (NARMS) and its value in critical decision-making. *Int J Infect Dis* **6**: S8–S15.
- Greenberg B (1965) Flies and disease. *Scientific American* **213**: 92-99.
- Gunn, J. (2008) The *Salmonella* PmrAB regulon: Lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends Microbiol* **16**: 284–290.

- Gunn, J., Lim, K., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S. (1998) PmrA–PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27**: 1171–1182.
- Hemmatinezhad, B., Ommi, D., Hafshejani, T., and Khamesipour, F. (2015) Molecular detection and antimicrobial resistance of *Pseudomonas aeruginosa* from houseflies (*Musca domestica*) in Iran. *J Venom Anim Toxins Incl Trop Dis* **21**: 18.
- Hicks, L., Bartoces, M., Roberts, R., Suda, K., Hunkler, R., Taylor Jr, T., and Schrag, S. (2015) US outpatient antibiotic prescribing variation according to geography, patient population, and provider specialty in 2011. *Clin Infect Dis* **60**: 1308–1316.
- Hocquet, D., Llanes, C., Thouverez, M., Kulasekara, HD., Bertrand, X., Plesiat, P., et al. (2012) Evidence for induction of integron-based antibiotic resistance by the SOS response in a clinical setting. *PLoS Pathog* **8**: e1002778.
- Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., and Coukell, A. (2017) Antimicrobial drug use in food-producing animals and associated human health risks: What, and how strong, is the evidence? *BMC Vet Res* **13**: 211.
- Iwasa, M., Makino, S-I., Asakura, H., Kobori, H., and Morimoto, Y. (1999) Detection of *Escherichia coli* O157:H7 from *Musca domestica* (Diptera: *Muscidae*) at a cattle farm in Japan. *J Med Entomol* **36**: 108–112.
- Jayol, A., Nordmann, P., Brink, A., and Poirel, L. (2015) Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother* **59**: 2780–2784.

- Jayol, A., Poirel, L., Brink, A., Villegas, MV., Yilmaz, M., and Nordmann, P. (2014) Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother* **58**: 4762–4766.
- Jernigan, D., Kargacin, L., Poole, A., and Kobayashi, J. (2001) Sentinel surveillance as an alternative approach for monitoring antibiotic-resistant invasive pneumococcal disease in Washington State. *Am J Public Health* **91**: 142–145.
- Johnson, T., Singer, R., Isaacson, R., Danzeisen, J., Lang, K., Kobluk, K., et al. (2015) In vivo transmission of an IncA/C plasmid in *Escherichia coli* depends on tetracycline concentration, and acquisition of the plasmid results in a variable cost of fitness. *Appl Environ Microbiol* **81**: 3561–3570.
- Joyner, C., Mills, M., and Nayduch, D. (2013) *Pseudomonas aeruginosa* in *Musca domestica* L.: temporospatial examination of bacteria population dynamics and house fly antimicrobial responses. *PloS one* **8**: e79224–e79224.
- Jutkina, J., Rutgersson, C., Flach, C., and Joakim, D. (2016) An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. *Sci Total Environ* **548–549**: 131–138.
- Khin Nwe, Oo., Sebastian, A., and Aye, T. (1989) Carriage of enteric bacterial pathogens by house flies in Yangon, Myanmar. *J Diarrhoeal Dis Res* **7**: 81–84.
- Knothe, H., Shah, P., Krcmery, V., Antal, M., and Mitsuhashi, S. (1983) Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. *Infection* **11**: 315–317.

- Lam, K., Babor, D., Duthie, B., Babor, E-M., Moore, M. and Gries, G. (2007) Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. *Animal Behaviour* **74**: 81–92.
- Li, J., Nation, R., Turnidge, J., Milne, R., Coulthard, K., Rayner, C., and Paterson, D. (2006) Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* **6**: 589–601.
- Liakopoulos, A., Mevius, D., and Ceccarelli, D. (2016) A Review of SHV Extended-Spectrum β -Lactamases: Neglected Yet Ubiquitous. *Front Microbiol* **7**: 1374.
- Lippa, A., and Goulian, M. (2009) Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet* **5**: e1000788.
- Liu, Y-Y., Wang, Y., Walsh, T., Yi, L-X., Zhang, R., Spence, J., et al. (2016) Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* **16**: 161–168.
- Ma, Q., Fonseca, A., Liu, W., Fields, A. T., Pimsler, M. L., Spindola, A. F., et al. (2012). *Proteus mirabilis* interkingdom swarming signals attract blow flies. *The ISME journal* **6**: 1356–1366.
- Ma, G., Zhu, Y., Yu, Z., Ahmad, A., and Zhang, H. (2016) High resolution crystal structure of the catalytic domain of MCR-1. *Sci. Rep* **6** (2016) 39540
- Macinga, D., and Rather, P. (1999) The chromosomal 2'-N-acetyltransferase of *Providencia stuartii*: physiological functions and genetic regulation. *Front Biosci* **4**: d132–140.
- Masterton, R. (2000) Surveillance studies: how can they help the management of infection? *J Antimicrob Chemother* **46**: 53–58.

- Masuda, N. and Ohya, S. (1992) Cross-resistance to meropenem, cepheems, and quinolones in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **36**: 1847–1851.
- Mather, A., Reeve, R., Mellor, D., Matthews, L., Reid-Smith, R., Dutil, L., et al. (2016) Detection of Rare Antimicrobial Resistance Profiles by Active and Passive Surveillance Approaches. *PloS one* **11**: e0158515–e0158515.
- McPhee, J., Bains, M., Winsor, G., Lewenza, S., Kwasnicka, A., Brazas, M., et al. (2006) Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg²⁺-induced gene regulation in *Pseudomonas aeruginosa*. *J Bacteriol* **188**: 3995–4006.
- Mitrophanov, A., Jewett, M., Hadley, T., and Groisman E. (2008) Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. *PLoS Genet* **4**: e1000233.
- Morero, N., Monti, M., and Argarana, C. (2011) Effect of ciprofloxacin concentration on the frequency and nature of resistant mutants selected from *Pseudomonas aeruginosa mutS* and *mutT* hypermutators. *Antimicrob Agents Chemother* **55**: 3668–3676.
- Morgan, D., Okeke, I., Laxminarayan, R., Perencevich, E., and Weisenberg, S. (2011) Non-prescription antimicrobial use worldwide: a systematic review. *Lancet Infect Dis* **11**: 692–701.
- Nayduch, D., Noblet, G., and Stutzenberger, F. (2005) Fate of bacteria, *Aeromonas caviae*, in the midgut of the housefly, *Musca domestica*. *Invertebrate Biology* **124**: 74–78.
- Nayduch, D., Pittman Noblet, G., and Stutzenberger, F. (2002) Vector potential of houseflies for the bacterium *Aeromonas caviae*. *Med Vet Entomol* **16**: 193–198.
- Nordmann, P., Jayol, A., and Poirel, L. (2016) Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis* **22**: 1038–1043.

- Nugent, G., Whitford, J., and Young, N. (2002) Use of released pigs as sentinels for *Mycobacterium bovis*. *J Wildl Dis* **38**: 665–677.
- O'Neill, J. (2016) Tackling drug-resistant infections globally: final report and recommendations. HM Government and the Wellcome Trust, London.
- Olaitan, A., Diene, S., Kempf, M., Berrazeg, M., Bakour, S., Gupta, S., et al. (2014) Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. *Int J Antimicrob Agents* **44**: 500–507.
- Onwugamba, FC., Fitzgerald, JR., Rochon, K., Guardabassi, L., Alabi, A., Kühne, S., et al. (2018) The role of 'filth flies' in the spread of antimicrobial resistance. *Travel Med Infect Dis* **2018**: 8–17.
- Otter, J., Doumith, M., Davies, F., Mookerjee, S., Dyakova, E., Gilchrist, M., et al. (2017) Emergence and clonal spread of colistin resistance due to multiple mutational mechanisms in carbapenemase-producing *Klebsiella pneumoniae* in London. *Sci Rep* **7**: 12711.
- Payie, K., Rather, P., and Clarke, A. (1995) Contribution of gentamicin 2'-N-acetyltransferase to the O acetylation of peptidoglycan in *Providencia stuartii*. *J Bacteriol* **177**: 4303–4310.
- Poirel, L., Gniadkowski, M., and Nordmann, P. (2002) Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum beta-lactamase CTX-M-15 and of its structurally related beta-lactamase CTX-M-3. *J Antimicrob Chemother* **50**: 1031–1034.

- Poirel, L., Jayol, A., Bontron, S., Villegas, M., Ozdamar, M., Turkoglu, S., et al. (2015) The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* **70**: 75–80.
- Poirel, L., Lebessi, E., Castro, M., Fevre, C., Foustoukou, M., and Nordmann, P. (2004) Nosocomial outbreak of extended-spectrum beta-lactamase SHV-5-producing isolates of *Pseudomonas aeruginosa* in Athens, Greece. *Antimicrob Agents Chemother* **48**: 2277–2279.
- Read, A., and Woods, R. (2014) Antibiotic resistance management. *Evol Med Public Health* **2014**: 147.
- Rhodes, G., Huys, G., Swing, J., McGann, P., Hiney, M., Smith, P., and Pickup, R. (2000) Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant *tetA*. *Appl Environ Microbiol* **66**: 3883–3890.
- Richards, M., Edwards, J., Culver, D., and Gaynes, R. (1999) Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit Care Med* **27**: 887–892.
- Roberts, J., Paul, S., Akova, M., Bassetti, M., Waele, J., Dimopoulos, G., et al. (2014) DALI: defining antibiotic levels in intensive care unit patients: are current beta-lactam antibiotic doses sufficient for critically ill patients? *Clin Infect Dis* **58**: 1072–1083.
- Rochon, K., Lysyk, T., and Selinger, L. (2004) Persistence of *Escherichia coli* in immature house fly and stable fly (Diptera: *Muscidae*) in relation to larval growth and survival. *J Med Entomol* **41**: 1082–1089.

- Rodríguez-Prieto, V., Vicente-Rubiano, M., Sánchez-Matamoros, A., Rubio-Guerri, C., Melero, M., Martínez-López, B., et al. (2015) Systematic review of surveillance systems and methods for early detection of exotic, new and re-emerging diseases in animal populations. *Epidemiol Infect* **143**: 2018–2042.
- Schluter, A., Krause, L., Szczepanowski, R., Goesmann, A., and Puhler, A. (2008) Genetic diversity and composition of a plasmid metagenome from a wastewater treatment plant. *J Biotechnol* **136**: 65–76.
- Schrag, S., Zell, E., Schuchat, A., and Whitney, C. (2002) Sentinel surveillance: a reliable way to track antibiotic resistance in communities? *Emerg Infect Dis* **8**: 496–502.
- Scott, T., Wright, S., Eldridge, B., and Brown, D. (2001) Cost effectiveness of three arbovirus surveillance methods in northern California. *J Am Mosq Control Assoc* **17**: 118–123.
- Sidorczyk, Z., ZÄHringer, U., and Rietschel, E. (1983) Chemical structure of the lipid A component of the lipopolysaccharide from a *Proteus mirabilis* Re-mutant. *Eur J Biochem* **137**: 15–22.
- Sougakoff, W., Goussard, S., Gerbaud, G., and Courvalin, P. (1988) Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes. *Rev Infect Dis* **10**: 879–884.
- Steinhaus, E. (1952) The Housefly: Its Natural History, Medical Importance, and Control. *Science* **115**: 154.
- Stoffolano, J. and Haselton, A. (2013) The adult dipteran crop: a unique and overlooked organ. *Annu Rev Entomol* **58**: 205–225.
- Storm, D., Rosenthal, K., and Swanson, P. (1977) Polymyxin and Related Peptide Antibiotics. *Annu Rev Biochem* **46**: 723–763.

- Sturenburg, E., Kuhn, A., Mack, D., and Laufs, R. (2004) A novel extended-spectrum beta-lactamase CTX-M-23 with a P167T substitution in the active-site omega loop associated with ceftazidime resistance. *J Antimicrob Chemother* **54**: 406–409
- Su, Z., Zhang, M., Liu, X., Tong, L., Huang, Y., Li, G. and Pang, Y. (2010) Comparison of bacterial diversity in wheat bran and in the gut of larvae and newly emerged adult of *Musca domestica* (Diptera: *Muscidae*) by use of ethidium monoazide reveals bacterial colonization. *J Econ Entomol* **103**: 1832–1841.
- Sun, S., Negrea, A., Rhen, M., and Andersson, D. (2009) Genetic analysis of colistin resistance in *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* **53**: 2298–2305.
- Szalanski, A., Owens, C., McKay, T., and Steelman, C. (2004) Detection of *Campylobacter* and *Escherichia coli* O157:H7 from filth flies by polymerase chain reaction. *Med Vet Entomol* **18**: 241–246.
- Tang, Q., Song, P., Li, J., Kong, F., Sun, L., and Xu, L. (2016) Control of antibiotic resistance in China must not be delayed: The current state of resistance and policy suggestions for the government, medical facilities, and patients. *Biosci Trends* **10**: 1–6.
- Touzé, T., Tran, AX., Hankins, JV., Mengin-Lecreulx, D., and Trent, MS, (2008) Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. *Mol microbiol* **67**: 264–277.
- U.S. Centers for Disease Control and Prevention. (2019) Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA, USA.

- Van Boeckel, T., Brower, C., Gilbert, M., Grenfell, B., Levin, S., Robinson, T., et al. (2015) Global trends in antimicrobial use in food animals. *Proc Natl Acad Sci U S A* **112**: 5649–5654.
- Van Boeckel, T., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B., Levin, S., and Laxminarayan, R. (2014) Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lancet Infect Dis* **14**: 742–750.
- Ventola, CL. (2015) The antibiotic resistance crisis. Part 1: causes and threats. *P T* **40**: 277–283.
- Wand, M., Bock, L., Bonney, L., and Sutton, J. (2016) Mechanisms of increased resistance to chlorhexidine and cross-resistance to colistin following exposure of *Klebsiella pneumoniae* clinical isolates to chlorhexidine. *Antimicrob Agents Chemother* **61**: e01162-16.
- Wang, X., Wang, Y., Zhou, Y., Li, J., Yin, W., Wang, S., et al. (2018) Emergence of a novel mobile colistin resistance gene, *mcr-8*, in NDM-producing *Klebsiella pneumoniae*. *Emerg Microbes Infect* **7**: 122–122.
- Wang, C., Feng, Y., Liu, L., Wei, L., Kang, M., and Zong, Z. (2020) Identification of novel mobile colistin resistance gene *mcr-10*. *Emerg Microbes Infect* **9**: 508–516.
- Wasala, L., Talley, J., DeSilva, U., Fletcher, J., and Wayadande, A. (2013) Transfer of *Escherichia coli* O157:H7 to spinach by house flies, *Musca domestica* (Diptera: Muscidae). *Phytopathology* **103**: 373–380.
- Wilke, M., Lovering, A., and Strynadka N. (2005) Beta-lactam antibiotic resistance: a current structural perspective. *Curr Opin Microbiol* **8**: 525–533.

- Xavier, BB., Lammens, C., Ruhai, R., Kumar-Singh, S., Butaye, P., Goossens, H., and Malhotra-Kumar, S. (2016) Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* **21**.
- Xi, C., Zhang, Y., Marrs, CF., Ye, W., Simon, C., Foxman, B., and Nriagu, J. (2009) Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Appl Environ Microbiol* **75**: 5714–5718.
- Yan, A., Guan, Z., and Raetz, C. (2007) An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in *Escherichia coli*. *J Biol Chem* **282**: 36077–36089.
- Yang, Y-Q., Li, Y-X., Lei, C-W., Zhang, A-Y., and Wang, H-N. (2018) Novel plasmid-mediated colistin resistance gene *mcr-7.1* in *Klebsiella pneumoniae*. *J Antimicrob Chemo* **73**: 1791–1795.
- Yin, W., Li, H., Shen, Y., Liu, Z., Wang, S., Shen, Z., et al. (2017) Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. *MBio* **8**: e00543–00517.
- Zhang, Y., Gu, AZ., He, M., Li, D., and Chen, J. (2017) Subinhibitory concentrations of disinfectants promote the horizontal transfer of multidrug resistance genes within and across genera. *Environ Sci Technol* **51**: 570–580.
- Zurek, K. and Nayduch, D. (2016) Bacterial associations across house fly life history: evidence for transstadial carriage from managed manure. *J Insect Sci* **16**: 2.
- Zurek, L., Schal, C., and Watson, D. (2000) Diversity and contribution of the intestinal bacterial community to the development of *Musca Domestica* (Diptera: *Muscidae*) larvae. *J Med Entomol* **37**: 924–928.

Table 1.1. Colistin resistance-associated mutations in PmrA

Mutation	Bacterial species	Reference
S42N	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
G53C	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
G53S	<i>K. pneumoniae</i>	Nordmann <i>et al.</i> , 2016
G15R	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
G53E	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
G53R	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
R81C	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
R81H	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009

Table 1.2. Colistin resistance-associated mutations in PmrB

Mutation	Bacterial species	Reference
L10P	<i>E. coli</i>	Cannatelli <i>et al.</i> , 2017
L10R	<i>E. coli</i>	Janssen <i>et al.</i> , 2019
L14R	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
C84R	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
C84Y	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
P94L	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
P94Q	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
P94S	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
E121K	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
E121Q	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
T147A	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
T156M	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
A159P	<i>E. coli</i>	Janssen <i>et al.</i> , 2019
A159V	<i>E. coli</i>	Janssen <i>et al.</i> , 2019
V161G	<i>E. coli</i>	Delannoy <i>et al.</i> , 2017
E166K	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
G206D	<i>E. coli</i>	Bourrel <i>et al.</i> , 2019
R14del	<i>K. pneumoniae</i>	Choi and Ko, 2014
L17Q	<i>K. pneumoniae</i>	Nordmann <i>et al.</i> , 2016
L82R	<i>K. pneumoniae</i>	Cannatelli <i>et al.</i> , 2014a
S85R	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
T140P	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
T157P	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
S208N	<i>K. pneumoniae</i>	Choi and Ko, 2014
Y209del	<i>K. pneumoniae</i>	Choi and Ko, 2014
*R256G	<i>K. pneumoniae</i>	Cheng <i>et al.</i> , 2015
L14F	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
L14S	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
L22P	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
S29R	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
T91A	<i>Salmonella enterica</i>	N/A
P94Q	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
E121A	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
S124P	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
N130Y	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
T147P	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
R155P	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
T156M	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
T156P	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
V161G	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
V161L	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
V161M	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
E166K	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
M186I	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
G206R	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
G206W	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009
S305R	<i>Salmonella enterica</i>	Sun <i>et al.</i> , 2009

* reported with CrrB mutation (S195N) in the same isolate.

Table 1.3. Colistin resistance-associated mutations in PhoP/PhoQ and MgrB

Mutation	Bacterial species	Reference
PhoP		
V3F	<i>K. pneumoniae</i>	Cheng <i>et al.</i> , 2015
D191Y	<i>K. pneumoniae</i>	Jayol <i>et al.</i> , 2015
I201N	<i>K. pneumoniae</i>	N/A*
PhoQ		
R16C	<i>K. pneumoniae</i>	Nordmann <i>et al.</i> , 2016
A20P	<i>K. pneumoniae</i>	Wand <i>et al.</i> , 2017
L26P	<i>K. pneumoniae</i>	Cheng <i>et al.</i> , 2015
L96P	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
S174N	<i>K. pneumoniae</i>	Choi and Ko, 2014
L348Q	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
G385S	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
L396Q	<i>K. pneumoniae</i>	Otter <i>et al.</i> , 2017
D438N	<i>K. pneumoniae</i>	N/A*
MgrB		
A14S	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
W20R	<i>K. pneumoniae</i>	Nordmann <i>et al.</i> , 2016
L24H	<i>K. pneumoniae</i>	Cannatelli <i>et al.</i> , 2014b
M27K	<i>K. pneumoniae</i>	Nordmann <i>et al.</i> , 2016
C28F	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
C28Y	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
D31N	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
F35I	<i>K. pneumoniae</i>	Olaitan <i>et al.</i> , 2014
G37S	<i>K. pneumoniae</i>	Cannatelli <i>et al.</i> , 2014b
C39Y	<i>K. pneumoniae</i>	Nordmann <i>et al.</i> , 2016
I45T	<i>K. pneumoniae</i>	Nordmann <i>et al.</i> , 2016
W47R	<i>K. pneumoniae</i>	Nordmann <i>et al.</i> , 2016
partial deletion	<i>K. pneumoniae</i>	Cannatelli <i>et al.</i> , 2014b
insertion sequences (<i>IS</i>)	<i>K. pneumoniae</i>	Cannatelli <i>et al.</i> , 2014b

* obtained from NCBI antimicrobial resistance genes database

Table 1.4. Amino acid identity of MCR enzymes

	MCR-1	MCR-2	MCR-3	MCR-4	MCR-5	MCR-6	MCR-7	MCR-8	MCR-9
MCR-1	100%	81%	33%	34%	36%	83%	35%	31%	36%
MCR-2		100%	32%	35%	35%	88%	34%	30%	34%
MCR-3			100%	49%	35%	34%	70%	40%	65%
MCR-4				100%	34%	33%	45%	38%	43%
MCR-5					100%	37%	36%	33%	33%
MCR-6						100%	33%	30%	34%
MCR-7							100%	37%	63%
MCR-8								100%	45%
MCR-9									100%

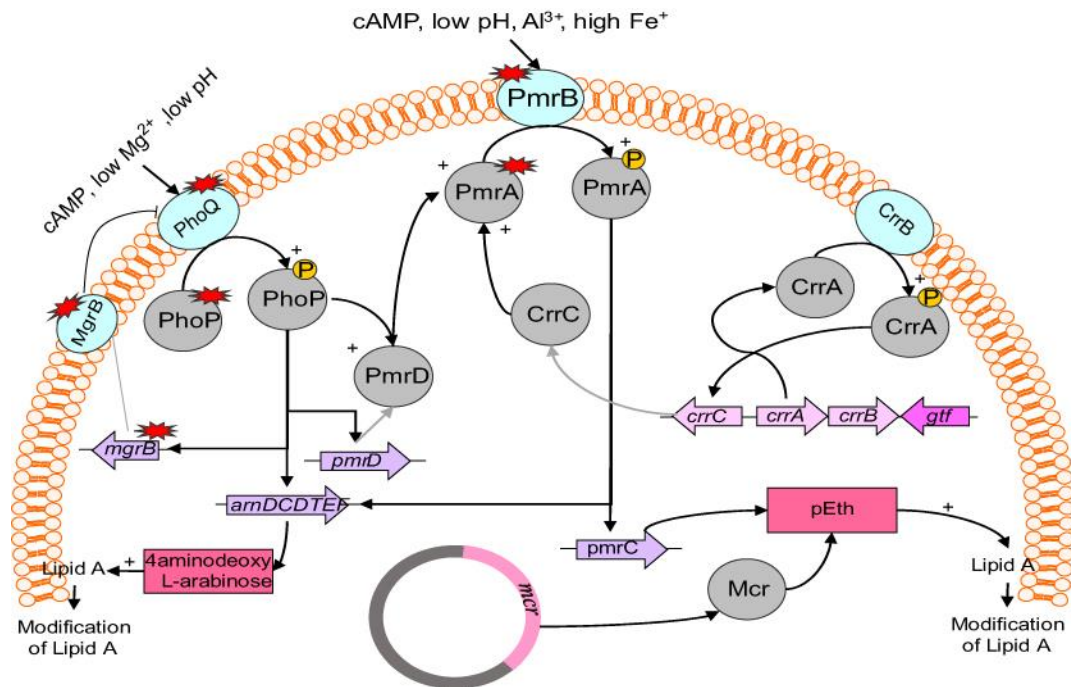


Figure 1.2. Colistin resistance mechanism. Colistin resistance is mediated by the activation of PmrA-PmrB, PhoP-PhoQ, and/or CrrA-CrrB two-component regulatory systems. The response regulators upregulate expression of LPS-modifying genes (*arnBCADTEF* and *pmrC*). Plasmid-mediated colistin resistance is triggered by MCR enzymes. LPS modifications include addition of L -Ara $4N$ and/or PEtN.

(figure obtained with no permission required from DOI <https://doi.org/10.2147/IDR.S199844>)

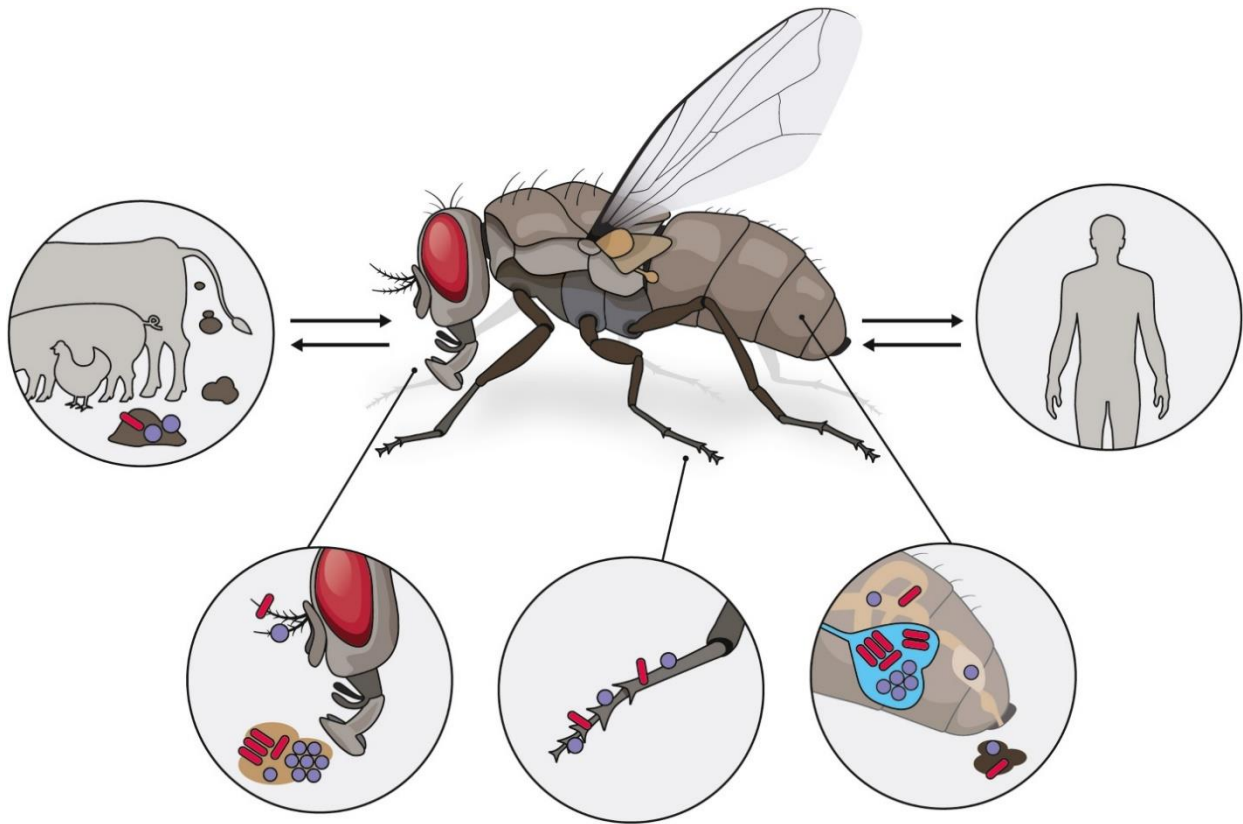


Figure 1.3. Mode of bacterial transmission by “filth” flies. Upon exposure of flies to bacteria from microbe-rich animal wastes, bacteria are transmitted to humans and animals through translocation, regurgitation, or excretion.

(figure obtained with permission from <https://doi.org/10.1016/j.tmaid.2018.02.007>)

Chapter 2

Flies and feces of sympatric animals: Isolation of antimicrobial-resistant bacteria and comparison of their microbiota

Abstract

Antimicrobial resistance is rising globally at an alarming rate. While multiple active surveillance programs have been established to monitor antimicrobial resistance, studies on the environmental link to spread of antimicrobial resistance are lacking. Flies are well-known vectors of bacterial pathogens, but there is little data on their role in spreading commensal bacteria that harbor antimicrobial resistance. A total of 493 flies were trapped from a dairy unit, a dog kennel, a poultry farm, a beef cattle unit, Auburn trash collection area, and Auburn downtown area to isolate *Escherichia coli* and *Klebsiella pneumoniae* for antimicrobial susceptibility testing. *E. coli* and *K. pneumoniae* were recovered from 38.0% and 16.7% of flies, respectively. In total, 35.3% of flies were found to harbor antimicrobial-resistant bacteria and 9.0% contained multidrug-resistant isolates including extended-spectrum beta lactamase (ESBL)-producing *E. coli* and *K. pneumoniae*. The bacterial communities and distribution of antimicrobial resistance genes (ARGs) and mobile genetic elements (MGE) in flies and feces from sympatric animals were investigated to determine whether flies can be used as sentinels for antimicrobial-resistant bacteria in the environment. *16S rRNA*-based metagenomic analysis of flies and fecal microbiota showed that both communities were over 98.0% similar at the bacterial phylum level. Forty-three of the 886 bacterial genera that were identified in flies and 33 of 596 genera in feces were potential human and/or veterinary pathogens. Flies and feces shared nine potentially pathogenic bacterial genera that showed significant relative abundance in flies and feces. Bray-Curtis dissimilarity analysis showed that the microbiota of the flies were more

similar to the microbiota of the feces of their sympatric animals than those of the feces from the three other locations studied. Microbiome source tracking determined that flies collected inside the poultry house shared 64.5% of the bacteria found in poultry feces, but less than 7.0% of the microbiota in urban flies matched fecal microbiota. Similarly, qPCR array-based resistome analysis showed that flies shared more than 71.7% of ARGs found in feces. Those findings suggest that flies share the bacterial communities and ARG and therefore may be used as sentinels in surveillance of antimicrobial resistance. However, long-term studies on more geospatially distinct populations as well as a fly challenge model are needed to fully evaluate the possibility of flies as sentinels for antimicrobial resistance.

2.1 Introduction

Despite decades of efforts to slow down the selection and transfer of antimicrobial resistance genes through judicious use of antimicrobials, infections with antimicrobial-resistant pathogens remain a serious public health threat causing more than 2.8 million infections and 35,000 deaths every year in the United States (Hoelzer *et al.*, 2017; U.S. Centers for Disease Control and Prevention, 2019). In total, antibiotic resistance is estimated to add up to \$20 billion annually to the direct healthcare costs in the United States, with additional costs for lost productivity as high as \$35 billion a year (Wallinga and Burch, 2013; Spellberg *et al.*, 2011). This threat has indeed reached animal husbandry and veterinary medicine. Multidrug-resistant *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus* spp., including methicillin-resistant *Staphylococcus aureus* (MRSA), have been reported in animals including asymptomatic carriers (Murphy *et al.*, 2009; Davis *et al.*, 2014; Courtice *et al.*, 2016).

Early detection of antimicrobial resistance is critical to understand the origin and to establish and implement prevention strategies. Surveillance of bacterial antimicrobial resistance is critically important because it provides necessary information to establish trends of resistance, identify new antimicrobial resistance, and develop infection control policies (Tacconelli *et al.*, 2018). Several active and passive surveillance methods have been developed for monitoring the prevalence and patterns of antimicrobial resistance of bacteria in humans and animals (Johnson 2015; Mather *et al.*, 2016; de Jong *et al.*, 2018). However, these current surveillance methods are limited by incomplete data collection in passive surveillance or cost and labor-intensive practices in active population-based methods. A variety of animals have been used as sentinels to indicate the presence of pathogens, but ethical, cost, and procedural problems also limit their usefulness (McCluskey, 2003; Bowser *et al.*, 2018). Flies seem to be a feasible candidate for a role as

sentinels for antimicrobial resistance. Flies have adapted to live in close association with animals and human. Female flies associate with microbe-rich organic materials such as manure for reproductive purposes. Upon exposure to animal wastes, flies can be involved in transmission of bacterial pathogens that they acquire and may play a role in dissemination of bacteria due to their unrestricted movements between different habitats and ability to travel long distances (Onwugamba *et al.*, 2018). To evaluate the potential roles flies might play as sentinels for antimicrobial resistance, multidrug-resistant bacteria were recovered from flies that were trapped at different locations including farms in Auburn AL. Also, the bacterial communities and ARGs/MGEs in flies were compared to those in feces of sympatric animals to investigate whether the microbiota and the ARGs/MGEs in flies mirrored those of animal feces.

2.2 Materials and Methods

2.2.1 Flies

Flies were trapped with non-toxic, insecticide and scent-free Raid™ fly ribbons (PIC Corporation, Linden, NJ, USA) as described below throughout the month of October 2017 from six locations in Auburn, AL including four animal facilities (dairy barn, dog kennel, poultry house, and beef cattle unit), Auburn downtown, and Auburn Environmental Services Facility trash collection area collection area (Figure 2.1). The daily high temperatures ranged from 20–27°C, and the average humidity and pressure during sampling were 48.3% and 30.1 mm Hg, respectively. The animal facilities were between 1.1 and 3.5 kilometers from Auburn downtown whereas the beef cattle barn was 16.1–20.9 kilometers in a direct line from the other five sampling locations. None of the facilities has had recent histories of disease outbreaks or antimicrobial use since 2017.

One trap ribbon was placed at each sampling site and left for two hours. Trapped flies were collected with sterile forceps, placed individually in a 1.5 mL micro-centrifuge tube containing 800 µL of sterile phosphate buffered saline (PBS), and transported to the laboratory within 30 minutes of collection. In the laboratory, flies were homogenized with a tissue homogenizer (Bertin Technologies, Rockville, MD, USA) at 5,000 RPM for 20 seconds, for three times, and homogenates were used for bacterial isolation and DNA extraction.

2.2.2 Fecal samples

Concurrently with the fly trapping, 150 mg fresh fecal samples were collected from all four farm locations, resuspended in 1 mL PBS and homogenized with a tissue homogenizer

(Bertin Technologies, Rockville, MD, USA) at 5,000 RPM for 20 seconds, for three times, and homogenates were used for DNA extraction.

2.2.3 Bacterial isolation and antimicrobial susceptibility testing

A 50- μ L aliquot of each of the fly homogenates was streaked onto MacConkey agar and incubated at 37°C for 18 hours. Colonies resembling *E. coli* and *K. pneumoniae* were selected for further identification using conventional biochemical tests consisting of oxidase reaction, urease utilization, and IMViC tests that included indole production, Methyl Red and Vogues-Proskauer tests, and citrate utilization.

Antimicrobial susceptibility testing was performed using the Kirby–Bauer agar disk diffusion test against 13 antimicrobial agents: ampicillin, amoxicillin-clavulanic acid, cefpodoxime, ceftazidime, amikacin, gentamicin, streptomycin, tetracycline, doxycycline, chloramphenicol, ciprofloxacin, polymyxin B, and trimethoprim-sulfamethoxazole. A single colony from one-day-old culture on MacConkey agar was suspended in normal saline to a turbidity equivalent to 0.5 McFarland Standard, spread as a uniform layer onto Mueller Hinton agar, and allowed to dry. Antibiotic impregnated disks were placed onto the agar surface, and the plates were incubated at 37°C for 18 hours. Zones of inhibition were read and interpreted as susceptible, intermediate, and resistant according to the Clinical and Laboratory Standards Institute standards (CLSI 2013). Multidrug-resistant (MDR) isolates were classified as having resistance to antimicrobial agents from three or more drug classes. Resistance to ampicillin in *K. pneumoniae* was not taken into consideration due to their intrinsic resistance to that drug. Extended-spectrum beta-lactamase (ESBL) production was identified in isolates resistant to the third-generation cephalosporins, ceftazidime and cefpodoxime.

2.2.4 Extraction of genomic DNA

Aliquots of fly homogenates and fecal suspensions were used for genomic DNA extraction with the High Pure PCR Template Preparation Kit (Roche Diagnostic, Indianapolis, IN) according to the manufacturer's instructions. Specifically, a volume of 400 μ L from the fly homogenates and fecal suspensions was mixed with 400 μ L Binding Buffer and 80 μ L proteinase K. Lysis was carried out by incubation at 72°C for 20 minutes with shaking at 600 rpm in a thermomixer (Eppendorf, Hamburg, Germany). Two hundred μ L isopropanol (Avantor Performance Materials, Center Valley, PA) was added, and the mixture was vortexed thoroughly. The aqueous solution was transferred into the upper reservoir of the filter of the kit inserted into a 2-ml collection tube and centrifuged at 8,000 g for 1 minute. The flow-through liquid was discarded; and Inhibitor Removal Buffer was added to the filter and centrifuged at 8,000 g for 1 minute. The flow-through liquid was discarded; and the filter was washed with 600 μ L Washing Buffer and centrifuged at 8,000 g for 1 minute. The washing was repeated with 400 μ L and followed by centrifugation at 13,000 g for 1 minute. The filter column was inserted in a new 2-mL collection tube. Nucleic acid was eluted with 50 μ L pre-warmed Elution Buffer added to the middle of the filter, incubated at 72°C for 5 minutes, and centrifuged at 8,000 g for 1 minute. The elution step was repeated to increase DNA yields.

2.2.5 Universal bacterial qPCR

Total number of bacteria in individual fly homogenate was estimated with qPCR performed in Roche Diagnostics LightCycler II 2 (Roche Molecular Systems, Inc., Pleasanton, CA). Primers were established to detect the bacterial *16S rRNA* by targeting the conserved

regions C3 and C4 with expected amplicon size of 331–339 bp (F: 5'-CGCTCGTTGCGGGACTTAACC-3'; R: 5'-GCAAACAGGATTAGATACCCTGGTAGTCC-3'). Copy numbers of amplicons were estimated using a standard curve of a positive control of the amplicon constructed in a plasmid. Each PCR reaction consisted of 20 mM Tris-HCl, 0.45 mM MgCl₂, 200 μM dNTPs, 50 mM KCl, 1X SYBR™ Green (Molecular Probes Invitrogen Detection Technologies, Carlsbad, California), 5 U of Platinum™ *Taq* DNA polymerase (ThermoFisher Scientific, Waltham, MA), 1 μM of each primer (Integrated DNA Technologies, Coralville, IA), and 5 μL of DNA template in a total volume of 20 μL. The PCR thermal conditions used were denaturation at 95 °C for 2 min, three high stringency step-down cycles of 15 s at 95°C, 60 s at 75°C, and 60 s at 72°C, and followed by 30 cycles of 95 °C for 0 s, 56 °C for 78 s, and 72 °C for 10 s. DNA extracts of *E. coli*, *Salmonella enterica*, *Chlamydia* spp., *Mycoplasma* spp., *Clostridium* spp., *Ehrlichia* spp., and *Anaplasma* spp. were used as positive controls. DNA extracts of *Theileria* spp. and *Babesia* spp. as well as DNAs extracted from whole blood of poultry, pigeons, water fowl, dogs, cattle, pigs and humans were used as negative controls in the amplification reactions.

2.2.6 16S rRNA gene-based microbiota analysis

Extracted nucleic acids from fly homogenates and fecal samples were subjected to 16S metagenomics analysis at Molecular Research, LLC (MR DNA, Shallowater, TX, and USA).

The variable region 4 (V4) of the *16S rRNA* was amplified using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) and the universal primers F515 (5'-GTGCCAGCMGCCGCGGTAA-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3'), with barcode on the forward primer. The PCR thermal conditions were 94°C for 3 minutes, followed

by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Amplicons from multiple samples were pooled in equal proportions based on their molecular weights and DNA concentrations, purified using calibrated Ampure XP beads, and used to prepare DNA libraries with the Illumina TruSeq DNA library preparation protocol. Paired-end sequencing was performed on Illumina 2×300 MiSeq platform as previously described (Chiodini *et al.*, 2018).

Sequencing data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Sequences below 150 bp as well as barcodes were depleted; and sequences were joined. Operational taxonomic units (OTUs) were assigned with using Usearch, defined by clustering at 3% divergence, and classified using BLASTn against a curated database derived from the Ribosomal Database Project (<http://rdp.cme.msu.edu>) and the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) as previously described (Mukherjee *et al.*, 2016).

2.2.7 Detection of ARG and MGE

Prior to detection of ARGs and MGEs, fly homogenates were pooled to create five pools for each location, while all fecal DNAs were used individually. A highly parallel qPCR array was performed on extracted nucleic acids from fly pools and feces at Michigan State University as previously described (Stedtfeld *et al.*, 2018). The qPCR array was designed to target 372 unique genes including 324 ARGs that encode 12 categories of genes conferring resistance to aminoglycosides (n=61), amphenicol (n=20), beta-lactams (n=55), fluoroquinolones (n=12), MDR (n=43), MLS_B (n=46), sulfonamide (n=6), tetracycline (n=27), trimethoprim (n=17), vancomycin (n=24), and other plasmid-mediated resistance (n=13) as well as MGEs (n=48). The

concentration and quality of the DNA samples were verified using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA). All samples had more than 5 ng of DNA. Samples were run in duplicate, and the qPCR detection occurred with a threshold cycle ≤ 30 .

Primer sets and samples from 384-well plates were loaded into a SmartChip with five 184-reaction wells by a microfluidic SmartChip Multisample Nanodispenser (Takara, USA) and amplified in SmartChip Real-Time PCR cycler (Takara, USA). Ct values were calculated using default parameters provided with the SmartChip analysis software.

2.2.8 Statistical analysis

The copy numbers of *16S rRNA* gene were Log_{10} transformed and compared between different samples with One-way ANOVA with Tukey Honestly Significant Difference (HSD). Chi-squared test was used to compare the recovery rate and prevalence of antimicrobial resistance across the sampling locations. Difference at $p \leq 0.05$ was considered significant.

Statistical analysis of differences in bacterial communities between flies and animals' feces was performed using Quantitative Insights Into Microbial Ecology (QIIME) for taxa assignments (genus and phylum) and diversity estimates (OTUs for bacterial richness and Shannon index for diversity) with using Bray Curtis distance and visualized with Principal Coordinate Analysis (PCoA) plots as described (Kim *et al.*, 2017). Relative abundance of bacterial taxa was calculated by dividing number of sequences that map to the designated taxonomic classification by total number of sequences within each sample. Absolute abundance of bacterial taxa was calculated by counting number of samples with more than 0.001% relative abundance for individual taxa. Comparison of alpha diversity was performed using nonparametric Kruskal–Wallis test because most microbiota data of samples within the same

group are not expected to meet the assumptions of normal distribution. Beta diversity significance was compared using pairwise permutation multivariate analysis of variance PERMANOVA. Dissimilarity in bacterial diversity in samples was compared with using Bray-Curtis Dissimilarity index. Sources of flies' microbiota were predicted with using SourceTracker implemented in QIIME at OTU level using a Bayesian approach (Knights *et al.*, 2011).

Comparison of ARG/MGE compositions between flies and fecal samples was performed to assess the potential acquisition of those genes by flies from feces. Quantification of detected ARG/MGE was normalized to the absolute abundance of each gene by dividing the copy number of the gene by the average copy number of the *16S rRNA* gene in that sample. Comparison of ARG/MGE between flies and fecal samples was performed by calculating the percentage of shared genes by dividing number of shared genes by total detected genes in flies and visualized in a heatmap.

2.3 Results

2.3.1 Samples

A total of 490 flies from the poultry (n = 58), dog kennel (n = 58), beef cattle barn (n = 108), dairy barn (n = 81), Auburn downtown (n = 101), and trash collection area (n = 84). A total of 221 flies were randomly selected from the dairy barn (n = 40), dog kennel (n = 27), poultry (n = 40), beef cattle unit (n = 29), trash collection area (n = 41) and downtown (n = 44) for bacterial isolation and antimicrobial susceptibility testing on the isolates; and 80 flies (dairy barn: 19; dog kennel: 13; poultry house: 12; beef cattle unit: 11; downtown: 13; and trash collection area: 12) were randomly selected for microbiota analysis and ARG/MGE detection. Five fecal samples were collected from each of the four farm locations and used for microbiota and ARG/MGE studies.

2.3.2 Bacterial isolation and antimicrobial resistance

To avoid selecting duplicate strains, only one bacterial strain belonging to each species (*E. coli* and *K. pneumoniae*) in an individual fly was selected for further analysis. Based on IMViC reactions of bacterial isolates, *E. coli* and *K. pneumoniae* were identified in 84 (38%) and 37 (16.7%) fly homogenates, respectively (Table 2.1). *E. coli* and *K. pneumoniae* were recovered together from four flies (1.8%). *E. coli* was most prevalent in flies trapped at the poultry farm (25/40) followed by dairy barn (16/40), beef unit (14/29), trash collection area (12/41), dog kennel (10/27), and Auburn downtown (7/44). On the other hand, *K. pneumoniae* was mostly isolated from the trash collection area (11/41) followed by dairy barn (10/40), poultry farm (6/40), beef unit and downtown (4/29 and 4/44, respectively), and dog kennel (2/27). The majority of the isolates were found to be susceptible to all tested antimicrobial agents. Only

35.7% of isolated *E. coli* (30/84) and 10.8% of *K. pneumoniae* (4/37) were resistant to at least one antimicrobial drug. Most of the resistant isolates, 16/30 of *E. coli* and 3/4 of *K. pneumoniae*, were classified as MDR. Moreover, three of the MDR *E. coli* and one *K. pneumoniae* isolates were phenotypically ESBL positive.

2.3.3 Relative abundance of bacteria in flies

The *16S rRNA*-based qPCR analysis of flies and fecal samples suggested that feces had statistically similar bacterial loads, whereas there is a difference in bacterial load between flies and feces and between flies from different locations (Figure 2.2). The average copy numbers of *16S rRNA* per fly trapped in the trash collection area ($10^{5.34 \pm 0.28}$) and downtown ($10^{5.70 \pm 0.25}$) were significantly higher than those in flies from the poultry house ($10^{4.61 \pm 0.33}$, $p < 0.05$), dog kennel ($10^{3.89 \pm 0.33}$, $p < 10^{-4}$), dairy ($10^{4.37 \pm 0.30}$, $p < 10^{-4}$), and beef cattle barn ($10^{4.12 \pm 0.24}$, $p < 10^{-4}$), but significantly lower than those in the fecal samples from chickens ($10^{7.81 \pm 0.51}$, $p < 0.05$), dogs ($10^{7.78 \pm 0.30}$, $p < 0.05$), cows ($10^{7.41 \pm 0.23}$, $p < 0.05$) and beef cattle ($10^{8.18 \pm 0.21}$, $p < 0.01$).

2.3.4 Microbiota comparison at a phylum level

The average sequence reads based on *16S rRNA* metagenomic analysis indicated a similar trend for bacterial abundance in different samples to the qPCR data. The total sequence reads for fecal samples (cows: $100,218.2 \pm 614.7$; dogs: $145,413.8 \pm 2,514.7$; poultry: $101,619.0 \pm 1,719.1$; beef cattle: $104,230.4 \pm 676.1$) and for flies from the trash collection area ($112,907.4 \pm 1251.2$) contained significantly more reads than for flies from the dairy barn ($64,585.5 \pm 272.6$), dog kennel ($54,074.4 \pm 366.4$), poultry house ($43,544.3 \pm 489.3$), beef cattle unit ($60,130.4 \pm 312.6$), and downtown Auburn ($61,133.7 \pm 350.2$) ($p < 0.05$).

Microbial analysis of the *16S rRNA* identified 22 bacterial phyla in flies. Four phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) accounted for more than 98% of the microbiome of flies trapped at all locations (Figure 2.3; Table 2.2). *Proteobacteria* was the most dominant phylum identified in flies trapped in the dog kennel (46.7%), beef cattle barn (43.9%) and the downtown Auburn (55.2%) (Table 2.2). *Firmicutes* was the predominant phylum in the trash collection area, dairy and poultry house with relative abundances of 47.4%, 41.4% and 64.8%, respectively. The highest abundance of *Bacteroidetes* was observed in flies from the dairy (16.7%) followed by flies from downtown (13.8%) and the dog kennel (11.1%). The highest abundance of *Actinobacteria* was observed in dog kennel flies (11.1%), followed by flies from the dairy barn (8.4%) and the poultry house (8.2%).

Fecal samples had 21 bacterial phyla, with five phyla (*Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria* and *Spirochaetes*) accounting for more than 98% of the microbiome of the fecal samples (Figure 2.3; Table 2.3). *Firmicutes* was the most dominant phylum identified in fecal samples from cows (37.1%), poultry (69.3%) and beef cattle (41.9%) (Table 2.3). In dogs' fecal samples, the predominant phylum was *Fusobacteria* (55.6%). The highest abundance of *Bacteroidetes* was observed in fecal samples of beef cattle (38.1%) followed by those from cows (24.4%). Similarly, the highest abundance of *Spirochaetes* was observed in fecal samples from beef cattle (5.1%) followed by cows (4.4%). The highest abundance of *Proteobacteria* was observed in fecal samples from poultry (12.7%) followed by cows (10.5%). All the phyla identified in the fecal samples were also found in flies except for the phyla *Ignavibacteriae* and *Nitrospirae* that were only found in a few flies from the dog kennel and the dairy barn, respectively (Table 2.2).

2.3.5 Microbiota comparison at a genus level

A total of 886 bacterial genera were identified in the flies, 47 of which are related to potential human and veterinary pathogens (Figure 2.4; Table 2.4). Furthermore, 23 out of these 47 genera showed relatively significant abundance (over 0.1% of the total bacterial community) in flies including *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Bacillus*, *Bartonella*, *Burkholderia*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Erysipelothrix*, *Morganella*, *Mycoplasma*, *Pseudomonas*, *Serratia*, *Shigella* (*Escherichia*), *Staphylococcus*, *Streptococcus*, *Treponema*, *Vibrio*, *Wohlfahrtiimonas*, and *Yersinia*. *Acinetobacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Pseudomonas*, *Shigella* (*Escherichia*), *Staphylococcus*, and *Streptococcus* were identified in flies sampled in all locations with 100% prevalence (Table 2.4). *Serratia* species were identified with 100% prevalence in flies sampled from all locations except downtown (91% prevalence). *Alcaligenes*, *Burkholderia*, *Morganella* and *Wohlfahrtiimonas* species were identified with at least 50% prevalence in flies sampled from all locations (Table 2.4). In flies trapped in the dairy barn, *Streptococcus* was the most abundant genus (7.1%) followed by *Acinetobacter* (6.9%) and *Pseudomonas* (3.7%). Flies from the poultry house showed the highest abundance of *Enterococcus* (19.4%). In flies from the beef cattle unit, *Serratia* species was the most abundant (13.6%) followed by *Enterobacter* (6.4%). In flies trapped from the trash collection area, *Enterobacter* (7.1%) and *Pseudomonas* (5.8%) were the most predominant genera. In the downtown flies, *Wohlfahrtiimonas* (2.0%) was the most abundant genus followed by *Enterococcus* (1.4%). Flies from the dog kennel showed the highest abundance of *Shigella* (*Escherichia*) (5.2%) followed by *Pseudomonas* (4.6%) and *Streptococcus* (4.4%).

In fecal samples, 596 bacterial genera were identified including 31 genera that contain potential pathogens in human and veterinary medicine (Figure 2.4; Table 2.5). Eleven of these 31 genera had relatively significant abundance ($\geq 0.1\%$ of total bacterial phyla in feces) including *Acinetobacter*, *Bacillus*, *Campylobacter*, *Clostridium*, *Enterococcus*, *Erysipelothrix*, *Pseudomonas*, *Shigella (Escherichia)*, *Streptococcus*, *Tannerella*, and *Treponema*. With the exception of *Erysipelothrix* and *Treponema*, the most abundant genera were found in all fecal samples (100% prevalence). *Clostridium* was the most abundant genus in all fecal samples (12.2% abundance in dairy cows, 2.3% in dogs, 6.0% in poultry, and 10.4% in beef unit cattle) followed by *Treponema* (2.4%) in feces from cows, *Acinetobacter* in feces from dogs (0.2%) and poultry (2.6%), and *Bacillus* (6.6%) in feces from beef cattle (Table 2.5). Nine bacterial genera of the abundant 11 genera in feces were also found in flies (*Acinetobacter*, *Bacillus*, *Clostridium*, *Enterococcus*, *Erysipelothrix*, *Shigella (Escherichia)*, *Streptococcus*, *Tannerella*, and *Treponema*). Some bacterial genera (*Actinobacillus*, *Aeromonas*, *Anaplasma*, *Ancylobacter*, *Bordetella*, *Borrelia*, *Chlamydia*, *Coxiella*, *Ehrlichia*, *Haemophilus*, *Klebsiella*, *Listeria*, *Moraxella*, *Nocardia* and *Pasteurella*) were only identified in flies.

2.3.6 Diversity of bacterial communities in flies and fecal samples

Similarly to analysis of bacterial load by *16S rRNA* qPCR and microbiota sequence reads, there was a significant difference in bacterial richness between the fly and fecal microbiota within and across four sampling locations ($p < 0.05$) (Figure 2.5A). A total of 190 to 1,668 OTUs were identified in flies. On average, 316.05 ± 185.7 OTUs were identified across locations. Flies trapped in the dairy showed the highest number of OTUs (447.4 ± 227.8), while flies trapped in the dog kennel showed the lowest number of OTUs (190.8 ± 65.3). Flies trapped at the poultry

house, beef unit, trash collection area, and downtown contained 240.7 ± 47.2 , 376.4 ± 206.4 , 361.1 ± 82.7 and 226.1 ± 169.2 OTUs, respectively. Flies trapped at the trash collection area had the most diverse bacterial community followed by flies trapped at the dairy (Figure 2.5B). The Shannon index of α -diversity in flies trapped at the poultry house, dog kennel, dairy, beef cattle barn, trash collection area, and downtown were 3.9 ± 0.8 , 3.1 ± 1.8 , 4.8 ± 1.6 , 4.7 ± 1.5 , 5.0 ± 0.8 and 3.0 ± 1.6 , respectively (Figure 2.5A).

Fecal samples had 508 to 3,341 OTUs. The average number of OTUs identified in fecal samples of cows, dogs, poultry and beef cattle were $1,084.8 \pm 393.5$, 345.4 ± 101.3 , 769.2 ± 453.2 , and 1662.8 ± 53.9 , respectively (Figure 2.5A). The beef cattle fecal microbiota were the most diverse among fecal samples. The Shannon diversity index of poultry, dog, cow, and beef cattle feces were 4.2 ± 1.7 , 4.0 ± 0.5 , 7.4 ± 1.1 , and 8.6 ± 0.3 , respectively (Figure 2.5B).

The compositional dissimilarity based on Bray Curtis distance metric suggested a high diversity in the bacterial community of flies and fecal samples. The three axes in the principal coordinate analysis (PCoA) based on Bray Curtis distance showed only 25.0% of variability in bacterial community structure of flies sampled across the different locations (Figure 2.6). The PCoA demonstrates that flies trapped at urban locations (trash collection area and downtown) and the poultry house had the largest segregate base. The least segregated microbiota were found between flies in the cow and beef cattle barns (Figure 2.6). Based on Bray Curtis dissimilarity metric, microbiota of the flies were more similar to those of the feces from the sympatric animals as compared to those from the three other animal species studied (Figure 2.7A). However, the SourceTracker analysis indicated a variable contribution of fecal microbiota to fly microbiota (Figure 2.7B). The largest proportion of sympatric animal microbiota was found in poultry flies with 64.5% shared microbiota with poultry feces. In contrast, the majority of the bacterial

communities in flies trapped in the kennel, dairy dairy and beef cattle unit were from unknown sources. Only 20.4% of the microbiota of flies from the dog kennel was shared with the fecal microbiota of the dogs' feces. Similarly, 18.2% of microbiota is shared between flies and feces from the dairy; and 10.6% is shared between flies and feces from the beef cattle unit.

2.3.7 ARGs and MGEs in feces and flies

The qPCR array was performed to detect a total of 372 genes including 324 ARGs and 48 MGEs. The qPCR array amplified a total of 276 genes in feces (237 ARGs and 39 MGEs) and 216 genes in flies (182 ARGs and 34 MGEs) in all 12 categories of tested antimicrobials. The most prevalent genes in feces were the aminoglycosides resistance genes, followed by MGEs, MDR and MLS_B. The MGEs were the most identified genes in flies from the kennel, poultry house, beef cattle unit, downtown, and trash collection area, while aminoglycoside resistance genes were the most identified in flies from the dairy dairy (Table 2.6). The most ARG/MGEs were identified in fecal samples from the dairy barn with 207 genes. Dairy flies carried 125 genes, 112 of which are shared with dairy feces (89.6% of flies' ARG/MGE) (Table 2.6). Poultry samples contained a total of 213 ARG/MGE (feces = 200; flies = 101), with 87.1% of flies' genes (n = 88/101) shared genes with poultry feces. Flies and feces from the dog kennel had 172 genes, 55 of which were shared (67.1% of flies' genes). Flies in the beef unit harbored 96 genes, with 86.5% (n = 83) shared with fecal samples collected from sympatric animals (n = 178). In total, 198 genes were shared between flies and feces, 91.7% of the ARGs and MGEs in flies (198/216).

In spite of the large proportions of ARGs/MGEs shared between all flies and feces from all locations, there was variability in fecal contribution into ARGs/MGEs in flies at a given

location. Only poultry and dairy flies were found to share most genes with poultry feces (88%) and dairy feces (88.5%), respectively (Figure 2.8). There were more ARGs and MGEs in flies from the beef unit and the dog kennel that were shared with poultry feces (beef = 92.9%; dogs = 86.9%) than sympatric animals (beef = 84.7%; dogs = 70.4%). In addition, 80.3% of ARG/MGEs in trash collection area flies were shared with poultry, whereas the other fecal samples shared an average of 62.8% of trash collection area flies genes.

2.4 Discussion

Flies with the synanthropic lifecycle cause major health problems (Onwugamba *et al.*, 2018). Flies can carry bacterial pathogens on the surface of their exoskeleton and in their alimentary tract. Consequently, flies can transmit bacteria through regurgitation, defecation, or translocation from the exoskeleton (Graczyk *et al.*, 2001). There are many reports of flies' role in transmission of various food-borne pathogens and antimicrobial-resistant bacteria (Ahmad *et al.*, 2011; Zurek *et al.*, 2014; Schaumburg *et al.*, 2016). Moreover, the gut of flies provides a suitable environment for horizontal gene transfer including antimicrobial resistance genes (Fukuda *et al.*, 2016). Here, the role of flies in dissemination of antimicrobial-resistant bacteria was examined by isolating MDR bacteria from flies and comparing flies' microbiota and ARG/MGE to those of sympatric animals.

Few studies have been conducted on flies in relation to microbiota and antimicrobial resistance in the United States. Graham *et al.* (2009) demonstrated the presence of antimicrobial-resistant enterococci and staphylococci in flies in poultry houses in Delaware. Another study demonstrated the presence of antimicrobial-resistant enterococci in houseflies in restaurants in Kansas (Macovei and Zurek, 2006).

In this study, *E. coli* and *K. pneumoniae* were recovered from 38.0% and 16.7% of the flies. Only a single representative colony resembling the bacterium of interest was selected from each culture of bacteria from flies to avoid analyzing potential duplicate isolates of the same clone. Of the isolated bacteria, 14 *E. coli* and 1 *K. pneumoniae* strains were found to be resistant to one or two antimicrobial agents, while 16 *E. coli* and 3 *K. pneumoniae* were multidrug-resistant. Three ESBL-producing *E. coli* isolates (1.4% prevalent in flies) were recovered from flies trapped in the dog kennel (n = 2) and the poultry house (n = 1). There was one ESBL-

producing *K. pneumoniae* (0.9% prevalent) from a fly trapped at the dog kennel. The overall 1.4% and 0.9% prevalence of *E. coli* and *K. pneumoniae* resistant to third generation cephalosporins was also similar to that of ESBL-producing *E. coli* and *K. pneumoniae* previously isolated from “filth flies” (Zurek *et al.*, 2014; Schaumburg *et al.*, 2016).

The *16S rRNA*-based microbial analysis showed high levels of similarity at both the phylum and genus levels in bacterial communities in flies and the feces of sympatric animals. The bacterial richness in flies and fecal samples in this study agrees with earlier reports in literature. Although the average number of observed OTUs were different, there was a significant difference between bacterial richness and diversity between microbiota of flies and microbiota of feces (Su *et al.*, 2010; Gupta *et al.*, 2012; Palavesam *et al.*, 2012; Junqueira *et al.*, 2017). While the bacterial community structure looked similar for most flies, the relative abundance of bacterial genera varied widely within and across locations, indicating the large influence of stochastic events in bacterial composition. The microbiota of flies trapped at the poultry house, the trash collection area, and downtown showed some segregation based on β -diversity in PCoA. However, flies from other locations clustered and shared their microbiota across locations. Consistent with PCoA, the microbiota source tracking indicated that the fly microbiota are composed of bacteria from various sources with the exception of flies from the poultry house, which can be explained by restricted movement of flies inside the enclosed house. The variation in proportion of shared microbiota among flies in different locations was probably due to their unrestricted movement that resulted in contacts with different environmental bacterial sources.

The qPCR array detected 276 ARGs and MGEs in feces and 216 in flies, with 198 genes shared between flies and feces. Dog feces were found to carry the lowest detected ARG/MGE

probably due to the low abundance of *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla (44%), which are the main reservoirs of ARGs/MGEs (Hu *et al.*, 2016). These four ARGs/MGEs-rich bacterial phyla accounted for more than 98.0% in flies and other fecal samples.

Based on general similarities in microbiota and ARG/MGE between flies and animals as well as the high prevalence of ARG/MGE in flies, it can be concluded that flies can be an important epidemiological tool in the surveillance of antimicrobial resistance and a potential sentinel for a one-health approach to antimicrobial resistance in different environments. However, detailed studies using optimized isolation protocols and epidemiological designs in addition to fly artificial challenge models with bacterial communities and acquired antimicrobial resistance genes will be necessary to clearly demonstrate a sentinel role for flies in documenting antimicrobial resistance.

References

- Ahmad, A., Ghosh, A., Schal, C., and Zurek, L. (2011) Insects in confined swine operations carry a large antibiotic resistant and potentially virulent enterococcal community. *BMC Microbiol* **11**: 23.
- Bowser, N., and Anderson, N. (2018) Dogs (*Canis familiaris*) as sentinels for human infectious disease and application to Canadian populations: a systematic review. *Vet Sci.* **5**: 83.
- Chiodini, R.J., Dowd, S.E., Barron, J.N., Galandiuk, S., Davis, B., and Glassing, A. (2018) Transitional and temporal changes in the mucosal and submucosal intestinal microbiota in advanced Crohn's disease of the terminal ileum. *J Med Microbiol* **67**: 549–559.
- Courtice, R., Sniatynski, M., and Rubin, J.E. (2016) Antimicrobial resistance and beta-lactamase production of *Escherichia coli* causing canine urinary tract infections: passive surveillance of laboratory isolates in Saskatoon, Canada, 2014. *Can Vet J* **57**: 1166–1168.
- Davis, J.A., Jackson, C.R., Fedorka-Cray, P.J., Barrett, J.B., Brousse, J.H., Gustafson, J., and Kucher, M. (2014) Carriage of methicillin-resistant staphylococci by healthy companion animals in the US. *Lett Appl Microbiol* **59**: 1–8.
- de Jong, A., Simjee, S., Garch, F.E., Moyaert, H., Rose, M., Youala, M., et al. (2018) Antimicrobial susceptibility of enterococci recovered from healthy cattle, pigs and chickens in nine EU countries (EASSA Study) to critically important antibiotics. *Vet Microbiol* **216**: 168–175.
- Fukuda, A., Usui, M., Okubo, T., and Tamura, Y. (2016) Horizontal transfer of plasmid-mediated cephalosporin resistance genes in the intestine of houseflies (*Musca domestica*). *Microb Drug Resist* **22**: 336–341.

- Graczyk, K., Knight, R., Gilman, H., and Cranfield, R. (2001) The role of non-biting flies in the epidemiology of human infectious diseases. *Microbes Infect* **3**: 231–235.
- Graham, P., Price, B., Evans, L., Graczyk, K., and Silbergeld, K. (2009) Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. *Sci Total Environ* **407**: 2701–2710
- Gupta, AK., Nayduch, D., Verma, P., Shah, B., Ghate, HV., Patole, MS., and Shouche, YS. (2012) Phylogenetic characterization of bacteria in the gut of houseflies (*Musca domestica* L.). *FEMS Microbiol Ecol* **79**: 581–93.
- Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., and Coukell, A. (2017) Antimicrobial drug use in food-producing animals and associated human health risks: What, and how strong, is the evidence? *BMC Vet Res* **13**: 211.
- Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., et al. (2016) The bacterial mobile resistome transfer network connecting the animal and human microbiomes. *Appl Environ Microbiol* **82**: 6672–6681.
- Johnson, AP. (2015) Surveillance of antibiotic resistance. *Philos Tans R Soc Lond B Biol Sci* **370**: 20140080.
- Junqueira, A., Ratan, A., Acerbi, E., Drautz-Moses, D., Premkrishnan, B., Costea, P., et al. (2017) The microbiomes of blowflies and houseflies as bacterial transmission reservoirs. *Sci Rep* **7**: 16324.
- Kim, BR., Shin, J., Guevarra, R., Lee, JH., Kim, DW., Seol, KH., et al. (2017) Deciphering Diversity Indices for a Better Understanding of Microbial Communities. *J Microbiol Biotechnol* **27**: 2089–2093.

- Knights, D., Kuczynski, J., Charlson, ES., Zaneveld, J., Mozer, MC., Collman, RG., et al. (2011) Bayesian community-wide culture-independent microbial source tracking. *Nat Methods* **8**: 761–763.
- Macovei, L., and Zurek, L. (2006) Ecology of antibiotic resistance genes: characterization of enterococci from houseflies collected in food settings. *Appl Environ Microbiol* **72**: 4028–4035.
- Mather, AE., Reeve, R., Mellor, DJ., Matthews, L., Reid-Smith, RJ., Dutil, L., et al. (2016) Detection of rare antimicrobial resistance profiles by active and passive surveillance approaches. *PLoS One* **11**: e0158515.
- McCluskey, B. (2003) Use of sentinel herds in monitoring and surveillance systems, *In Animal Disease Surveillance and Survey Systems: Methods and Applications*. Ames, IA: Iowa State Press: 119–133.
- Mukherjee, N., Bartelli, D., Patra, C., Chauhan, BV., Dowd, SE., and Banerjee, P. (2016) Microbial diversity of source and point-of-use water in rural Haiti - a pyrosequencing-based metagenomic survey. *PLoS One* **11**: e0167353.
- Murphy, C., Reid-Smith, RJ., Prescott, JF., Bonnett, BN., Poppe, C., Boerlin, P., et al. (2009) Occurrence of antimicrobial-resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. *Can Vet J* **50**: 1047–1053.
- Onwugamba, FC., Fitzgerald, JR., Rochon, K., Guardabassi, L., Alabi, A., Kühne, S., et al. (2018) The role of 'filth flies' in the spread of antimicrobial resistance. *Travel Med Infect Dis* **2018**: 8–17.

- Palavesam, A., Guerrero, FD., Heekin, AM., Wang, J., Dowd, SE., et al. (2012) Pyrosequencing-Based Analysis of the Microbiome Associated with the Horn Fly, *Haematobia irritans*. *PLoS ONE* **7**: e44390.
- Schaumburg, F., Onwugamba, F., Akulenko, R., Peters, G., Mellmann, A., Köck, R., and Becker, K. (2016) A geospatial analysis of flies and the spread of antimicrobial-resistant bacteria. *Int J Med Microbiol.* **306**: 566–571.
- Spellberg, B., Blaser, M., Guidos, RJ., Boucher, H., Bradley, J., Eisenstein, B., et al. (2011) Combating antimicrobial resistance: policy recommendations to save lives. *Clin Infect Dis* **52**: S397–428.
- Stedtfeld, RD., Guo, X., Stedtfeld, TM., Sheng, H., Williams, MR., Hauschild, K., et al. (2018) Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. *FEMS Microbiol Ecol.* **94**: fiy130.
- Su, Z., Zhang, M., Liu, X., Tong, L., Huang, Y., Li, G., and Pang, Y. (2010) Comparison of bacterial diversity in wheat bran and in the gut of larvae and newly emerged adult of *Musa domestica* (Diptera: Muscidae) by use of ethidium monoazide reveals bacterial colonization. *J Econ Entomol* **103**: 1832–41.
- Tacconelli, E., Sifakis, F., Harbarth, S., Schrijver, R., van Mourik, M., Voss, A., et al. (2018) Surveillance for control of antimicrobial resistance. *Lancet Infect Dis* **18**: e99–e106.
- U.S. Centers for Disease Control and Prevention (2019) Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA, USA.
- Wallinga, D. and Burch D. (2013) Does adding routine antibiotics to animal feed pose a serious risk to human health? *BMJ* **347**: f4214.

Zurek, L., and Ghosh, A. (2014) Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. *Appl Environ Microbiol* **80**: 3562–3567

Table 2.1. Prevalence of isolated *E. coli* and *K. pneumoniae* isolated from flies

Location (<i>N</i>)	<i>E. coli</i>			<i>K. pneumoniae</i>		
	total	resistant	MDR	total	resistant	MDR
Downtown (44)	7	1	0	4	0	0
Trash collection area (41)	12	4	1	11	0	0
Dairy barn (40)	16	12	10	10	2	1
Poultry house (40)	25	6	1	6	1	1
Beef unit (29)	14	1	1	4	0	0
Dog kennel (27)	10	6	3	2	1	1
Total	84	30	16	37	4	3

N = number of individual fly homogenates used for culture; MDR = multidrug-resistant.

Table 2.2. The most relatively abundant phyla in fly microbiota

Phylum	Relative abundance of bacterial phylum in flies (%)					
	cow	dog	poultry	beef	trash	downtown
<i>Acidobacteria</i>	0.085	0.015	0.001	0.345	0.013	0.007
<i>Actinobacteria</i>	8.431	11.066	8.171	6.105	1.391	0.879
<i>Armatimonadetes</i>	0.001	0.000	0.000	0.000	0.000	0.000
<i>Bacteroidetes</i>	16.694	11.130	1.419	8.989	5.113	13.786
<i>Candidatus saccharibacteria</i>	0.036	0.000	0.000	0.173	0.002	0.000
<i>Chlamydiae</i>	0.042	0.000	0.000	0.030	0.011	0.008
<i>Chloroflexi</i>	0.104	0.005	0.016	0.073	0.020	0.003
<i>Cyanobacteria</i>	0.083	0.013	0.012	0.034	0.008	0.008
<i>Deinococcus thermus</i>	0.044	0.001	0.000	0.031	0.010	0.010
<i>Elusimicrobia</i>	0.002	0.000	0.000	0.000	0.000	0.000
<i>Fibrobacteres</i>	0.013	0.000	0.000	0.000	0.000	0.000
<i>Firmicutes</i>	41.410	30.103	64.825	37.797	47.385	30.001
<i>Fusobacteria</i>	0.254	0.929	0.163	1.881	0.248	0.067
<i>Gemmatimonadetes</i>	0.005	0.004	0.000	0.018	0.002	0.002
<i>Ignavibacteriae</i>	0.000	0.001	0.000	0.000	0.000	0.000
<i>Lentisphaerae</i>	0.015	0.000	0.000	0.033	0.020	0.000
<i>Planctomycetes</i>	0.073	0.009	0.000	0.066	0.016	0.008
<i>Proteobacteria</i>	31.685	46.697	25.285	43.954	45.696	55.213
<i>Spirochaetes</i>	0.339	0.004	0.016	0.136	0.005	0.001
<i>Synergistetes</i>	0.000	0.001	0.000	0.008	0.002	0.000
<i>Tenericutes</i>	0.340	0.003	0.085	0.024	0.030	0.001
<i>Verrucomicrobia</i>	0.343	0.020	0.006	0.303	0.032	0.006

Average relative abundance of $\geq 0.001\%$ used as cut-off;

The relative abundance was calculated by dividing number of sequences that map to the designated phylum by total number of sequences within each sample.

Table 2.3. The most relatively abundant phyla in fecal microbiota

Phylum	Relative abundance of bacteria at phylum level (% mean)			
	cow	dog	poultry	beef
<i>Acidobacteria</i>	0.177	0.003	0.090	1.245
<i>Actinobacteria</i>	0.915	0.277	0.293	0.220
<i>Bacteroidetes</i>	24.445	7.214	10.851	38.096
<i>Candidatus saccharibacteria</i>	0.028	0.000	0.008	0.079
<i>Chlamydiae</i>	0.000	0.000	0.000	0.001
<i>Chlorobi</i>	0.001	0.000	0.003	0.001
<i>Chloroflexi</i>	0.000	0.001	0.006	0.028
<i>Cyanobacteria</i>	1.419	0.009	0.219	1.094
<i>Deferribacteres</i>	0.001	0.001	0.040	0.002
<i>Deinococcus_thermus</i>	0.001	0.000	0.000	0.002
<i>Elusimicrobia</i>	0.006	0.001	0.033	0.091
<i>Fibrobacteres</i>	0.025	0.000	0.002	0.061
<i>Firmicutes</i>	37.085	34.056	69.355	41.894
<i>Fusobacteria</i>	0.073	55.648	5.203	0.072
<i>Lentisphaerae</i>	0.091	0.002	0.076	0.866
<i>Planctomycetes</i>	0.009	0.002	0.027	0.178
<i>Proteobacteria</i>	10.472	2.728	12.716	8.072
<i>Spirochaetes</i>	4.369	0.022	0.449	5.145
<i>Synergistetes</i>	0.000	0.000	0.027	0.001
<i>Tenericutes</i>	0.569	0.021	0.139	0.285
<i>Verrucomicrobia</i>	0.313	0.013	0.463	2.569

Average relative abundance of $\geq 0.001\%$ used as cut-off;

The relative abundance was calculated by dividing number of sequences that map to the designated phylum by total number of sequences within each sample.

Table 2.4. Top bacterial genera including human and veterinary pathogens in flies

Genus	Cow		Dog		Poultry		Beef cattle		Trash collection area		Downton	
	Prevalence (%)	Abundance (%)	Prevalence	Abundance	Prevalence	Abundance	Prevalence	Abundance	Prevalence	Abundance	Prevalence	Abundance
<i>Acinetobacter</i>	100	6.891	100	1.243	100	1.401	100	3.754	100	3.652	100	0.084
<i>Actinobacillus</i>	0.05	0.011	-	-	-	-	-	-	-	-	9.091	0.000
<i>Actinomyces</i>	63.158	0.023	30.769	0.005	50.000	0.033	63.636	0.060	66.667	0.004	54.545	0.003
<i>Aeromonas</i>	52.632	0.006	23.077	0.002	41.667	0.003	27.273	0.006	83.333	0.133	72.727	0.014
<i>Alcaligenes</i>	73.684	0.071	61.538	0.711	75.000	0.048	100	0.066	91.667	0.134	72.727	0.009
<i>Anaplasma</i>	-	-	7.692	-	-	-	-	-	8.333	0.003	-	-
<i>Ancylobacter</i>	-	-	-	-	-	-	9.091	0.003	25.000	0.002	-	-
<i>Bacillus</i>	100	0.813	100	0.179	100	1.079	100	0.497	100	0.664	100	0.016
<i>Bartonella</i>	26.316	0.014	38.462	0.746	66.667	0.003	18.182	0.004	66.667	0.003	72.727	0.002
<i>Bordetella</i>	15.789	0.001	-	-	0.000	-	9.091	0.002	-	-	-	-
<i>Borrelia</i>	5.263	0.008	-	-	8.333	0.000	9.091	0.000	-	-	9.091	0.000
<i>Brucella</i>	15.789	0.027	-	-	8.333	0.000	9.091	0.000	-	-	-	-
<i>Burkholderia</i>	94.737	1.001	84.615	1.963	58.333	0.004	81.818	0.022	83.333	0.012	100	0.004
<i>Campylobacter</i>	63.158	0.043	30.769	0.001	16.667	0.063	72.727	0.195	16.667	0.001	36.364	0.002
<i>Chlamydia</i>	10.526	0.001	0.000	-	-	-	9.091	0.001	16.667	0.002	18.182	0.005
<i>Citrobacter</i>	89.474	0.025	61.538	0.030	83.333	0.049	81.818	0.048	100	0.054	72.727	0.002
<i>Clostridium</i>	100	2.068	100	0.534	100	1.804	100	2.187	100	0.694	100	0.443
<i>Corynebacterium</i>	100	1.701	100	3.504	100	5.426	100	1.500	100	1.051	100	0.116
<i>Coxiella</i>	10.526	0.003	-	-	-	-	0.000	-	-	-	9.091	0.001
<i>Ehrlichia</i>	5.263	0.001	-	-	-	-	9.091	0.003	-	-	-	-
<i>Enterobacter</i>	100	3.164	100	3.493	100	6.252	100	6.370	100	7.034	100	0.111
<i>Enterococcus</i>	100	0.930	100	0.450	100	19.407	100	2.906	100	2.173	100	1.351
<i>Erysipelothrix</i>	78.947	0.055	7.692	0.000	16.667	0.005	45.455	0.112	66.667	0.024	45.455	0.003
<i>Haemophilus</i>	26.316	0.015	23.077	0.046	16.667	0.005	18.182	0.001	25.000	0.004	18.182	0.001
<i>Helicobacter</i>	15.789	0.000	15.385	0.003	8.333	0.004	9.091	0.000	-	-	18.182	0.000
<i>Klebsiella</i>	-	-	-	-	-	-	-	-	16.667	0.001	-	-
<i>Legionella</i>	42.105	0.091	30.769	0.015	8.333	0.000	63.636	0.091	75.000	0.052	63.636	0.012
<i>Listeria</i>	10.526	0.001	-	-	16.667	0.000	18.182	0.000	50.000	0.002	36.364	0.003
<i>Moraxella</i>	15.789	0.005	-	-	-	-	-	-	-	-	-	-
<i>Morganella</i>	52.632	0.124	84.615	1.125	83.333	0.166	90.909	0.453	100	0.585	100	0.016
<i>Mycoplasma</i>	63.158	0.252	15.385	0.001	-	-	18.182	0.004	-	-	-	-
<i>Myxococcus</i>	5.263	0.000	7.692	0.000	-	-	-	-	8.333	0.000	9.091	0.000
<i>Neisseria</i>	10.526	0.004	23.077	0.004	25.000	0.008	9.091	0.007	16.667	0.001	-	-
<i>Nocardia</i>	10.526	0.000	15.385	0.001	-	-	-	-	8.333	0.002	9.091	0.006
<i>Pasteurella</i>	10.526	0.012	-	-	-	-	27.273	0.001	8.333	0.000	18.182	0.000
<i>Pseudomonas</i>	100	3.743	100	4.639	100	0.617	100	2.651	100	5.800	100	1.234
<i>Rhodococcus</i>	47.368	0.035	23.077	0.003	-	-	63.636	0.051	66.667	0.014	36.364	0.010
<i>Rickettsia</i>	-	-	-	-	41.667	0.035	-	-	50.000	0.002	27.273	0.001
<i>Serratia</i>	100	0.031	100	1.835	100	0.043	100	13.578	100	0.614	90.909	0.111
<i>Shigella</i>	100	1.756	100	5.211	100	6.983	100	2.361	100	0.776	100	0.054
<i>Staphylococcus</i>	100	0.671	100	1.720	100	6.801	100	1.158	100	0.213	100	0.192
<i>Streptococcus</i>	100	7.058	100	4.378	100	0.863	100	1.422	100	1.105	100	0.057
<i>Tannerella</i>	47.368	0.009	15.385	0.012	16.667	0.000	9.091	0.000	33.333	0.001	45.455	0.000
<i>Treponema</i>	100	0.218	46.154	0.004	33.333	0.001	72.727	0.071	25.000	0.005	27.273	0.001
<i>Vibrio</i>	15.789	0.006	61.538	0.007	100	0.032	9.091	0.001	100	2.423	81.818	0.007
<i>Wohlfahrtiimonas</i>	68.421	0.026	100	1.015	91.667	0.104	100	0.518	100	0.631	100	2.031
<i>Yersinia</i>	27.701	0.000	46.154	0.003	58.333	0.003	27.273	0.008	100	0.341	81.818	0.002

Table 2.5. Top bacterial genera including human and veterinary pathogens in feces

Genus	Cow		Dog		Poultry		Beef cattle	
	Prevalence (%)	Abundance (%)	Prevalence	Abundance	Prevalence	Abundance	Prevalence	Abundance
<i>Acinetobacter</i>	100	0.265	100	0.155	100	2.614	100	3.624
<i>Actinomyces</i>	100	0.000	40	0.001	60	0.002	100	0.002
<i>Alcaligenes</i>	40	0.001	40	0.002	80	0.006	60	0.001
<i>Bacillus</i>	100	0.499	100	0.044	100	1.231	100	6.627
<i>Bartonella</i>	20	0.000	40	0.001	-	-	-	-
<i>Brucella</i>	60	0.002	20	0.000	80	0.010	100	0.054
<i>Burkholderia</i>	60	0.004	100	0.003	100	0.004	100	0.003
<i>Campylobacter</i>	100	0.094	100	0.021	100	0.053	100	0.260
<i>Citrobacter</i>	100	0.024	100	0.007	100	0.050	100	0.051
<i>Clostridium</i>	100	12.218	100	2.260	100	5.956	100	10.437
<i>Corynebacterium</i>	100	0.022	100	0.012	100	0.053	100	0.016
<i>Enterobacter</i>	100	0.025	100	0.026	100	0.034	100	0.023
<i>Enterococcus</i>	100	0.247	100	0.045	100	0.180	100	0.019
<i>Erysipelothrix</i>	100	0.360	80	0.007	100	0.038	100	0.198
<i>Helicobacter</i>	60	0.001	100	0.021	80	0.083	80	0.002
<i>Legionella</i>	100	0.002	20	0.000	80	0.001	80	0.001
<i>Morganella</i>	100	0.002	100	0.002	40	0.001	100	0.002
<i>Mycoplasma</i>	100	0.015	40	0.001	80	0.004	100	0.036
<i>Neisseria</i>	-	-	-	-	-	-	20	0.000
<i>Pseudomonas</i>	100	0.024	100	0.062	100	0.509	100	0.097
<i>Rhodococcus</i>	20	0.000	40	0.000	-	-	20	0.000
<i>Rickettsia</i>	20	0.000	40	0.001	60	0.003	100	0.024
<i>Serratia</i>	100	0.011	100	0.007	100	0.009	100	0.007
<i>Shigella</i>	100	0.270	100	0.034	100	0.578	100	0.200
<i>Staphylococcus</i>	100	0.007	100	0.007	100	0.013	100	0.039
<i>Streptococcus</i>	100	0.271	100	0.021	100	0.077	100	0.093
<i>Tannerella</i>	100	0.279	100	0.099	100	0.104	100	0.004
<i>Treponema</i>	100	2.417	80	0.006	100	0.088	100	1.134
<i>Vibrio</i>	20	0.000	60	0.002	40	0.002	-	-
<i>Wohlfahrtiimonas</i>	80	0.001	40	0.003	80	0.002	100	0.001
<i>Yersinia</i>	-	-	40	0.001	-	-	-	-

Table 2.6. Abundance of ARG/MGE in flies and feces

Class (<i>N</i>)	Total	Flies	Feces	Shared	Trash	Downtown	Poultry			Dog			Beef			Dairy		
							feces	flies	shared	feces	flies	shared	feces	flies	shared	feces	flies	shared
Aminoglycoside (61)	48	34	45	32	18	6	37	18	18	33	7	5	33	10	9	40	25	23
Amphenicol (20)	16	11	16	6	8	2	8	4	1	3	4	1	6	3	3	8	5	4
Beta-lactamase (55)	44	27	42	27	18	5	27	10	7	9	10	3	26	13	8	17	9	8
Fluoroquinolones (12)	10	7	9	6	5	1	6	1	1	2	2	1	3	4	3	6	4	3
MDR* (43)	35	32	34	32	26	8	29	15	13	20	13	10	25	15	13	25	16	15
MGEs (48)	40	34	39	32	27	13	34	19	19	28	22	18	28	21	19	32	22	20
MLS _B * (46)	37	26	34	24	16	6	24	11	9	14	6	2	24	7	7	28	14	12
Other (13)	7	4	6	4	3	1	2	1	1	2	3	2	3	1	1	6	2	2
Sulfonamide (6)	5	4	5	4	3	1	4	3	3	4	2	2	4	3	3	5	3	3
Tetracyclines (27)	26	25	25	22	17	12	23	15	15	22	12	11	20	17	16	21	19	18
Trimethoprim (17)	12	6	10	3	5	0	2	2	1	4	1	0	2	0	0	8	4	2
Vancomycin (24)	14	6	11	6	1	2	4	2	0	4	0	0	4	2	1	11	2	2
Total (372)	294	216	276	198	147	57	200	101	88	145	82	55	178	96	83	207	125	112

N = number of primer pairs in the qPCR array

* MDR: multidrug resistance; MLS_B : macrolides, lincosamide and streptogramins B.

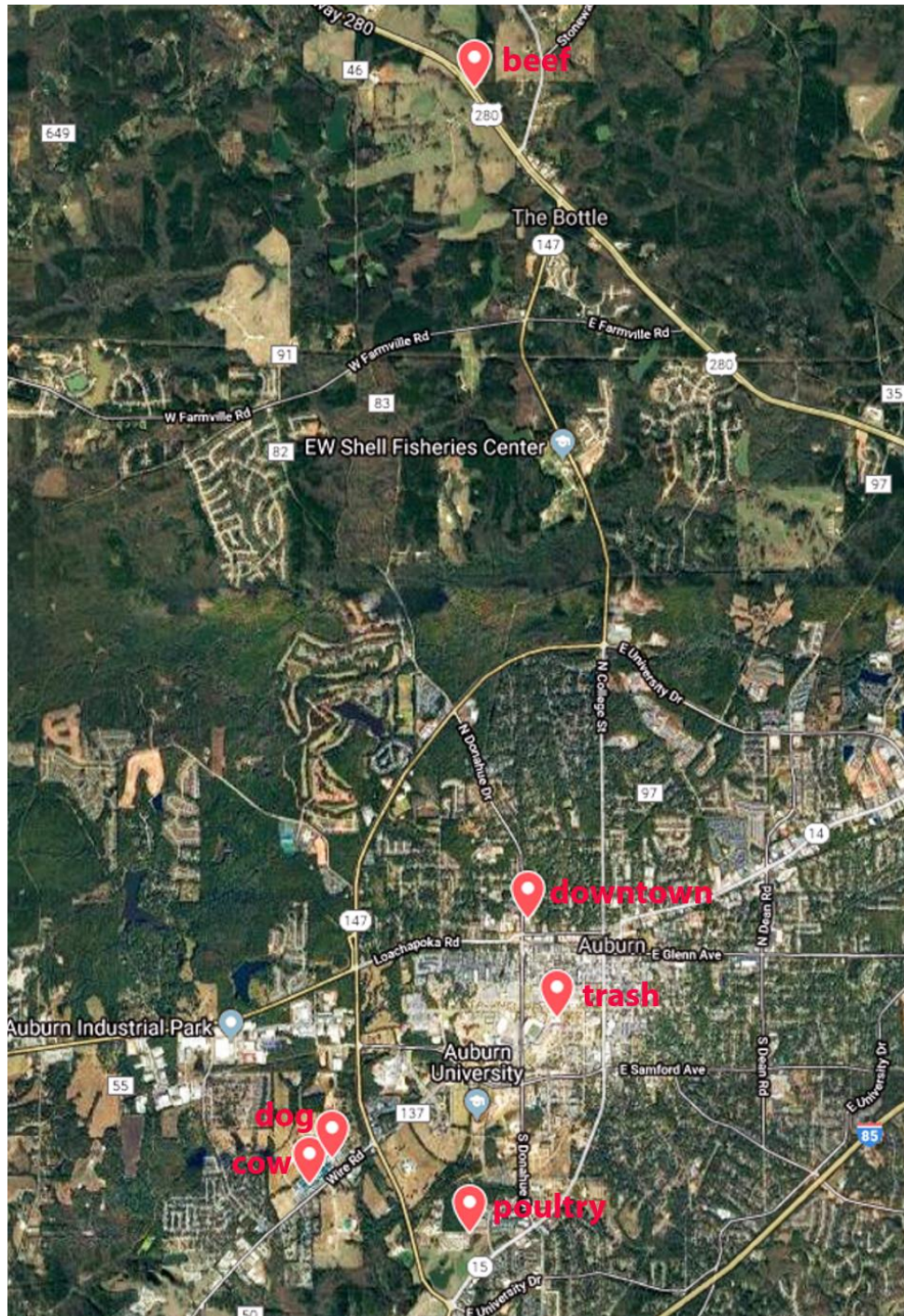


Figure 2.1. Locations for trapping flies in this study. The map was generated by using Google Maps. Sampling sites consisted of beef cattle barn, a dog kennel, a poultry house, and a dairy barn as well as urban areas such as a trash facility and downtown. The GPS coordinates of the trapping sites were 32.58416°/ -85.49615°, 32.590700°/ -85.512799°, 32.583858°/ -85.497973°, 32.589491°/ -85.513361°, 32.612404°/ -85.491943° and 32.606633°/ -85.481586° latitude and longitude, respectively.

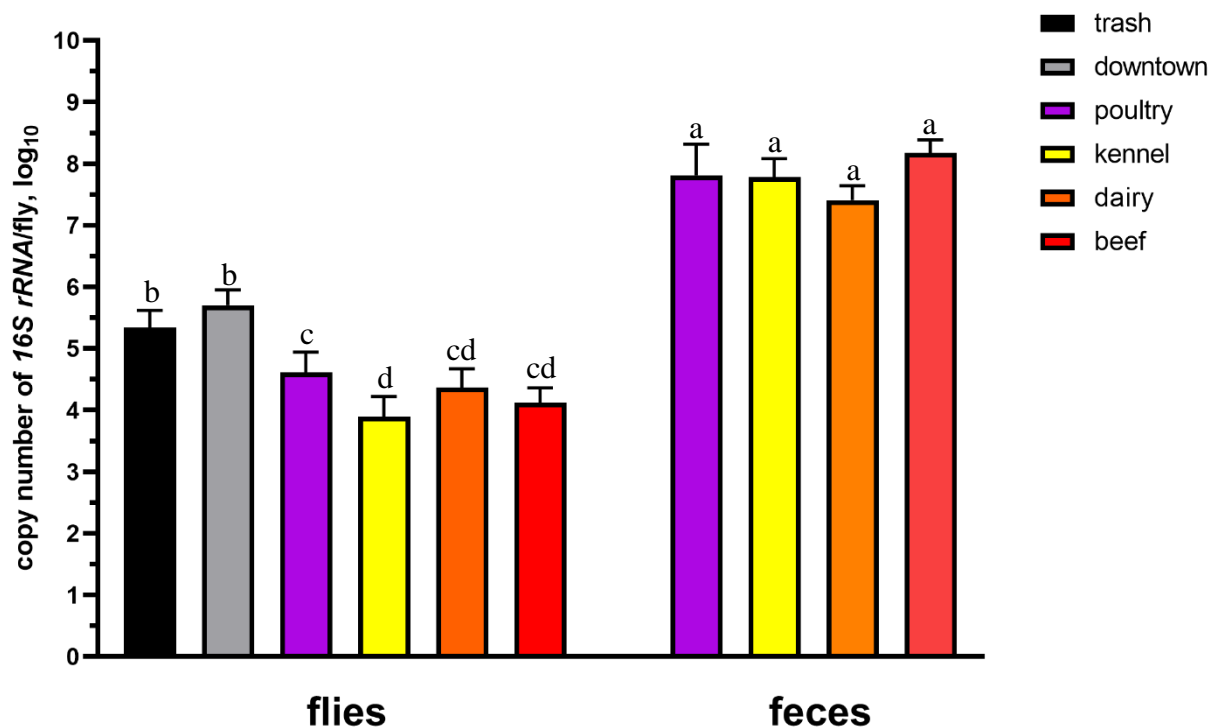


Figure 2.2. Copy number of bacterial *16S rRNA* in flies and feces based on qPCR. A quantitative universal bacteria PCR was used to determine the relative number of bacteria in whole fly homogenates. The average copy numbers of *16S rRNA* in feces (poultry $10^{7.81 \pm 0.51}$; dogs $10^{7.78 \pm 0.3}$; dairy $10^{7.41 \pm 0.23}$; beef $10^{8.18 \pm 0.21}$) were significantly higher than flies ($p < 10^{-4}$). The copy numbers in the trash facility flies ($10^{5.34 \pm 0.28}$) and downtown ($10^{5.7 \pm 0.25}$) were significantly higher than those of flies trapped in the poultry house ($10^{4.61 \pm 0.33}$, $p < 0.01$), dog kennel ($10^{3.89 \pm 0.34}$, $p < 10^{-4}$), dairy ($10^{4.37 \pm 0.28}$, $p < 10^{-4}$) and beef cattle barn ($10^{4.11 \pm 0.25}$, $p < 10^{-4}$). One-way ANOVA with Tukey HSD was used to compare the log₁₀ transformed average copy numbers of *16S rRNA* between different samples. Different letters indicate $P < 0.05$. Error bars indicate the standard error of the mean.

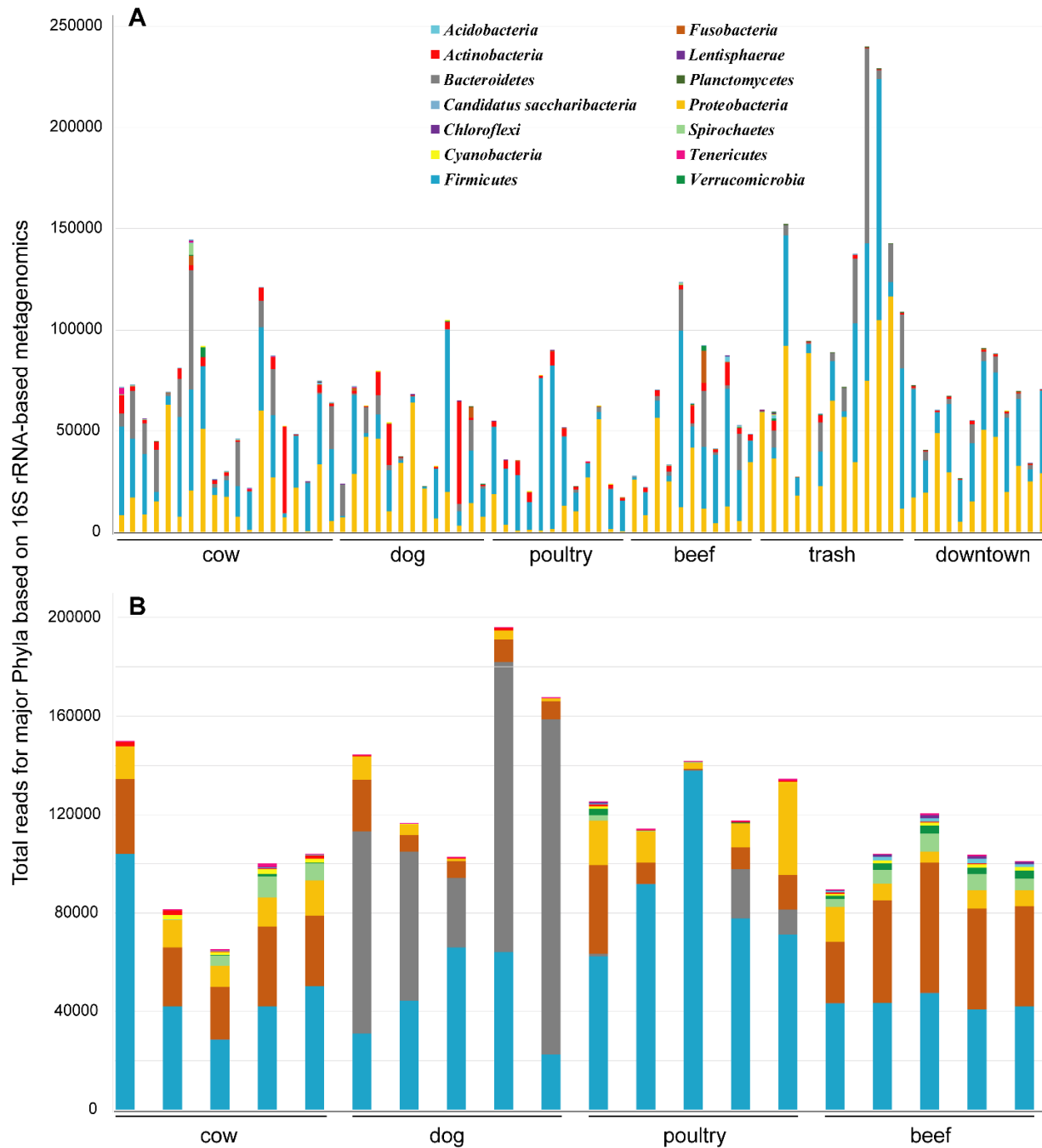


Figure 2.3. Bacterial composition of flies and feces at phylum level. A total of 14 bacterial phyla, top-11 phyla from flies (A) and top-12 phyla from feces (B), showed a significant abundance ($\geq 0.1\%$ of the total reads). *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* accounted for more than 98% of the microbiota of flies trapped at all locations. *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria* and *Spirochaetes* accounted for more than 98% of the microbiota of all fecal samples.

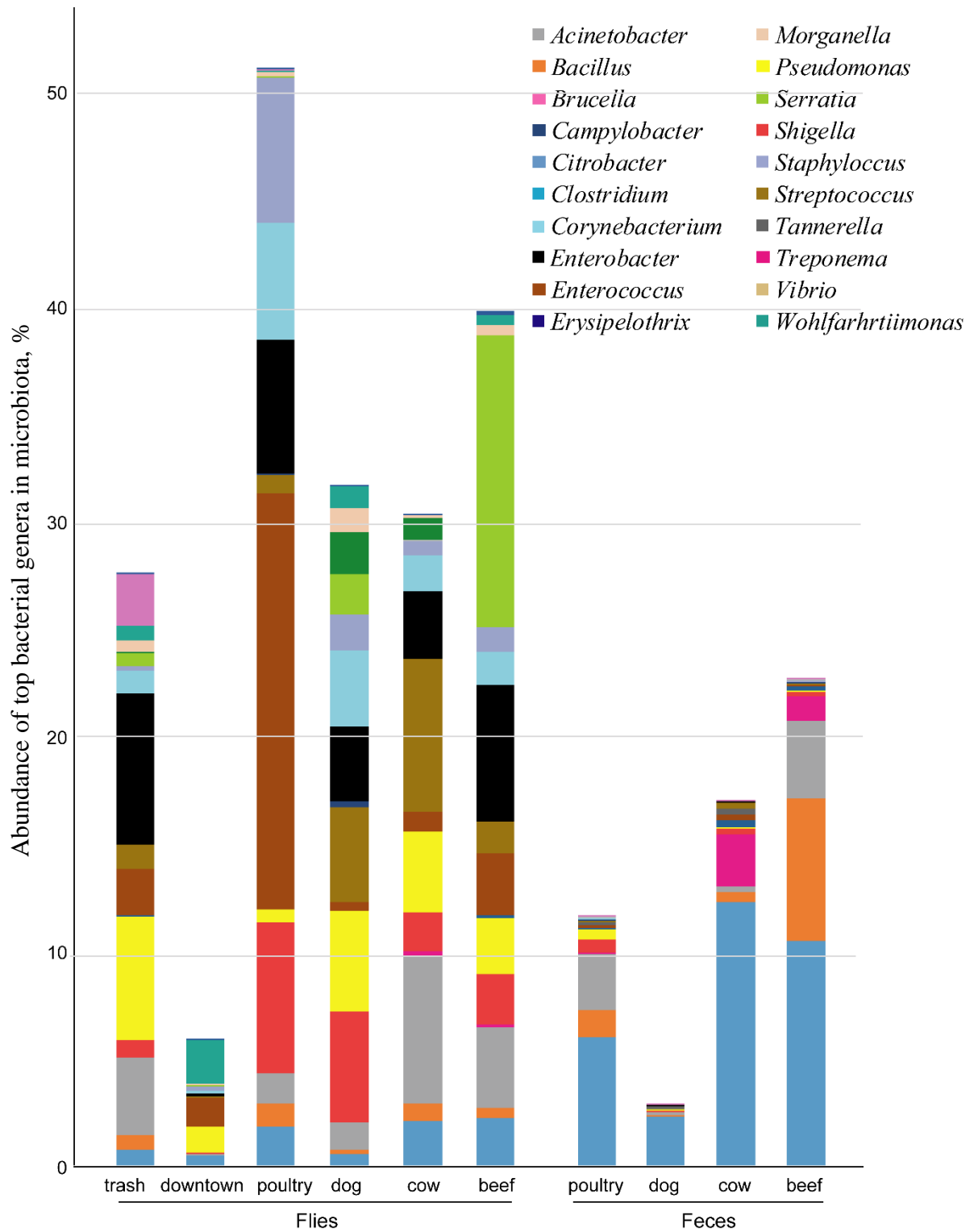


Figure 2.4. Relative abundance of bacterial genera containing potential pathogens in flies and fecal samples. Ten most abundant genera that contain potential pathogens identified in each group of flies or fecal samples are shown, resulting in a total of 20 genera of potential pathogens with >0.1% abundance in total bacterial community.

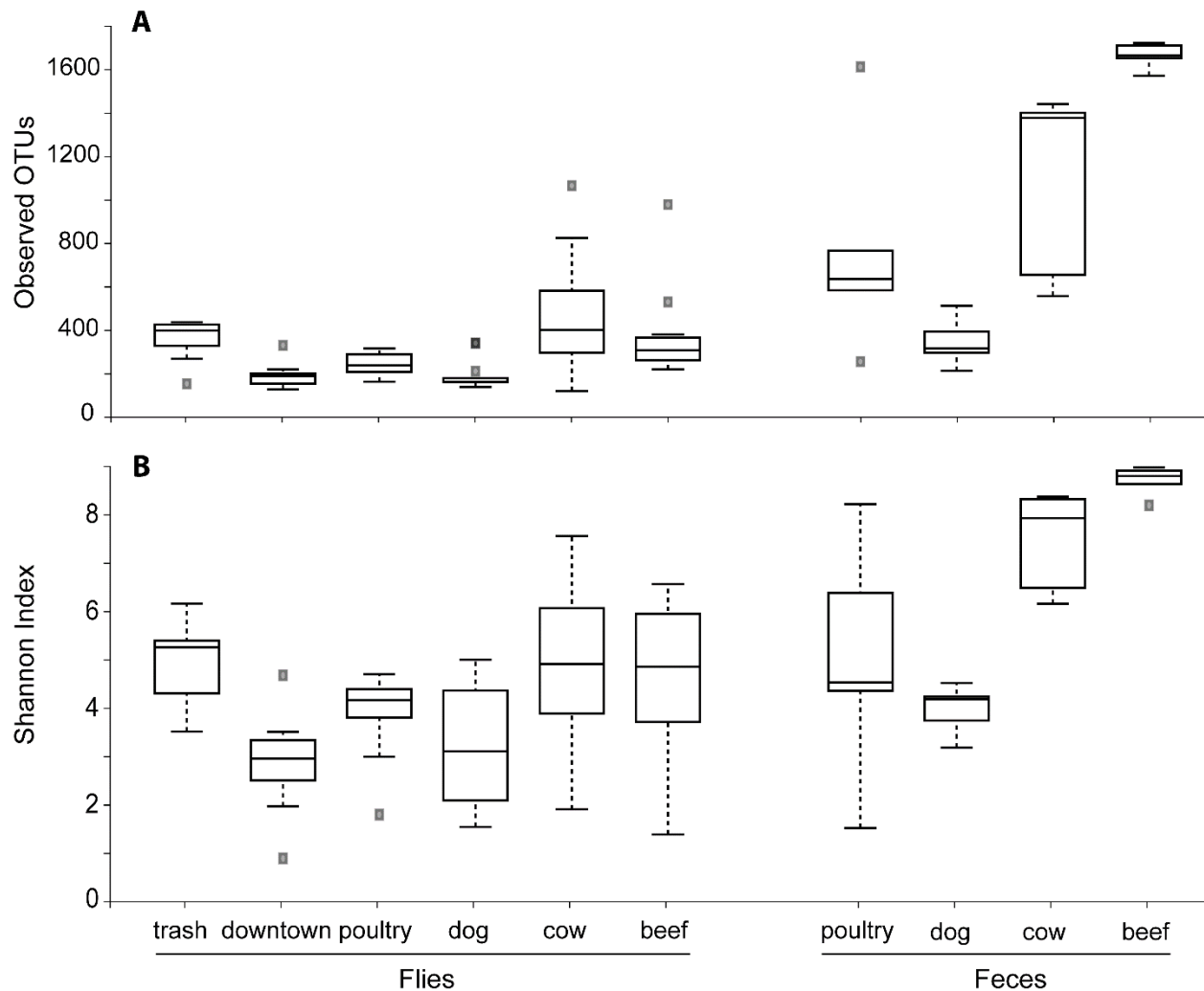


Figure 2.5. Richness and diversity of the bacterial communities of flies and feces. The richness of bacteria communities was estimated by analyzing number of observed OTUs (A), and abundance and evenness of bacteria were indicated by Shannon index (B). The lower and upper whiskers of the box plots denote 9th and 91st percentiles, respectively. The lower and upper extents are 25th and 75th percentiles, and the horizontal line in the middle of box indicates median value. Analysis of number of observed OTUs and Shannon index demonstrated significant difference in bacterial diversity between flies and feces, between flies from the different locations and within same locations, and between feces from the different animal hosts and within the same animal host.

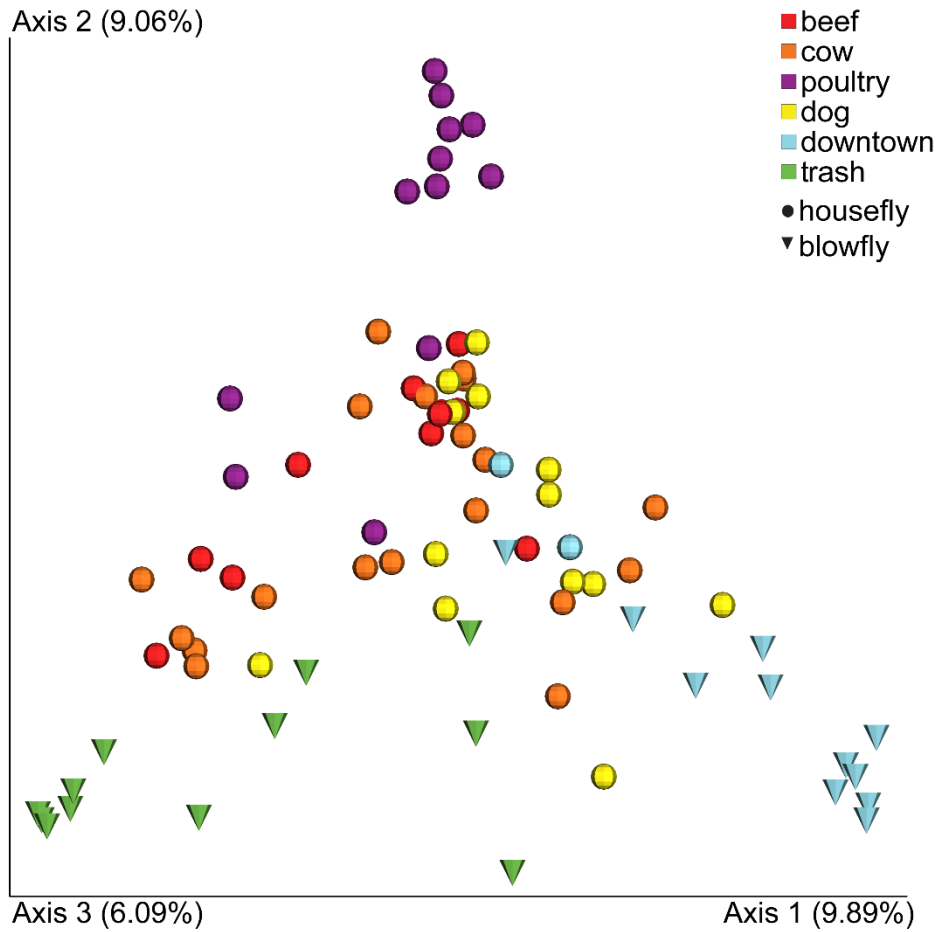


Figure 2.6. Dissimilarity of microbiota of flies across locations. PCoA using Bray Curtis distance metric showing flies sampled from different locations can be separated based on their microbiome complexity. A few flies from farm locations and urban locations clustered across locations indicating shared microbiomes.

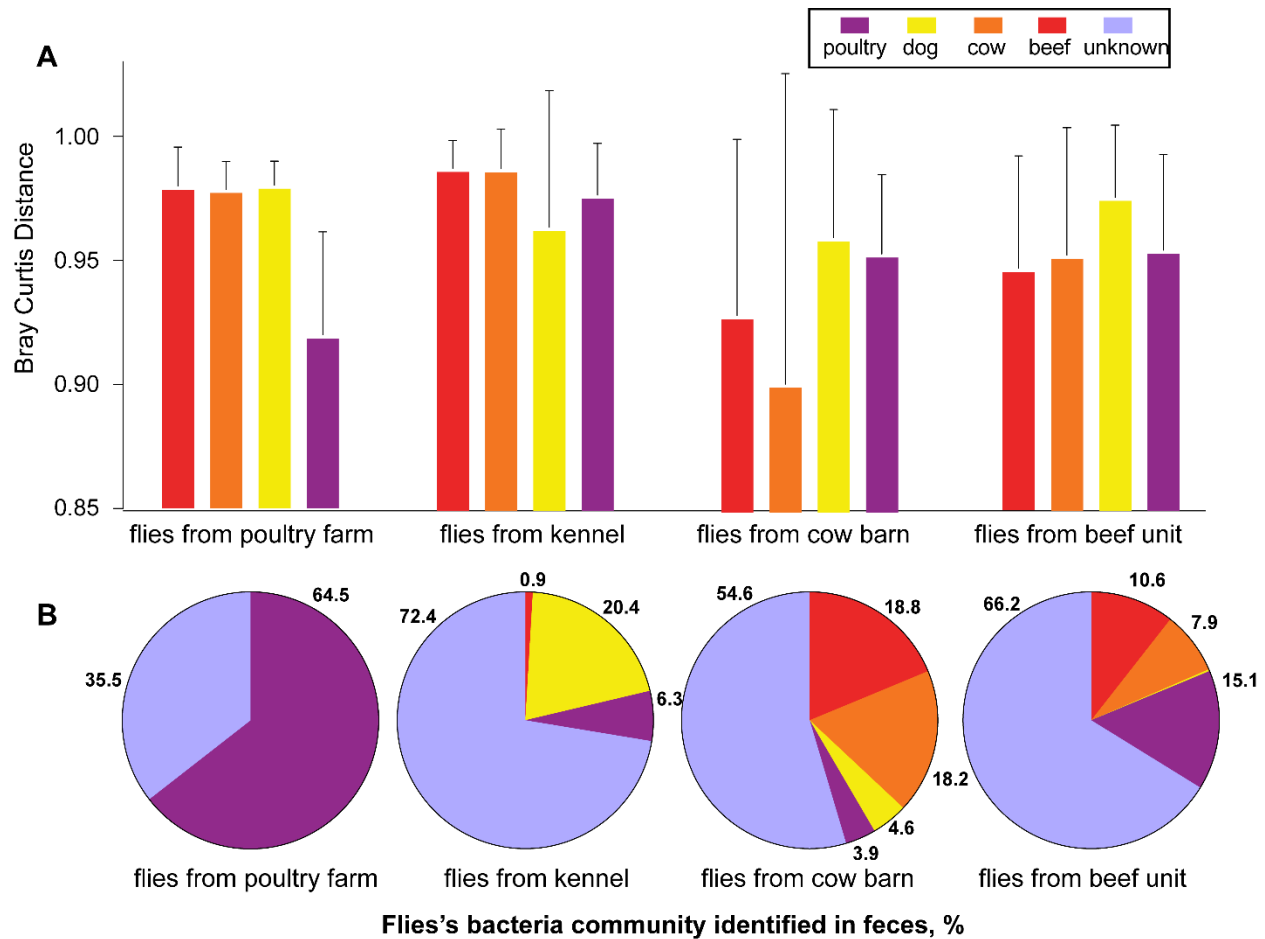


Figure 2.7. Source tracking of microbiota of flies trapped from four animal facilities. (A) Comparison of bacterial diversity of flies sampled from four locations with those of sympatric animal feces. Bray-Curtis dissimilarity analysis shows the bacterial composition of flies is more similar to that found in fecal samples from sympatric animals than to the compositions found in three other animal species. **(B)** Source tracking shows that bacterial communities of poultry flies were dominated by those in poultry feces (64.5%). However, unknown sources contributed most to the communities of bacteria in flies trapped in the kennel, dairy dairy and beef cattle unit. Poultry feces were second in contributing to the bacterial communities in flies trapped in the kennel, dairy dairy and beef cattle unit. The bars indicate standard deviation.

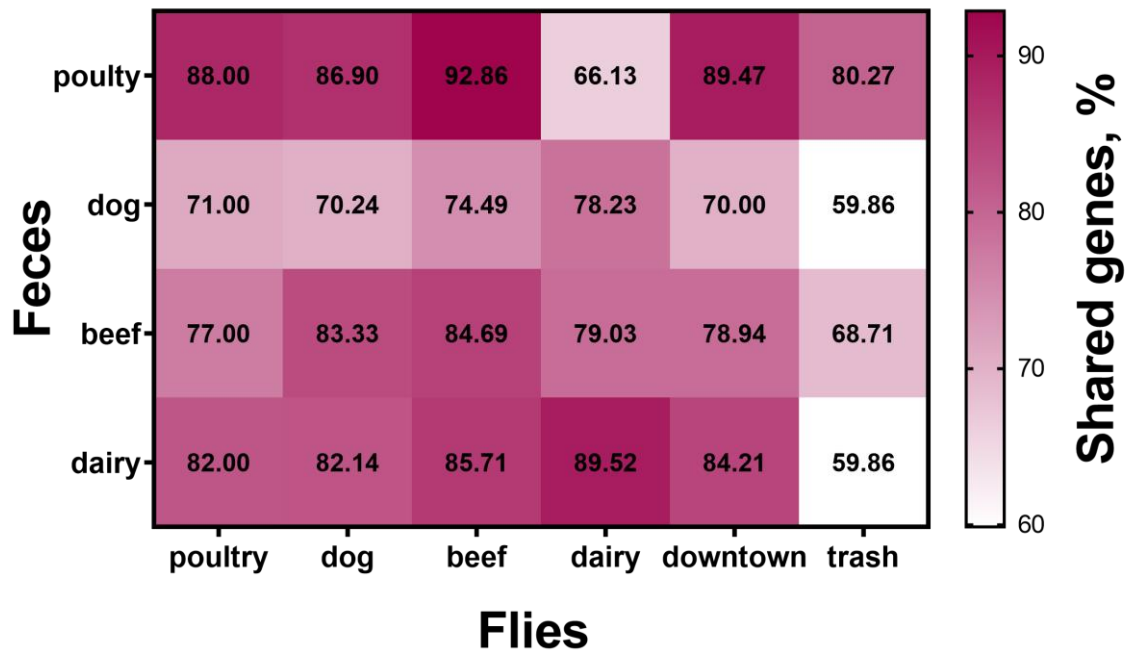


Figure 2.8. ARGs and MGEs shared between flies and feces. A heatmap showing the rate of shared percentage of flies' ARG/MGE with feces across locations. Only poultry and dairy flies were found to share most genes with poultry feces (88%) and dairy feces (88.5%), respectively. There were more ARGs and MGEs in flies from beef unit and the dog kennel that were shared with poultry feces than sympatric animals. Majority of ARG/MGEs in trash facility flies was shared with poultry feces, whereas the other fecal samples shared an average of 62.8% of flies' genes.

Chapter 3

Genomic analysis of ESBL-producing *Escherichia coli* isolates from houseflies in a dog kennel

Abstract

There are numerous global reports on increased prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* in dogs as reservoirs. However, studies have tended to focus on isolation of ESBL-producing bacteria from humans and pet dogs; studies on the environmental link to spread of ESBL-producing *E. coli* from dog kennels are lacking. Flies are well known mechanical vectors of bacterial pathogens including antimicrobial-resistant ones. Here, two ESBL-producing *E. coli* isolates that were recovered from flies trapped at a dog kennel were subjected to whole genome sequencing. *In silico* multilocus sequence typing (MLST) and serotyping show that both isolates belong to ST68 and O25:H6. Moreover, both isolates carry the same antimicrobial resistance genes and virulence determinants, indicating that they are closely related. A total of 12 antimicrobial resistance genes were found in the genome, encoding AmpC/CMY-2 extended-spectrum beta-lactamase, 3 resistance-nodulation-division (RND) efflux pumps, and 2 major facilitator superfamily (MFS) efflux pumps as well as their coupled outer membrane channel TolC. Virulome analysis reveals that the strains harbor various virulence determinants that are theoretically involved in bacterial adhesion, iron acquisition, T6SS, and production of autotransporters and hemolysin/cytolysin A. Taken together, the results suggest that the two related ESBL-producing *E. coli* strains isolated from different flies from the same source and that flies may contribute to bacterial dissemination from source location to the community due to their unrestricted movement and ability to travel long distances.

3.1 Introduction

Despite decades of effort to slow down the selection and transfer of resistance genes through judicious use of antimicrobials, infections with antimicrobial-resistant pathogens remain a serious public health threat, causing more than 2.8 million infections and resulting in 35,000 deaths every year in the United States (Hoelzer *et al.*, 2017; U.S. Centers for Disease Control and Prevention, 2019). In total, antibiotic resistance is estimated to add up to \$20 billion annually to direct healthcare costs in the USA, with additional costs for lost productivity as high as \$35 billion a year (Wallinga and Burch, 2013; Spellberg *et al.*, 2011).

Antimicrobial resistance is rising globally at an alarming rate. While multiple active surveillance programs have been established to monitor antimicrobial resistance, studies on the environmental link to spread of antimicrobial resistance are lacking. Flies are well-known mechanical vectors of bacterial pathogens including antimicrobial-resistant ones, but there is little data on their role in spreading microbial communities and antimicrobial resistance (Poudel *et al.*, 2019). Flies have adapted to live in a close association with animals and humans. Female flies associate with microbe-rich organic materials such as manure for reproductive purposes. Upon exposure to animal wastes, flies can be involved in transmission of bacterial pathogens that they acquire and may play a role in dissemination of bacteria due to their unrestricted movements between different habitats and ability to travel long distances (Onwugamba *et al.*, 2018).

Escherichia coli is a common commensal colonizer of the intestinal tract, but some isolates are some of the most medically important pathogens causing intestinal and extraintestinal infections. Such isolates harbor various virulence factors such as adhesins, invasins, siderophores, and toxins (Sannes *et al.*, 2004). ESBL-production in *E. coli* has been

reported worldwide (Bush, 2008; Sader *et al.*, 1998; Shen *et al.*, 1999). *Enterobacteriaceae* producing ESBLs is resistant to third- and fourth-generation cephalosporins and monobactams (Einhorn *et al.*, 2002). Moreover, many reported ESBL-producing *E. coli* strains demonstrate a multidrug resistance pattern, limiting treatment options for infections with these pathogens (Pitout, 2013).

Unfortunately, investigating antimicrobial-resistant bacteria has tended to focus on clinical isolates from human and animals. Studies on the environmental link between the two populations are limited. Only a few reports have described isolation of ESBL-producing *E. coli* from environmental compartments such as surface water and houseflies (Chen *et al.*, 2010; Blaak *et al.*, 2014). In this study, two ESBL-producing *E. coli* strains were isolated from different houseflies in a dog kennel in Auburn, AL and subjected to whole genome sequencing. *In silico* MLST, serotyping, and detection of virulence and antimicrobial resistance genes were performed.

3.2 Materials and Methods

3.2.1 Bacterial isolates

During the work described in chapter 2 of this dissertation (pages 64-65), three multidrug-resistant *E. coli* isolates and one *K. pneumoniae* strain were found to be phenotypically ESBL positive. Two of the *E. coli* (hereafter, Ec4703 and Ec4706) and the one *K. pneumoniae* strains were isolated from flies collected at the dog kennel with Fly ID's: K50, K52 and K2, respectively. Ec4703 and Ec4706 were selected for genomic DNA extraction and subjected to whole genome sequencing.

3.2.2 Extraction of genomic DNA

Genomic DNA extraction was performed using the High Pure PCR Template Preparation Kit (Roche Diagnostic, Indianapolis, IN) according to the manufacturer's instructions. Specifically, prior to extraction, bacteria were grown in 2 mL Lysogeny Broth at 37°C for 18 hours. Bacteria in 400 µL of the culture were washed once and resuspended in 400 µL PBS and mixed with 400 µL Binding Buffer and 80 µL proteinase K. Lysis was carried out by incubation at 72°C for 20 minutes with shaking at 600 rpm in a thermomixer (Eppendorf, Hamburg, Germany). Two hundred µL isopropanol (Avantor Performance Materials, Center Valley, PA) was added, and the mixture was vortexed thoroughly. The aqueous solution was transferred into the upper reservoir of the filter of the kit inserted into a 2-ml collection tube and centrifuged at 8,000 g for 1 minute. The flow-through liquid was discarded; and Inhibitor Removal Buffer was added to the filter and centrifuged at 8,000 g for 1 minute. The flow-through liquid was discarded; and the filter was washed with 600 µL Washing Buffer and centrifuged at 8,000 g for 1 minute. The washing was repeated with 400 µL and followed by centrifugation at 13,000 g for

1 minute. The filter column was inserted in a new 2-mL collection tube. Nucleic acid was eluted with 50 μ L pre-warmed Elution Buffer added to the middle of the filter, incubated at 72°C for 5 minutes, and centrifuged at 8,000 g for 1 minute. The elution step was repeated to increase DNA yields.

3.2.3 Whole genome sequencing

The extracted genomic DNA of Ec4703 and Ec4706 samples were sent to the Iowa State University Veterinary Diagnostic Laboratory for next-generation sequencing using the Illumina MiSeq platform according to the manufacturer's instructions and sequencing provider's protocol. WGS-based multilocus sequence typing was carried out using MLST 2.0 (Larsen *et al.*, 2012). Core genome MLST was determined with cgMLSTFinder 1.1 (Zhou *et al.*, 2020). Serotypes were predicted with SerotypeFinder 2.0 (Joensen *et al.*, 2015). Typing of plasmid replicons was determined by using PlasmidFinder 2.1 (Carattoli *et al.*, 2014). Antimicrobial resistance genes were predicted with Comprehensive Antibiotic Resistance Genes Database (CARD) with a threshold e-value of 1e-10 (McArthur *et al.*, 2013) and ResFinder 3.2 with a threshold sequence similarity of 90% (Zankari *et al.*, 2012). The genomes were screened for virulence genes using Virulence Factors of Pathogenic Bacteria Database (VFDB) with a threshold e-value of 1e-10 (Chen *et al.*, 2005) and determined using VirulenceFinder 2.0 (Joensen *et al.*, 2014).

3.3 Results

3.3.1 Sequencing data

The raw genome sequences consisted of 90,282 and 98,971 read-pairs for Ec4703 and Ec4706, respectively (Table 3.1). The draft sequences of Ec4703 were composed of 1,029 contigs with an average length of 1,738.7 bp and total of 1,026 contigs containing more than 200 bp. Ec4706 sequences, on the other hand, comprised 1,310 contigs with an average length of 3,143.2 bp and 1,304 contigs having more than 200 bp (Table 3.1).

3.3.2 Genomic relationship

The *in silico* MLST and serotyping data indicated that Ec4703 and Ec4706 belong to the sequence type 68 (ST68) and serotype O25:H6. Not only sharing the same MLST and serotype, Ec4703 and Ec4706 were found to carry the same type of plasmid replicon, IncII (Table 3.2). Core-genome MLST of Ec4703 compared total 279 loci against 2513 loci in the database (11.1%) and 276 allele matches, resulting in cgST 132275, while Ec4706 was found to belong to cgST 138352 with 1186 loci that were called and 1175 allele matches.

3.3.3 Antimicrobial resistance and virulence genes

Ec4703 and Ec4706 share not only the same sequence type and serotype but they also contain the same antimicrobial resistance genes and virulence determinants. The β -lactamase gene that is responsible for ESBL phenotype was found to *bla_{CMY-2}*. No other acquired antimicrobial resistance genes were found in the genome. The genomes of Ec4703 and Ec4706 encode three resistance-nodulation-cell division (RND) antibiotic efflux systems including AcrEF, AcrAB, and MdtEF and their transcriptional regulators (H-NS, AcrS, MarA, and GadX).

Additionally, genes encoding biosynthesis of two major facilitator superfamily (MFS) antibiotic efflux pumps were found, for EmrKY and EmrAB. The genome also harbors *tolC* encoding the outer membrane channel that couples with MFS and RND efflux pumps.

WGS-based virulome analysis of Ec4703 and Ec4706 revealed that the genomes of the isolates harbor a total of 54 virulence-associated genes, theoretically producing 11 virulence factors, including adherence factors, siderophores, type VI secretion systems, autotransporters, invasion factors, and hemolysin (Table 3.3). There were 24 adherence genes responsible for synthesis, assembly, and regulation of three types of fimbriae: type-1 fimbriae, type-4 fimbriae, and *E. coli* common pilus (ECP) as well as the putative attaching and effacing protein EaeH. The genomes also harbor 13 *aec* genes theoretically encoding synthesis and assembly of ATP-dependent type VI secretion system (ACE T6SS). Iron acquisition is predicted to be mediated by a total of 11 genes encoding heme uptake (*chu*) and iron/manganese transport systems (*sitABCD*). Other virulence factors include invasion proteins (Ibe and Tia), autotransporters (UpaG and Cah), and hemolysin/cytolysin A.

3.4 Discussion

Flies have adapted to live in close association with animals and humans. Female flies associate with microbe-rich organic materials such as manure for reproductive purposes. Upon exposure to animal wastes, flies can be an indirect route of transmission of bacterial pathogens that they acquire and may play a role in dissemination of bacteria due to their unrestricted movements between different habitats and ability to travel long distances (Onwugamba *et al.*, 2018). More importantly, it is likely that flies can also spread antimicrobial resistance between animals and humans due to ubiquity of antimicrobial resistant bacteria in animal waste (Agga *et al.*, 2015; Argudín *et al.*, 2017). Several studies have reported isolation of bacteria, including those with antimicrobial resistance, from flies (Iwasa *et al.*, 1999; Allen *et al.*, 2004; Usui *et al.*, 2013; Blaak *et al.*, 2014). The description of flies as a mechanical vector of bacterial transmission and antimicrobial resistance spread indicates that flies may be useful as an alternative one-health indicator of prevalence of antimicrobial resistance by mirroring the habitat. In this study, two ESBL-producing *E. coli* strains (Ec4703 and Ec4706) were isolated from houseflies trapped in a dog kennel in Auburn, AL and subjected to WGS to investigate clonal spread of antimicrobial-resistant bacteria from animal husbandry to flies.

Although multilocus sequence typing and serotyping are widely used in epidemiological studies, they have low discriminatory power, and non-related bacteria can be clustered within the same group if they have identical sequence type (Chin *et al.*, 2011). Analysis of whole genome sequences is the ideal approach for robust phylogenetic analysis and genomic diversity. The raw genome sequences of Ec4703 and Ec4706 consisted of 90,282 and 98,971 read-pairs composing draft sequences with 1,029 and 1,310 contigs, respectively. *In silico* MLST, cgMLST, and serotyping of Ec4703 and Ec4706 indicate that they are closely related, with the same sequence

type and serotype. Further, Ec4703 and Ec4706 contain identical antimicrobial resistance and virulence genes profiles. ST68 is the most identified sequence type in asymptomatic and symptomatic animals including dogs and described as a global multidrug-resistant *E. coli* clone in several studies (Ho *et al.*, 2012; Chen *et al.*, 2014; Rincón *et al.*, 2014). Recovery of ST68 that produce ESBL enzymes from flies is indeed a health threat to animals and humans due to the lifestyle of flies. CMY-2 β -lactamase is one of the most detected cephalosporinases in *E. coli* isolated from dogs globally (Damborg *et al.*, 2011; Rocha-Gracia *et al.*, 2015; Hong *et al.*, 2020). Mattioni Marchetti *et al.* (2020) reported that *bla*_{CMY-2}-carrying *E. coli* infections were related to the deaths of eight dogs in Italy. Pathogenic *E. coli* isolates harbor various virulence factors such as adhesins, invasins, siderophores, and toxins. The distribution of virulence-associated genes in Ec4703 and Ec4706 shows that most of the genes were found to contribute to bacterial adhesion mediated by type 1 fimbriae, type 4 fimbriae, ECP, and EaeH. Other virulence-associated genes included those encoding iron uptake systems, and T6SS. Although most detected genes are carried by commensal strains of *E. coli*, they are crucial virulence factors in pathogenic strains (Bahrani-Mougeot *et al.*, 2002; Ludwig *et al.*, 2004; Rendon *et al.*, 2007; Boyer *et al.*, 2009; Pusz *et al.*, 2014).

Taken together, isolation of two closely related bacterial strains from different flies in the same habitat supports the role of flies as an indirect route of transmission of potential bacterial pathogens including antimicrobial-resistant bacteria, and suggests that flies may be useful as an alternative one-health indicator of prevalence of antimicrobial resistance.

References

- Agga, G., Arthur, T., Durso, L., Harhay, D., and Schmidt, J. (2015) Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. *PLoS One* **10**: e0132586.
- Allen, S., Thomas, J., Alexander, N., Bailey, R., and Emerson, P. (2004) Flies and *Helicobacter pylori* infection. *Arch Dis Child* **89**: 1037–1038.
- Argudín, M., Deplano, A., Meghraoui, A., Dodémont, M., Heinrichs, A., Denis, O., et al. (2017). Bacteria from animals as a pool of antimicrobial resistance genes. *Antibiotics (Basel, Switzerland)* **6**: 12.
- Bahrani-Mougeot, F., Buckles, E., Lockett, C., Hebel, J., Johnson, D., Tang, C., Donnenberg, M. (2002) Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic *Escherichia coli* virulence determinants in the murine urinary tract. *Mol Microbiol* **45**:1079–1093.
- Blaak, H., Hamidjaja, R., van Hoek, A., de Heer, L., de Roda Husman, A., and Schets, F. (2014) Detection of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* on flies at poultry farms. *Appl Environ Microbiol* **80**: 239–246.
- Bush, K. (2008) Extended-spectrum beta-lactamases in North America. *Clin Microbiol Infect* **14** Suppl 1: 134–143.
- Carattoli, A., Zankari, E., García-Fernández, A., Larsen, M., Lund, O., Villa, L., et al. (2014) *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* **58**: 3895–3903.

- Chen, C., Ke, S., Li, C., Chiou, C., and Chang, C. (2014) Prolonged clonal spreading and dynamic changes in antimicrobial resistance of *Escherichia coli* ST68 among patients who stayed in a respiratory care ward. *J Med Microbiol* **63**: 1531–1541.
- Chen, H., Shu, W., Chang, X., Chen, J., Guo, Y., and Tan, Y. (2010). The profile of antibiotics resistance and integrons of extended-spectrum beta-lactamase producing thermotolerant coliforms isolated from the Yangtze River basin in Chongqing. *Environ. Pollut* **158**: 2459–2464.
- Chen, L., Yang, J., Yu, J., Yao, Z., Sun, L., Shen, Y., and Jin, Q. (2005) VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res* **33**: D325–D328.
- Chin, C-S., Sorenson, J., Harris, J., Robins, W., Charles, R., Jean-Charles, R., et al. (2011) The origin of the Haitian cholera outbreak strain. *N Engl J Med* **364**: 33–42.
- Damborg, P., Gaustad, I., Olsen, J., and Guardabassi, L. (2011) Selection of CMY-2 producing *Escherichia coli* in the faecal flora of dogs treated with cephalexin. *Vet Microbiol* **151**: 404–408.
- Einhorn, A., Neuhauser, M., Bearden, D., Quinn, J., and Pendland, S. (2002) Extended-spectrum beta-lactamases: frequency, risk factors, and outcomes. *Pharmacotherapy* **22**: 14–20.
- Ho, P., Yeung, M., Lo, W., Tse, H., Li, Z., Lai, E., et al. (2012) Predominance of pHK01-like incompatibility group FII plasmids encoding CTX-M-14 among extended-spectrum beta-lactamase-producing *Escherichia coli* in Hong Kong, 1996-2008. *Diagn Microbiol Infect Dis* **73**: 182–186.
- Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., and Coukell, A. (2017) Antimicrobial drug use in food-producing animals and associated human health risks: What, and how strong, is the evidence? *BMC Vet Res* **13**: 211.

- Hong, J., Song, W., Park, H., Oh, J., Chae, J., Jeong, S., and Jeong, S. (2020). Molecular characterization of fecal extended-spectrum β -lactamase- and Ampc β -lactamase-producing *Escherichia coli* from healthy companion animals and cohabiting humans in South Korea. *Front Microbiol* **11**: 674.
- Iwasa, M., Makino, S-I., Asakura, H., Kobori, H., and Morimoto, Y. (1999) Detection of *Escherichia coli* O157:H7 from *Musca domestica* (Diptera: *Muscidae*) at a cattle farm in Japan. *J Med Entomol* **36**: 108–112.
- Joensen, K., Scheutz, F., Lund, O., Hasman, H., Kaas, R., Nielsen, E., and Aarestrup, F. (2014) Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J Clin Microbiol* **52**: 1501–1510.
- Joensen, K., Tetzschner, A., Iguchi, A., Aarestrup, F., and Scheutz, F. (2015) Rapid and easy *in silico* serotyping of *Escherichia coli* using whole genome sequencing (WGS) data. *J Clin Microbiol* **53**: 2410–2426.
- Larsen, M., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. et al. (2012) Multilocus Sequence Typing of Total Genome Sequenced Bacteria. *J Clin Microbiol* **50**: 1355–1361.
- Ludwig, A., von Rhein, C., Bauer, S., Hüttinger, C., and Goebel, W. (2004) Molecular analysis of cytolysin A (ClyA) in pathogenic *Escherichia coli* strains. *J Bacteriol* **186**: 5311–5320.
- Mattioni Marchetti, V., Bitar, I., Mercato, A., Nucleo, E., Marchesini, F., Mancinelli, M. (2020) Deadly puppy infection caused by an MDR *Escherichia coli* O39 *bla*_{CTX-M-15}, *bla*_{CMY-2}, *bla*_{DHA-1}, and *aac(6)-Ib-cr* – positive in a breeding kennel in central Italy. *Front Microbiol* **11**: 584.

- McArthur, A., Waglechner, N., Nizam, F., Yan, A., Azad, M., Baylay, A., et al. (2013) The Comprehensive Antibiotic Resistance Database. *Antimicrob Agents Chemother* **57**: 3348–3357.
- Onwugamba, F., Fitzgerald, J., Rochon, K., Guardabassi, L., Alabi, A., Kühne, S., et al. (2018) The role of 'filth flies' in the spread of antimicrobial resistance. *Travel Med Infect Dis* **2018**: 8–17.
- Pitout, J. (2013) *Enterobacteriaceae* that produce extended-spectrum β -lactamases and AmpC β -lactamases in the community: the tip of the iceberg? *Cur Pharm Des* **19**: 257–263.
- Poudel, A. Hathcock, T., Butaye, P., Kang, Y., Price, S., Macklin, K., et al. (2019) Multidrug-resistant *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus* spp. in houseflies and blowflies from farms and their environmental settings. *Int J Environ Res Public Health* **16**: 3583.
- Pusz, P., Bok, E., Mazurek, J., Stosik, M., and Baldy-Chudzik, K. (2014) Type 1 fimbriae in commensal *Escherichia coli* derived from healthy humans. *Acta Biochim Pol* **61**: 389–392.
- Rendón, M., Saldaña, Z., Erdem, A., Monteiro-Neto, V., Vázquez, A., Kaper, J., et al. (2007) Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization. *Proc Natl Acad Sci U S A* **104**: 10637–10642.
- Rincón, G., Radice, M., Giovanakis, M., Di Conza, J., and Gutkind G. (2014) First report of plasmid-mediated fluoroquinolone efflux pump QepA in *Escherichia coli* clinical isolate ST68, in South America. *Diagn Microbiol Infect Dis* **79**: 70–72.

- Rocha-Gracia, R., Cortés-Cortés, G., Lozano-Zarain, P., Bello, F., Martínez-Laguna, Y., and Torres, C. (2015) Faecal *Escherichia coli* isolates from healthy dogs harbour CTX-M-15 and CMY-2 β -lactamases. *Vet J* **203**: 315–319.
- Sader, H., Jones, R., Gales, A., Winokur, P., Kugler, K., Pfaller, M., and Doern, G. (1998) Antimicrobial susceptibility patterns for pathogens isolated from patients in Latin American medical centers with a diagnosis of pneumonia: analysis of results from the sentry antimicrobial surveillance program (1997). SENTRY Latin America study group. *Diagn Microbiol Infect Dis* **32**: 289–301.
- Sannes, M., Kuskowski, M., Owens, K., Gajewski, A., and Johnson, J. (2004) Virulence factor profiles and phylogenetic background of *Escherichia coli* isolates from veterans with bacteremia and uninfected control subjects. *J Infect Dis* **190**: 2121–2128.
- Shen, D., Biedenbach, D., Winokur, P., Pfaller, M., and Jones, R. (1999). Phenotypic and genotypic characterizations of Chinese strains of *Escherichia coli* producing extended-spectrum β -lactamases. *Diagn Microbiol Infect Dis* **34**: 159–164.
- Spellberg, B., Blaser, M., Guidos, R.J., Boucher, H., Bradley, J., Eisenstein, B., et al. (2011) Combating antimicrobial resistance: policy recommendations to save lives. *Clin Infect Dis* **52**: S397–S428.
- U.S. Centers for Disease Control and Prevention (2019) Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA, USA.
- Usui, M., Iwasa, T., Fukuda, A., Sato, T., Okubo, T., and Tamura, Y. (2013) The role of flies in spreading the extended-spectrum β -lactamase gene from cattle. *Microb Drug Resist* **19**: 415–420.

- Wallinga, D. and Burch D. (2013) Does adding routine antibiotics to animal feed pose a serious risk to human health? *BMJ* **347**: f4214.
- Zankari, E., Hasman, H., Kaas, R., Seyfarth, A., Agersø, Y., Lund, O., et al. (2013) Genotyping using whole-genome sequencing is a realistic alternative to surveillance based on phenotypic antimicrobial susceptibility testing. *J Antimicrob Chemother* **68**: 771–777.
- Zhou, Z., Alikhan, NF., Mohamed, K., Fan, Y., Agama Study Group, and Achtman, M. (2020) The Enterobase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. *Genome Res* **30**: 138–152.

Table 3.1. Summary of the sequencing data

	Ec4703	Ec4706
raw data reads	90,282	98,971
total contigs (n)	1,029	1,310
> 200 bp contigs	1,026	1,304
≥ 1000 bp contigs	661	1,094
longest contig length (bp)	11,795	30,930
average contig length (bp)	1,738.7	3,143.2
N50	2,223	4,202

N50, minimum contig length to cover 50%;
bp, base pair.

Table 3.2. Multilocus sequence typing and serotyping of Ec4703 and Ec4706

Comparison scheme	Ec4703	Ec4706
MLST	68	68
<i>adk</i>	33	33
<i>fumC</i>	26	26
<i>gyrB</i>	2	2
<i>icd</i>	31	31
<i>mdh</i>	5	5
<i>purA</i>	16	16
<i>recA</i>	19	19
cgMLST	132275	138352
O antigen	O25	O25
H antigen	H6	H6
Plasmid replicon type	IncII	IncII

MLST was performed based on allelic variations of 7 housekeeping genes in MLST 2.0;

cgMLST was performed based on allelic variations compared to 2513 total loci in

cgMLST 1.1;

O- and H- serotypings were performed in SerotypeFinder 2.0 based on sequences of *wzx*

and *fliC*;

Typing of plasmid replicons was determined by using PlasmidFinder 2.1.

Table 3.3. Virulence determinants in the ESBL-producing *E. coli* identified in this study

Virulence class	Virulence factors	Genes (n)
adhesion	type 1 fimbriae, type 4 fimbriae, ECP, EaeH	24
secretion systems	T6SS	13
iron acquisition	hemin uptake, iron manganese transporter	11
invasion	Ibe, Tia	3
autotransporter	UpaG, Cah	2
toxins	hemolysin/cytolysin A	1
Total	22	54

ECP = *E. coli* common pilus; n = number; T6SS = type VI secretion system

Chapter 4

Isolation and molecular characterization of colistin-resistant bacteria from flies

Abstract

Colistin is the last-resort drug used to treat carbapenem-resistant Gram-negative bacteria. Resistance to colistin increases the fear of losing our battle against pan-drug resistant bacteria. Several reports and articles have discussed colistin resistance in clinical isolates, but the environmental link to the emergence and spread of the resistance is still lacking. In this study, 485 colistin-resistant bacteria were recovered from flies trapped at a trash collection area in Auburn, AL. *Providencia* species were the most prevalent colistin-resistant bacteria (64.0%) followed by *Serratia* (16.9%), *Enterobacter* (6.7%), and *Klebsiella* (5.6%). A *Klebsiella pneumoniae* isolate was subjected to whole genome analysis. Complete genome sequencing resulted in a total genome size of 5,337,408 bp for this isolate with a plasmid of 224,442 bp. *In silico* MLST, O- and K- antigens classify the isolate as ST784, O1, and KL146. The genome contains various virulence determinants involved in adhesion, iron acquisition and, most importantly, the regulator gene *magA*, which contributes to *K. pneumoniae* virulence by hypermucoviscosity phenotype. The findings in this work indicate that flies can be an important environmental link to spread of colistin-resistant bacteria with clinical importance including naturally resistant organisms such as *Providencia*, *Proteus*, and *Serratia*, which certainly results in health threats for human and animals.

4.1 Introduction

Antimicrobial resistance remains a serious public health threat despite decades of efforts to slow down the selection and transfer of resistance genes through judicious use of antimicrobials (Hoelzer *et al.*, 2017). In the United States, more than 2.8 million illnesses and 35,000 deaths are attributed to infections with antimicrobial-resistant bacteria every year (U.S. Centers for Disease Control and Prevention, 2019).

Colistin, polymyxin E, is a cationic polypeptide antibiotic that is used as the last-resort treatment option for infections with carbapenem-resistant Gram-negative bacteria (Bergen *et al.*, 2006). Due to extensive use of colistin as growth promoters, resistance to colistin has drastically increased in naturally susceptible bacteria (Rhouma *et al.*, 2016). Moreover, increased prevalence of infections caused by naturally colistin-resistant bacteria such as *Providencia* spp., *Proteus* spp., and *Serratia* spp. has been reported in several articles (Hayakawa *et al.*, 2012; Merkier *et al.*, 2013; Samonis *et al.*, 2014). However, despite all efforts in studying the prevalence of colistin resistance, almost all reports have discussed colistin resistance in bacterial isolates from infected human or animals, and studies on the environmental link to the spread of antimicrobial resistance are lacking.

Flies are common in and around livestock operations, easily making effective contacts with animals, manure and the environment. Throughout all life stages, flies associate with microbe-rich organic materials such as manure for various purposes including reproductive (West, 1951). Consequently, they are involved in transmission of bacterial pathogens that they acquire from animal waste and may disseminate bacteria due to their unrestricted movements between different habitats and ability to travel long distances (Winpisinger *et al.*, 2005; Nazni *et al.*, 2005). More importantly, it is likely that flies can also spread antimicrobial resistance

between animals and humans due to ubiquity of antimicrobial resistant bacteria in animal waste (Agga *et al.*, 2015; Argudín *et al.*, 2017). Schaumburg *et al.* (2016) reported that flies carried multidrug-resistant bacteria belonging to specific clonal lineages that were identical to those found in animal manure. Moreover, the gut of flies provides a suitable environment to harbor antimicrobial resistance genes and for horizontal gene transfer (Akhtar *et al.*, 2009; Fuduka *et al.*, 2016). Despite the ubiquitous nature of flies and reports of their role in the spread of medically important pathogens (Zurek and Ghosh, 2014; Zhang *et al.*, 2018; Yang *et al.*, 2019), few studies have explored the extent to which flies transmit antimicrobial resistance especially to the last-resort antimicrobial agent, colistin.

Klebsiella pneumoniae is an opportunistic pathogen that is a leading cause of nosocomial infections (Nordmann *et al.*, 2009). Emergence of colistin-resistant *K. pneumoniae* has led to fear of pan-drug resistance. Colistin resistance in *K. pneumoniae* has been reported in numerous outbreaks globally including the United States (Sutherland and Nicolau, 2015), Canada (Walkty *et al.*, 2009), and South America (Perez, 2015). The most common mechanism of resistance in *Enterobacteriaceae* is mediated by change in the negative charge of the bacterial lipopolysaccharides (LPS) through adding 4-amino-4-deoxy-L-arabinose ($_L$ -Ara4N) and/or phosphoethanolamine (pEtN) to phosphate groups of the lipid A component of LPS. An overlapping network of a large panel of genes is involved in the LPS remodeling, including LPS-modifying enzymes such as the pEtN-phosphotransferase PmrC (Gunn, 2008) and products of the *armBCADTEF* operon, which is responsible for synthesis and addition of $_L$ -Ara4N to the lipid A (Yan *et al.*, 2007). Those genes, *pmrC* and *arn*, are highly regulated by three two-component regulatory systems: PmrA-PmrB, PhoP-PhoQ, and CrrA-CrrB, which are normally activated inside macrophages or by environmental stimuli such as Fe^{3+} and low pH, allowing bacterial

survival through LPS modifications (Gunn, 2008). However, in the case of colistin resistance, constitutive activation of the described two-component regulatory systems. Such activation can be achieved by certain mutations in operons *pmrAB*, *phoPQ*, and *crrAB* and/or the negative feedback regulator *mgrB* (Poirel *et al.*, 2017). It was thought that colistin resistance in *Enterobacteriaceae* was exclusively chromosomally mediated until the identification of 10 variants of plasmid-harbored mobile colistin resistance, *mcr*, genes (Liu *et al.*, 2016).

Studies of colistin resistance in *K. pneumoniae* has been mainly performed on clinical isolates. Monitoring of environmental prevalence and spread of colistin-resistance remain poorly understood. In this study, 485 colistin-resistant bacteria were isolated from 255 flies trapped at a trash collection area in Auburn, AL. A complete genome sequencing of a colistin-resistant *K. pneumoniae* strain was performed, and the genome sequence was released in GenBank.

4.2 Material and methods

4.2.1 Fly trapping

Flies were trapped from the trash collection area in the trash collection area in Auburn, AL throughout the month of August 2018. The daily high temperatures ranged from 27–33 °C, and the average humidity and pressure during flies' collection were 86.5 % and 30.5 mm Hg, respectively.

One trap ribbon was placed close to trash trucks and left for two hours. Trapped flies were collected with sterile forceps, placed individually in a 1.5 mL micro-centrifuge tube containing 800 µL of sterile phosphate buffered saline (PBS), and transported to the laboratory within 30 minutes of collection. In the laboratory, flies were homogenized with a tissue homogenizer (Bertin Technologies, Rockville, MD, USA) at 5,000 RPM for 20 seconds, for three times, and homogenates were used for bacterial isolation.

4.2.2 Isolation of colistin-resistant bacteria

A 50 µL aliquot of each fly homogenate was diluted 2-fold with PBS. The total 100 µL was spread on CHROMagar™ COL-APSE (CHROMagar, Paris, France). Plates were incubated at 37 °C for 18 hours. Colonies were selected based on different morphologies, sub-cultured on MacConkey agar supplemented with 3.5 µg/mL colistin sulfate (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37 °C for 18 hours. On the following day, colonies were grown in Lysogeny Broth (LB) supplemented with 3.5 µg/mL colistin sulfate and incubated at 35 °C for 18 hours with shaking at 220 rpm. Bacteria were harvested from a volume of 400 µL of culture by centrifugation at 6,000 g for 1 minute and resuspended in 400 µL PBS to be used for genomic DNA extraction. The remaining cultured bacteria were stored in 80% glycerol at -80 °C.

4.2.3 Extraction of genomic DNA

Genomic DNA extraction of selected bacteria was performed using the High Pure PCR Template Preparation Kit (Roche Diagnostic, Indianapolis, IN, USA) according to the manufacturer's instructions. Specifically, prior to extraction, bacteria were grown in 2 mL Lysogeny Broth at 37°C for 18 hours. Bacteria in 400 µL of the culture were washed once and resuspended in 400 µL PBS and mixed with 400 µL Binding Buffer and 80 µL proteinase K. Lysis was carried out by incubation at 72°C for 20 minutes with shaking at 600 rpm in a thermomixer (Eppendorf, Hamburg, Germany). Two hundred µL isopropanol (Avantor Performance Materials, Center Valley, PA) was added, and the mixture was vortexed thoroughly. The aqueous solution was transferred into the upper reservoir of the filter of the kit inserted into a 2-ml collection tube and centrifuged at 8,000 g for 1 minute. The flow-through liquid was discarded; and Inhibitor Removal Buffer was added to the filter and centrifuged at 8,000 g for 1 minute. The flow-through liquid was discarded; and the filter was washed with 600 µL Washing Buffer and centrifuged at 8,000 g for 1 minute. The washing was repeated with 400 µL and followed by centrifugation at 13,000 g for 1 minute. The filter column was inserted in a new 2-mL collection tube. Nucleic acid was eluted with 50 µL pre-warmed Elution Buffer added to the middle of the filter, incubated at 72°C for 5 minutes, and centrifuged at 8,000 g for 1 minute. The elution step was repeated to increase DNA yields.

4.2.4 Isolation of plasmid DNA

Plasmid DNA was extracted with PureLink™ Quick Plasmid Miniprep Kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Specifically, 2 mL of

cultured bacteria were harvested by centrifugation at 13,000 g for 1 minute. Cells were then resuspended in 250 μ L Resuspension Buffer (R3), lysed by adding 250 μ L Lysis Buffer (L7), and neutralized with 350 μ L Precipitation Buffer (N4). The lysate was centrifuged at 13,000 g for 10 minutes. The supernatant was transferred onto a spin column, which was inserted in a 2-mL collection tube and centrifuged at 13,000 g for 1 minutes. The spin column was washed twice with 700 μ L Wash Buffer (W9); each wash was followed by centrifugation at 13,000 g for 1 minute. The plasmid DNA was eluted with 75 μ L pre-heated TE Buffer added to the center of the column, incubated at room temperature for 1 minute, and centrifuged at 13,000 g for 1 minute.

4.2.5 *16S rRNA*-based bacterial identification

PCR amplification of the whole *16S rRNA* gene was performed with universal primers 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3' as previously described (Weisburg *et al.*,1991). The PCR was performed with an initial denaturation step at 94°C for 3 minutes and thirty cycles of: 1) denaturation at 94°C for 30 seconds, 2) annealing at 56°C for 40 seconds, and 3) extension at 72°C for 1 minute. Finally, an elongation step was performed at 72°C for 5 minutes. Amplicons were sent to ELIM Biopharmaceuticals, Inc. (San Francisco, CA, USA) for purification and Sanger sequencing. The obtained sequences were identified using NCBI Nucleotide Basic Local Alignment Search Tool (BLASTn).

4.2.6 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer agar disk diffusion test against 16 agents: ampicillin, imipenem, amoxicillin-clavulanic acid, cefepime, cefpodoxime, ceftazidime, amikacin, gentamicin, streptomycin, doxycycline, chloramphenicol, ciprofloxacin, polymyxin B, nitrofurantoin, fosfomycin, and trimethoprim-sulfamethoxazole. A single colony from a one-day-old culture on MacConkey agar was suspended in saline to obtain a turbidity equivalent of a 0.5 McFarland Standard; then spread as a uniform layer onto Mueller Hinton agar plate, and allowed to dry. Antibiotic impregnated disks were placed onto the agar surface, and the plates were incubated at 37 °C for 18 hours. Zones of inhibition were read and interpreted as susceptible, intermediate, and resistant according to the Clinical and Laboratory Standards Institute standards (CLSI 2013).

Minimal inhibitory concentration (MIC) of colistin was determined for selected bacterial isolates. A 2-fold serial dilution series (16–0.5 µg/mL) of colistin sulfate was prepared with cation-adjusted Mueller Hinton broth (Millipore Sigma, St. Louis, MO, USA), and 1,980 µL of each concentration was aliquoted in a 15-mL test tube. A negative control of growth medium without bacterial inoculation was included as well as a growth medium without colistin as a positive control. The strains ATCC 25922 *E. coli* and NCTC 13846 *E. coli* were used as susceptible and resistant controls, respectively. A single colony of bacteria from MacConkey agar was inoculated and grown in cation-adjusted Mueller Hinton broth at 37°C for 18 hours. Cultured bacteria were diluted in cation-adjusted Mueller Hinton broth to the optical density at 600 nm (OD₆₀₀) of 1.0 followed by a 10-fold dilution to obtain a final OD₆₀₀ concentration of 0.1. From the diluted bacterial suspension, 20 µL was transferred into 1,980 µL of the medium containing antimicrobials and incubated at 37°C for 24 hours without shaking. Susceptibility was determined by inhibited bacterial growth (lack of turbidity).

4.2.7 Conjugation Assay

Broth mating conjugation assay was performed to determine the transferability of colistin resistance to streptomycin-resistant recipient *E. coli* 4810. Bacterial strains used in the conjugation assay are listed in Table 4.1. Donor bacteria were grown in LB broth supplemented with 2 µg/mL colistin sulfate at 37°C for 18 hours, whereas recipient bacteria were grown in LB broth supplemented with 10 µg/mL streptomycin sulfate (Sigma Aldrich, St. Louis, MO, USA). On the following day, both donor and recipient were washed twice with fresh LB broth and mixed at 1:1, 1:2, 1:3, and 1:4 donor:recipient ratios. The mixtures were diluted 1:10 with LB broth without antibiotic pressure in a total volume of 2 mL and incubated at 37 °C for 18 hours without shaking. The following day, bacteria were harvested by centrifugation at 6,000 rpm for 1 minute, resuspended in 1 mL of fresh LB broth, and spread on LB agar containing 2 µg/mL colistin and 10 µg/mL streptomycin. The plates were incubated at 37 °C for 18 hours; and transconjugants that grew under double antibiotics pressure were selected and sub-cultured on MacConkey agar supplemented with both antibiotics and CHROMagar™ COL-APSE. The colistin MIC for transconjugants was measured as described above.

4.2.8 Whole genome sequencing

The bacterial DNA of the selected isolate was sent to the sequencing provider OE Biotech Co., Limited (Shanghai, China) for third-generation single-molecule real-time (SMRT) sequencing and genome assembly. Briefly, the library was constructed with SMRTbell template prep kit 1.0 (Pacific Biosciences, Menlo Park, CA, USA), and SMRT sequencing of the library was performed on the PacBio Sequel platform. Reads with low quality were removed with

SMRT Analysis 2.3.0 (Pacific Biosciences). Self-correction of sequencing reads and preliminary *de novo* assembly of sequencing data were performed with Falcon software as previously described (Chin *et al.*, 2016). The corrected subreads were used as an input for the single pass read accuracy improver Sprai software 0.9.9.23 (Miyamoto *et al.*, 2014) to correct sequencing errors, and each single DNA sequence contig was circularized by using Circlator software (Hunt *et al.*, 2015). The plasmid replicon type was identified by PlasmidFinder (Carattoli and Hasman, 2020).

4.2.9 Gene predictions and annotations

Predictions of *tRNA*, *rRNA*, and non-coding RNA (*sRNA*) genes were performed with tRNAscan-SE 1.3.1, RNAmmer 1.2, and Rfam 10.0, respectively (Lowe and Eddy, 1997; Griffiths *et al.*, 2003; Lagesen *et al.*, 2007). Repetitive DNA sequences were detected with RepeatMasker 4.0.7 (Tarailo-Gravovac and Chen, 2009). PILER-CR 1.06 and CRT1.2-CLI were used to predict CRISPR sequences (Edgar, 2007; Bland *et al.*, 2007). Prediction of prophages was performed with PhiSpy 2.3 (Akhter *et al.*, 2012). Prediction of coding sequences in the assembled genome was performed with Prokaryotic Dynamic Programming Genefinding Algorithm (Prodigal) 2.6.3 software (Hyatt *et al.*, 2010). *In silico* MLST prediction based on allelic numbers of 7 housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) was performed with MLST 2.0 (Larsen *et al.*, 2012). Serotypes of O- and K-antigens were predicted based on genetic components of *rfb* and *cps* loci, respectively, with using Kaptive-Web database (Wick *et al.*, 2018).

Predicted genes were annotated for functional classification as well as pathogenesis and antimicrobial resistance by using DIAMOND software (Buchfink *et al.*, 2015) against various

databases. First, genes were annotated against NCBI Non-Redundant Protein Database (NCBI NR) and Protein Knowledgebase (Swiss-Prot) for general annotations with a threshold e-value of $1e-5$ (Boutet *et al.*, 2007; Tatusova *et al.*, 2016). Second, genes were functionally classified with Clusters of Orthologous Groups (COG) database with a cutoff e-value of $1e-5$ (Tatusov *et al.*, 2000). In addition, functional and cellular pathways were predicted with using Kyoto Encyclopedia of Genes and Genomes (KEGG) with a cutoff e-value of $1e-5$ (Kanehisa and Goto, 2000). Third, annotations for bacterial pathogenicity were performed against Pathogen-Host Interactions (PHI) and Virulence Factors of Pathogenic Bacteria (VFDB) databases with a threshold e-value of $1e-10$ (Chen *et al.*, 2005; Winnenburg *et al.*, 2006). Finally, screening for antimicrobial resistance genes was performed with Comprehensive Antibiotic Resistance Genes Database (CARD) with a threshold e-value of $1e-10$ (McArthur *et al.*, 2013). A comprehensive display of the genomic circular map was created using Circos 0.69 (Krzywinski *et al.*, 2009).

4.3 Results

4.3.1 Colistin-resistant bacteria in flies

A total of 255 flies were trapped in this study. Prior to homogenization, pools of five flies each were established and each pool assigned a designated ID (AU/42 – AU/91). From the 40 pools of flies, 485 colistin-resistant bacterial isolates were recovered, and 89 of the isolates were selected based on their morphologies for *16S rRNA*-based identification. The majority of those bacterial isolates was identified as *Providencia* spp. (n=57), followed by *Serratia* spp. (n=15), *Enterobacter* spp. (n=6), *Klebsiella* spp. (n=5), *Proteus* spp. (n=3), *Hafnia alvei* (n=1), *Kluyvera georgiana* (n=1), and *Pantoea ananatis* (n=1).

Phenotypic antimicrobial resistance profiles of selected *Enterobacter* spp., all *Klebsiella* spp., *Hafnia alvei*, *Kluyvera georgiana*, and *Pantoea ananatis* were determined with the disk-diffusion method. The tested *Enterobacter* spp., *Hafnia alvei*, and *Pantoea ananatis* were susceptible to all antimicrobial agents in the study. *Klebsiella* species were all resistant to ampicillin due to intrinsic resistance. *K. pneumoniae* 7401 was resistant to trimethoprim-sulfamethoxazole and exhibited intermediate resistance to doxycycline. *Kluyvera georgiana* showed resistance against ampicillin, clavulanic acid, doxycycline, and chloramphenicol and intermediate resistance activity against streptomycin and ciprofloxacin (Table 4.2).

The minimal inhibitory concentration of colistin on the isolates showed variability even between the same bacterial species (Table 4.2). For example, *K. pneumoniae* 8601 and 9002 had higher resistance (16 µg/mL) than 7401 and 8701 (4 µg/mL), while *K. variicola* resistance to colistin was the highest among *Klebsiella* species with 256 µg/mL. *Enterobacter* species also showed variability in resistance with greater than 256 µg/mL for *E. asburiae* and 128 µg/mL for

E. cloacae. Finally, *Pantoea ananatis*, *Kluyvera georgiana*, and *Hafnia alvei* were resistant to colistin at concentrations of 4 µg/mL.

4.3.2 Transferability of colistin resistance

Plasmids of *Klebsiella pneumoniae* (7401, 8601, 8701, 9002), *Klebsiella variicola* (8501), and *E. coli* 4801 (recipient) were extracted and run on a FlashGel™ DNA Cassette, 1.2% agarose (Lonza Group AG, Basel, Switzerland). *K. pneumoniae* 9002 and *E. coli* 4810 did not show any bands, while each of *K. pneumoniae* 7401 and 8601 and *K. variicola* 8501 had one band (Figure 4.1). The transferability of colistin resistance was successful only with donor *K. pneumoniae* 8701. The MIC of colistin for transconjugants was measured at 4 µg/mL. In order to confirm the success of transferability of colistin resistance from *K. pneumoniae* to *E. coli*, transconjugants were cultured on CHROMagar™ COL-APSE, on which they grew as typical *E. coli* small pink colonies (Figure 4.2). The genomic DNA of *K. pneumoniae* 8701 was sent to the sequencing company for WGS and genome-based further analysis.

4.3.3 Genome features of Kp8701

SMRT sequencing of *K. pneumoniae* 8701 resulted in three datasets with roughly 3.65 Gb total bases available for *de novo* assembly (Table 4.3). The bases created 397,836 total reads with a minimum contig length of 51 bp and a maximum length of 70,234 bp (average length of 9,162 bp). Assuming an average genome size of 5 Mb for *K. pneumoniae*, the total bases obtained from SMRT sequencing theoretically had 730-fold coverage of the genome.

The *de novo* assembly of the genome of *K. pneumoniae* 8701 consisted of two contigs: one contig belonged to the chromosome with a size of 5,337,408 bp and a GC content of 57.4%,

and the other contig belonged to a conjugative plasmid with an IncFIB (K) replicon type, size of 224,442 bp, and a GC content of 52.4% (Table 4.3).

The chromosomal genome was predicted to contain 89 *tRNA* genes encoding all 21 amino acids as well as selenocysteine. There were 25 genes predicted for *rRNA* biogenesis consisting of 9 genes for *5S rRNA*, 8 for *16S rRNA*, and 8 for *23S rRNA*. Six genes were predicted as non-coding RNA's (sRNA). Repetitive DNA sequences were found to represent 0.8% of the chromosome with 465 sequences. Eleven repeated sequences were found in the plasmid, representing 0.3% of the total length of the plasmid (Table 4.4). Sequences for CRISPR were not found in the chromosomal or plasmid genome.

The chromosome harbors 4,944 protein-coding sequences representing 88.1% of the whole chromosome with a gene length of 951.2 bp on average. On the other hand, the plasmid contained 245 protein-coding sequences representing 82.7% with an average gene length of 757.89 bp. There was a 53.2 kb prophage region found in the plasmid containing 68 genes. Comprehensive circular genome maps of the chromosome and plasmid are shown in Figure 4.3.

In silico MLST of Kp8701 showed that the isolate belongs to ST-784 with 100% identity to detected alleles (Table 4.5). O- and K-antigens serotypes were predicted to be O1v2 and KL146 with 98.4% and 95.2% similarity to reference *rfb* and *cps* loci (100% coverage for both).

4.3.4 Functional analysis of Kp8701 genome

A total of 4,204 protein-coding sequences in the chromosome were functionally classified based on COG into 21 out of 25 COG functional groups (Table 4.6). The four functional classes that were not found in the genome were chromatin structure (B), extracellular structure (W), nuclear structure (Y), and cytoskeleton (Z). The most abundant genes were classified in Class E

for amino acid transport and metabolism and Class G for carbohydrate transport and metabolism with 14.1% and 13.3%, respectively, while the least abundant class was in class A for RNA processing and modification with one gene in the chromosome (0.02%).

Plasmid genes were classified in 17 COG functional classes (Table 4.6). Class L for genes involved in replication, recombination and repair showed higher relative abundance, with 39 genes out of 139 being classified. As expected, genes for biogenesis of essential metabolism components were found least in the plasmid.

In order to understand cellular pathways, a total of 3,258 genes in the chromosome and 44 genes in the plasmid were annotated and classified based on reference genomes in the KEGG database into four categories: 1) metabolism; 2) environmental information processing; 3) genetic information processing; and 4) cellular community. The most abundant genes in the chromosome were found to be involved in membrane transport under the KEGG category of environmental information processing, with 385 genes (14.4%) followed by carbohydrate metabolism with 383 genes (14.3%) (Figure 4.4). Similarly, membrane transport genes were found mostly in the plasmid, with 14 genes (35.9%) followed by 8 genes involved in signal transduction (20.5%) (Figure 4.5).

4.3.5 Antimicrobial resistance determinants in Kp8701

Antimicrobial resistance genes were detected by annotating the genome against the CARD database. The plasmid was found to harbor only *vgaC*, which encodes an efflux protein that confers resistance to streptogramin A. In the chromosome, 12 genes were predicted to contribute to antimicrobial resistance with different mechanisms including efflux biogenesis (n = 9), antibiotics modification (n = 2), and reduced permeability (n = 1). Four efflux pumps were

predicted in the genome including two belonging to major facilitator superfamily (KpnGH and KpnEF) and two of resistance-nodulation-division (AdeFGH and OqxAB). Six genes encoding resistance transcriptional regulators of efflux genes were found in the genome (*crp*, *marA*, *marR*, *emrR*, *baeR*, and *hns*). Two antibiotic modifying enzymes were predicted in the genome to be encoded by *bla_{SHV-1}* and *fosA6* conferring resistance to ampicillin and fosfomycin. Finally, the *ompC* allele that encodes narrow-pore OmpK37 porin was found in the genome theoretically leading to resistance against β -lactams via reducing the uptake of drugs through passive diffusion (Doménech-Sánchez *et al.*, 1999).

Genes that are involved in resistance to polycationic compounds including colistin (*arn*, and *pmrC*) were found in the genome. Wild-type genes of colistin resistance-associated two-component regulatory systems (*pmrAB*, *phoPQ*, *crrAB*) and *mgrB* were found in the genome.

4.3.6 Virulence factors in Kp8701

There are a total of 81 genes that were predicted to be involved in biogenesis and regulation of virulence factors in Kp8701, including biosynthesis of type 3 and type 1 fimbriae, biosynthesis of capsular polysaccharide, iron uptake (enterobactin siderophores and salmochelin siderophores), AcrAB efflux system, type VI secretion system, and the virulence-associated RcsAB two-component regulatory system (Table 4.7).

4.4 Discussion

Female flies associate with microbe-rich organic materials such as manure for reproductive purposes. Upon exposure to animal wastes, flies can be involved in transmission of bacterial pathogens that they acquire and may play a role in dissemination of bacteria due to their unrestricted movements between different habitats and ability to travel long distances. More importantly, it is likely that flies can also spread antimicrobial resistance between animals and humans due to ubiquity of antimicrobial resistant bacteria in animal waste. Moreover, the gut of flies provides a suitable environment to harbor bacteria carrying antimicrobial resistance genes and for horizontal gene transfer. Colistin is the last-resort drug used to treat carbapenem-resistant Gram-negative bacteria. Resistance to colistin increases the fear of losing our battle against pan-drug resistant bacteria. Several reports and articles have discussed colistin resistance in clinical isolates, but the environmental link to the emergence and spread of the resistance is still lacking (Vakili *et al.*, 2014; Pormohammad *et al.*, 2020). In this study, 485 colistin-resistant bacteria were recovered from flies trapped at the trash collection area in Auburn, AL. Eighty nine isolates were subjected to *16S rRNA*-based bacterial identification. *Providencia* species were the most prevalent colistin-resistant bacteria (64.0%) followed by *Serratia* spp. (16.9%), *Enterobacter* spp. (6.7%), and *Klebsiella* spp. (5.6%).

Minimal inhibitory concentration of colistin was measured on bacteria that do not possess naturally intrinsic resistance. The MIC of colistin on *Klebsiella*, *Enterobacter*, *Hafnia alvei*, *Kluyvera georgiana*, and *Pantoea ananatis* showed variability in level of resistance, probably due to several factors. Although colistin resistance in Gram-negative bacteria is mainly caused by remodeling of the lipid A component of LPS, a higher resistance level in the same species can be caused by a complete loss of lipid A (Moffatt *et al.*, 2010), presence of more than one

mutation in genes encoding regulators such as *pmrB* leading to higher constitutive expression of *pmrC* (Moskowitz *et al.*, 2012; Huang *et al.*, 2019), or mutations in both regulators *pmrA* and *phoP* resulting in co-addition of pEtN and Ara4N to lipid A phosphates through PmrC and ArnA (Gutu *et al.*, 2013).

It was important to investigate whether the resistance was chromosomally-encoded or plasmid-mediated in our isolates. Transfer of phenotypic resistance was observed with *K. pneumoniae* 8701 as a donor to the recipient *E. coli* 4810. FRET-qPCR detection of *mcr* genes in Kp8701 was negative (data not shown). The genomic DNA of *K. pneumoniae* was sent for whole genome sequencing for full investigation. None of the chromosomal genes encoding colistin resistance mechanisms had mutations leading to overexpression of LPS-modifying genes including *arn*, *pmr*, and *pho* operons. Molecular experiments are indeed needed to understand the mechanism of resistance and the phenomenon of transferable colistin resistance. However, those experiments could not be envisioned in this study because of the complexity and large number of genes that are known to be involved in LPS biosynthesis and remodeling. Colistin resistance-associated genes were detected in the recipient genome (data not shown). Therefore, a novel regulator of those operons might have been harbored in the plasmid leading to overexpression of LPS-modifying genes. Another possible explanation of the phenotype, based on the genome sequences, is the presence of efflux systems. However, none of the known genes encoding efflux pumps that confer colistin resistance were found in the plasmid. A novel efflux system or a transcriptional regulator for expression of efflux system genes might be in the plasmid, which confers resistance against colistin in *K. pneumoniae* and transconjugants *E. coli*. The genome of Kp8701 encodes 4 efflux pumps that may contribute to a multidrug resistance phenotype. However, the antimicrobial susceptibility profile of the isolate shows that Kp8701 is resistant

only to colistin as well as intrinsic ampicillin resistance due to SHV-1. Regulation of expression and biosynthesis of efflux systems are complex processes that need further investigation, which is beyond the scope of this study.

The WGS-based MLST of *K. pneumoniae* 8701 classified the organism as ST784 *K. pneumoniae*, which has not been reported in clinical infections of humans and animals. The O antigen of Kp8701 was *in silico*-typed as O1 serotype, which is one of the most frequently isolated serotypes in clinical isolates (Hansen *et al.*, 1999). Although Kp8701 harbors capsular type KL146, which has not been clinically reported, the genome contains the regulator gene *magA* (Table 3.7). Capsular polysaccharide and MagA contribute to *K. pneumoniae* hypervirulence by mucoviscosity phenotype, which provides resistance to phagocytosis (Fang *et al.*, 2004; Chuang *et al.*, 2006). In addition, the pathogenicity of *K. pneumoniae* strains is caused by other multiple virulence factors including fimbrial biosynthesis contributing to adhesion and siderophore biosynthesis (Russo and Marr, 2019). Genes involved in biogenesis and regulation of virulence factors were found in the genome of *K. pneumoniae* 8701.

In recent years, several articles have discussed and highlighted colistin resistance in human and animal infections, but information is still scarce regarding mechanical and biological vectors. The findings in this work indicate that flies can be an important environmental link to spread of colistin-resistant bacteria with clinical importance including naturally resistant organisms such as *Providencia*, *Proteus*, and *Serratia*, which certainly results in health threats for human and animals.

4.5 Genome announcement

Raw sequencing reads of *K. pneumoniae* 8701 were deposited in the Sequence Read Archive (SRA) under accession number SRR11212804 (BioProject number PRJNA609348). The *de novo* genome assembly of the chromosome and plasmid was deposited in GenBank under accession numbers CP049604 and CP049605, respectively.

References

- Agga, G., Arthur, T., Durso, L., Harhay, D., and Schmidt, J. (2015) Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. *PLoS One* **10**: e0132586.
- Akhtar, M., Hirt, H., and Zurek, L. (2009) Horizontal transfer of the tetracycline resistance gene *tetM* mediated by pCF10 among *Enterococcus faecalis* in the house fly (*Musca domestica* L.) alimentary canal. *Microb Ecol* **58**: 509–518.
- Akhter, S., Aziz, R., and Edwards, R. (2012) PhiSpy: a novel algorithm for finding prophages in bacterial genomes that combines similarity-and composition-based strategies. *Nucleic Acids Res* **40**: e126–e126.
- Argudín, M., Deplano, A., Meghraoui, A., Dodémont, M., Heinrichs, A., Denis, O., et al. (2017). Bacteria from animals as a pool of antimicrobial resistance genes. *Antibiotics (Basel, Switzerland)* **6**: 12.
- Bergen, P., Li, J., Rayner, C., and Nation, R. (2006) Colistin methanesulfonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **50**: 1953–1958.
- Bland, C., Ramsey, T., Sabree, F., Lowe, M., Brown, K., Kyrpides, N., and Hugenholtz, P. (2007) CRISPR recognition tool (CRT): a tool for automatic detection of clustered regularly interspaced palindromic repeats. *BMC Bioinformatics* **8**: 209.
- Boutet, E., Lieberherr, D., Tognolli, M., Schneider, M., and Bairoch, A. (2007) UniProtKB/Swiss-Prot. *Methods Mol Biol* **406**: 89–112.
- Buchfink, B., Xie C., and Huson, D. (2015) Fast and sensitive protein alignment using DIAMOND. *Nat Methods* **12**: 59–60.

- Carattoli, A. and Hasman, H. (2020) PlasmidFinder and In Silico pMLST: Identification and Typing of Plasmid Replicons in Whole-Genome Sequencing (WGS). *Methods Mol Biol* **2075**: 285–294.
- Chen, L., Yang, J., Yu, J., Yao, Z., Sun, L., Shen, Y., and Jin, Q. (2005) VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res* **33**: D325–D328.
- Chin, C., Peluso, P., Sedlazeck, F., Nattestad, M., Concepcion, G., Clum, A. et al. (2016) Phased diploid genome assembly with single-molecule real-time sequencing. *Nat Methods* **13**: 1050.
- Chuang, Y., Fang, C., Lai, S., Chang, S., and Wang, J. (2006) Genetic determinants of capsular serotype K1 of *Klebsiella pneumoniae* causing primary pyogenic liver abscess. *J Infect Dis* **193**: 645–654.
- Doménech-Sánchez, A., Hernández-Allés, S., Martínez-Martínez, L., Benedí V., and Albertí, S. (1999) Identification and characterization of a new porin gene of *Klebsiella pneumoniae*: its role in beta-lactam antibiotic resistance. *J Bacteriol* **181**: 2726-2732.
- Edgar, R. (2007) PILER-CR: fast and accurate identification of CRISPR repeats. *BMC Bioinformatics* **8**: 18.
- Fang, C., Chuang, Y., Shun, C., Chang, S., and Wang, J. (2004) A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J Exp Med* **199**: 697–705.
- Fukuda, A., Usui, M., Okubo, T., and Tamura, Y. (2016) Horizontal transfer of plasmid-mediated cephalosporin resistance genes in the intestine of houseflies (*Musca domestica*). *Microb Drug Resist* **22**: 336–341.

- Griffiths-Jones, S., Bateman, A., Marshall, M., Khanna, A., and Eddy, S. (2003) Rfam: an RNA family database. *Nucleic Acids Res* **31**: 439–441.
- Gunn, J. (2008) The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends Microbiol* **16**: 284–290.
- Gutu, A., Sgambati, N., Strasbourger, P., Brannon, M., Jacobs, M., Haugen, E. et al. (2013) Polymyxin resistance of *Pseudomonas aeruginosa* *phoQ* mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother* **57**: 2204–2215.
- Hansen, D., Mestre, F., Alberti, S., Hernandez-Alles, S., Alvarez, D., Doménech-Sánchez, A., et al. (1999) *Klebsiella pneumoniae* lipopolysaccharide O typing: revision of prototype strains and O-group distribution among clinical isolates from different sources and countries. *J Clin Microbiol* **37**: 56-62.
- Hayakawa, K., Marchaim, D., Divine, G., Pogue, J., Kumar, S., Lephart, P. et al. (2012) Growing prevalence of *Providencia stuartii* associated with the increased usage of colistin at a tertiary health care center. *Int J Infect Dis* **16**: e646–e648.
- Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., and Coukell, A. (2017) Antimicrobial drug use in food-producing animals and associated human health risks: What, and how strong, is the evidence? *BMC Vet Res* **13**: 211.
- Huang, J., Dai, X., Ge, L., Shafiq, M., Shah, J., Sun, J. et al. (2019) Sequence duplication within *pmrB* gene contribute to high-level colistin resistance in avian pathogenic *Escherichia coli*. *Microb Drug Resist* [abstract].
- Hunt, M., Silva, N., Otto, T., Parkhill, J., Keane, J., and Harris, S. (2015) Circlator: automated circularization of genome assemblies using long sequencing reads. *Genome Biol* **16**: 294.

- Hyatt, D., Chen, G., LoCascio, P., Land, M., Larimer, F., and Hauser, L. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC bioinformatics* **11**: 119.
- Kanehisa, M. and Goto, S. (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* **28**: 27–30.
- Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R. et al. (2009). Circos: an information aesthetic for comparative genomics. *Genome Res* **19**:1639–1645.
- Lagesen, K., Hallin, P., Rødland, E., Staerfeldt, H., Rognes, T., and Ussery, D. (2007) RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res* **35**: 3100–3108.
- Larsen, M., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R., et al. (2012) Multilocus Sequence Typing of Total Genome Sequenced Bacteria. *J. Clin. Microbiol.* **50**: 1355-1361.
- Liu, Y., Wang, Y., Walsh, T., Yi, L., Zhang, R., Spencer, J. et al. (2016) Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* **16**: 161–168.
- Lowe, T. and Eddy, S. (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* **25**: 955–964.
- McArthur, A., Wagleichner, N., Nizam, F., Yan, A., Azad, M., Baylay, A. et al. (2013) The Comprehensive Antibiotic Resistance Database. *Antimicrob Agents Chemother* **57**: 3348–3357.

- Merkier, A., Rodriguez, M., Togneri, A., Brengi, S., Osuna, C., Pichel, M. et al. (2013) Outbreak of a cluster with epidemic behavior due to *Serratia marcescens* after colistin administration in a hospital setting. *J Clin Microbiol* **51**: 2295–2302.
- Miyamoto, M., Motooka, D., Gotoh, K., Imai, T., Yoshitake, K., Goto, N. et al. (2014) Performance comparison of second- and third-generation sequencers using a bacterial genome with two chromosomes. *BMC Genomics* **15**: 699.
- Moffatt, J., Harper, M., Harrison, P., Hale, J., Vinogradov, E., Seemann, T. et al. (2010) Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother* **54**: 4971–4977.
- Moskowitz, S., Brannon, M., Dasgupta, N., Pier, M., Sgambati, N., Miller, A. et al. (2012) PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother* **56**: 1019–1030.
- Nazni, W., Luke, H., Wan Rozita, W., Abdullah, A., Sa'diyah, I., Azahari, A. et al. (2005) Determination of the flight range and dispersal of the house fly, *Musca domestica* (L.) using mark release recapture technique. *Trop Biomed* **22**: 53–61.
- Nordmann, P., Cuzon, G., and Naas, T. (2009) The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis* **9**: 228–236.
- Perez, L. (2015) Evaluation of polymyxin susceptibility profile among KPC-producing *Klebsiella pneumoniae* using Etest and MicroScan WalkAway automated system. *APMIS* **123**: 951–954.
- Poirel, L., Jayol, A., and Nordmann, P. (2017) Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* **30**: 557–596.

- Pormohammad, A., Mehdinejadani, K., Gholizadeh, P., Nasiri, M., Mohtavinejad, N., Dadashi, M., et al. (2020) Global prevalence of colistin resistance in clinical isolates of *Acinetobacter baumannii*: a systematic review and meta-analysis. *Microb Pathog* **139**: 103887.
- Rhouma, M., Beaudry, F., and Letellier, A. Resistance to colistin: what is the fate for this antibiotic in pig production? *Int J Antimicrob Agents* **48**:119–126.
- Russo, T. and Marr, C. (2019) Hypervirulent *Klebsiella pneumoniae*. *Clin Microbiol Rev* **32**: e00001-19.
- Samonis, G., Korbila, I., Maraki, S., Michailidou, I., Vardakas, K., Kofteridis, D. et al. (2014) Trends of isolation of intrinsically resistant to colistin *Enterobacteriaceae* and association with colistin use in a tertiary hospital. *Eur J Clin Microbiol Infect Dis* **33**: 1505–1510.
- Schaumburg, F., Onwugamba, F., Akulenko, R., Peters, G., Mellmann, A., Köck, R., and Becker, K. (2016) A geospatial analysis of flies and the spread of antimicrobial-resistant bacteria. *Int J Med Microbiol* **306**: 566–571.
- Sutherland C. and Nicolau D. (2015) Susceptibility profile of ceftolozane/tazobactam and other parenteral antimicrobials against *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* From US Hospitals. *Clin The* **37**: 1564–1571.
- Tarailo-Graovac, M. and Chen N. (2009) Using RepeatMasker to identify repetitive elements in genomic sequences. *Curr Protoc Bioinformatics* **4**: Unit 4.10.
- Tatusov, R., Galperin, M., Natale, D., and Koonina E. (2000) The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res* **28**: 33–36.
- Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E., Zaslavsky, L. et al. (2016) NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res* **44**: 6614–6624.

- U.S. Centers for Disease Control and Prevention (2019) Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA, USA.
- Vakili, B., Fazeli, H., Shoaee, P., Yaran, M., Ataei, B., Khorvash, F., and Khaleghi, M. (2014) Detection of colistin sensitivity in clinical isolates of *Acinetobacter baumannii* in Iran. *J Res Med Sci* **19**: S67–S70.
- Walkty, A., DeCorby, M., Nichol, K., Karlowsky, J., Hoban, D., and Zhanel, G. (2009) In vitro activity of colistin (polymyxin E) against 3,480 isolates of Gram-negative bacilli obtained from patients in Canadian hospitals in the CANWARD study, 2007-2008. *Antimicrob Agents Chemother* **53**: 4924–4926.
- Weisburg, W., Barns, S., Pelletier D., and Lane, D. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.
- West L. (1951). The housefly. Its natural history, medical importance, and control. Comstock Publishing Co. Inc., Ithaca, NY.
- Wick, R., Heinz, E., Holt, K., and Wyres, K. (2018) Kaptive Web: User-Friendly Capsule and Lipopolysaccharide Serotype Prediction for *Klebsiella* Genomes. *J Clin Microbiol* **56**: e00197-18.
- Winnenburg, R., Baldwin, T., Urban, M., Rawlings, C., Köhler J., and Hammond-Kosack, K. (2006) PHI-base: a new database for pathogen host interactions. *Nucleic Acids Res* **34**: D459–D464.
- Winpisinger, K., Ferketich, A., Berry, R., and Moeschberger, M. (2005) Spread of *Musca domestica* (Diptera: muscidae), from two caged layer facilities to neighboring residences in rural Ohio. *J Med Entomol* **42**: 732–738.

- Yan, A., Guan, Z., and Raetz, C. (2007) An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in *Escherichia coli*. *J Biol Chem* **282**: 36077–36089.
- Yang, Q., Tansawai, U., Andrey, D., Wang, S., Wang, Y., Sands, K. et al. (2019) Environmental dissemination of *mcr-1* positive *Enterobacteriaceae* by *Chrysomya* spp. (common blowfly): An increasing public health risk. *Environ Int* **122**: 281–290.
- Zhang, J., Wang, J., Chen, L., Yassin, A., Kelly, P., Butaye, P. et al. (2018) Housefly (*Musca domestica*) and blow fly (*Protophormia terraenovae*) as vectors of bacteria carrying colistin resistance genes. *Appl Environ Microbiol* **84**: pii: e01736–17.
- Zurek, L. and Ghosh, A. (2014) Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. *Appl Environ Microbiol* **80**: 3562–3567.

Table 4.1. Bacterial strains used in conjugation assay

Isolate	Phenotype	Genotype	Source
Donor			
<i>K. pneumoniae</i> 7401	COL ^R	Unknown	This study
<i>K. pneumoniae</i> 8601	COL ^R	Unknown	This study
<i>K. pneumoniae</i> 8501	COL ^R	Unknown	This study
<i>K. variicola</i> 8501	COL ^R	Unknown	This study
<i>E. coli</i> 13846 *	COL ^R	<i>mcr-1</i>	NCTC
Recipient			
<i>E. coli</i> 4810	STR ^R		Wang lab (2017)

* *mcr-1* positive control;

COL: colistin;

STR: streptomycin;

NCTC: National Collection of Type Cultures.

Table 4.2. Antimicrobial Susceptibility profiles of colistin-resistant isolates

Lab ID	Species	AMP	IPM	AMC	FEP	CAZ	CPD	AMK	GEN	STR	DOX	CHL	CIP	PMB	NIT	FOF	SXT	COL µg/mL
4201	<i>Enterobacter asburiae</i>	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	≥256
4210	<i>Enterobacter asburiae</i>	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	≥256
6515	<i>Enterobacter cloacae</i>	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	128
6516	<i>Enterobacter cloacae</i>	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	128
8911	<i>Hafnia alvei</i>	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	4
7401	<i>K. pneumoniae</i>	R*	S	S	S	S	S	S	S	S	I	S	S	S	S	S	R	4
8601	<i>K. pneumoniae</i>	R*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	16
8701	<i>K. pneumoniae</i>	R*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	4
9002	<i>K. pneumoniae</i>	R*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	16
8501	<i>Klebsiella variicola</i>	R*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	256
7501	<i>Kluyvera georgiana</i>	R	S	R	S	S	S	S	S	I	R	R	I	S	S	S	S	4
7503	<i>Pantoea ananatis</i>	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	4

* Intrinsic resistance;

AMP: ampicillin; IMP: imipenem; AMC: amoxicillin-clavulanic acid; FEP: cefepime; CAZ: ceftazidime; CPD: cefpodoxime; AMK: amikacin; GEN: gentamycin; STR: streptomycin; DOX: doxycycline; CHL: chloramphenicol; CIP: ciprofloxacin; PMB: polymyxin B; NIT: nitrofurantoin; FOF: fosfomicin; SXT: trimethoprim-sulfamethazole; col: colistin;

S: susceptible;

I: intermediate;

R: resistant.

Table 4.3. Statistics of genome sequencing of Kp8701

Total bases (Gb)	3.65	
Total reads	397,836	
Minimum length (bp)	51	
Maximum length (bp)	70,234	
Mean length (bp)	9,162	
Contigs (n)	2	
Length (bp)	Chromosome	5,337,408
	Plasmid	224,442
GC %	Chromosome	57.42
	Plasmid	52.37

Gb: giga base ($\times 10^9$ bases);
bp: base pair;
n: number after *de novo* assembly

Table 4.4. Repetitive DNA sequences in the genome of Kp8701

Class	Chromosome			Plasmid		
	Number	Length	Percentage	Number	Length	Percentage
Interspersed repeats	63	4610	0.09	2	192	0.09
Small RNA	63	15,797	0.3	0	0	0
Satellites	0	0	0	0	0	0
Simple repeats	329	14,177	0.27	9	686	0.31
Low complexity	10	487	0.01	0	0	0
Total	465	35,071	0.76	11	686	0.31

Table 4.5. WGS-based MLST analysis

Locus	Allele	Allele Length (bp)	Alignment (%)
<i>gapA</i>	2	450	100
<i>infB</i>	1	318	100
<i>mdh</i>	1	477	100
<i>pgi</i>	1	432	100
<i>phoE</i>	27	420	100
<i>rpoB</i>	5	501	100
<i>tonB</i>	4	414	100

Table 4.6. COG functional classification

Category/class	Genes (n)	
	Chromosome	Plasmid
Cellular processing and signaling	814	29
[D] Cell cycle control, cell division, and chromosome partitioning	35	3
[M] Cell wall/membrane/envelope biogenesis	236	6
[N] Cell motility	55	0
[O] Post-translational modification, protein turnover, and chaperones	156	3
[T] Signal transduction mechanisms	172	13
[U] Intracellular trafficking, secretion, and vesicular transport	102	3
[V] Defense mechanisms	58	1
[W] Extracellular structures	0	0
[Y] Nuclear structure	0	0
[Z] Cytoskeleton	0	0
Information storage and processing	773	57
[A] RNA processing and modification	1	0
[B] Chromatin structure and dynamics	0	0
[J] Translation, ribosomal structure and biogenesis	128	0
[K] Transcription	459	18
[L] Replication, recombination and repair	185	39
Metabolism	2,378	58
[C] Energy production and conversion	304	1
[E] Amino acid transport and metabolism	591	24
[F] Nucleotide transport and metabolism	94	1
[G] Carbohydrate transport and metabolism	559	8
[H] Coenzyme transport and metabolism	187	1
[I] Lipid transport and metabolism	128	0
[P] Inorganic ion transport and metabolism	392	21
[Q] Secondary metabolites biosynthesis, transport, and catabolism	123	2
Poorly characterized	1,032	19
[R] General function prediction only	653	13
[S] Function unknown	379	6

Table 4.7. Virulence genes identified in Kp8701

Virulence class	Virulence factor	Gene(s)
Adherence	type 3 fimbriae	<i>mrk</i>
	type 1 fimbriae	<i>fim</i>
Antiphagocytosis	capsule	<i>cps</i> locus, <i>magA</i>
Efflux	AcrAB efflux	<i>acrAB</i>
Iron uptake	enterobactin siderophores	<i>ent</i> , <i>fep</i> , <i>fes</i>
	salmochelinsiderophores	<i>iroE</i> , <i>iroN</i>
Secretion systems	type VI secretion system-I	<i>tss</i> locus (12 genes), <i>ompA</i> , <i>tle</i> , <i>tli</i>
	type VI secretion system-III	<i>dotU</i> , <i>icmF</i> , <i>impA</i> , <i>impF</i> , <i>impG</i> , <i>impH</i> , <i>impJ</i> , <i>lysM</i> , <i>ompA</i> , <i>sciN</i> , <i>vgrG</i> .

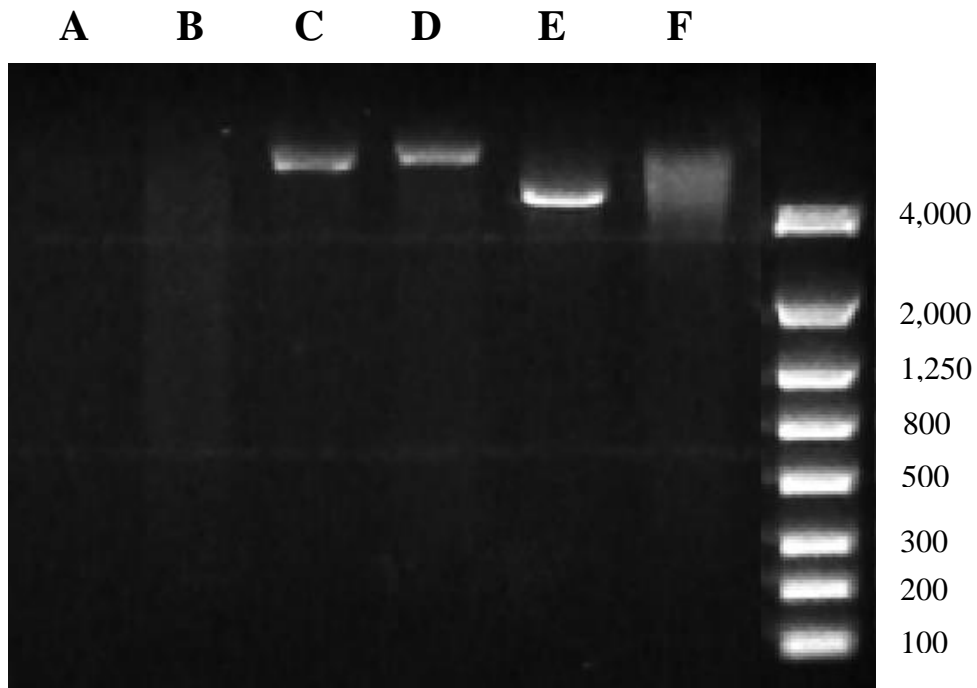


Figure 4.1. Electrophoresis of plasmids used in conjugation assay. Plasmids of *E. coli* 4810 (A), *K. pneumoniae* 9002 (B), *K. pneumoniae* 8701 (C), *K. pneumoniae* 8601 (D), *K. variicola* 8501 (E), *K. pneumoniae* 7401 (F), and FlashGel™ DNA marker (Lonza, Cat. 50473).

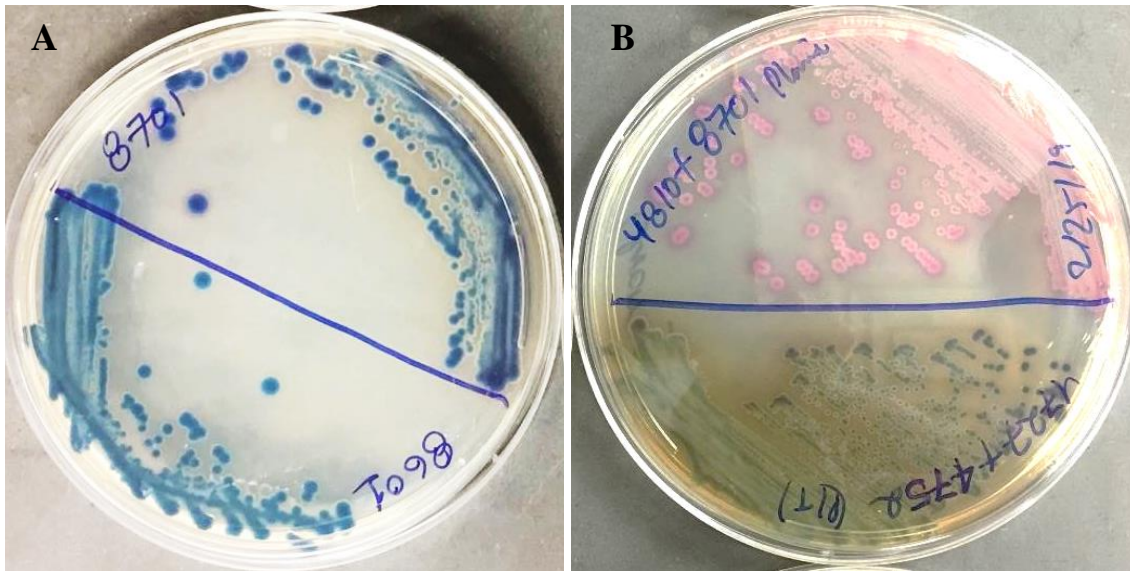


Figure 4.2. Morphology of *K. pneumoniae* 8701 (A) and trans-conjugant (B). The morphology of coliforms including *Klebsiella* on CHROMagar™ COL-APSE is metallic blue, while *E. coli* grows as pink colonies. Ability of trans-conjugants to grow on the medium indicates the success of conjugation.

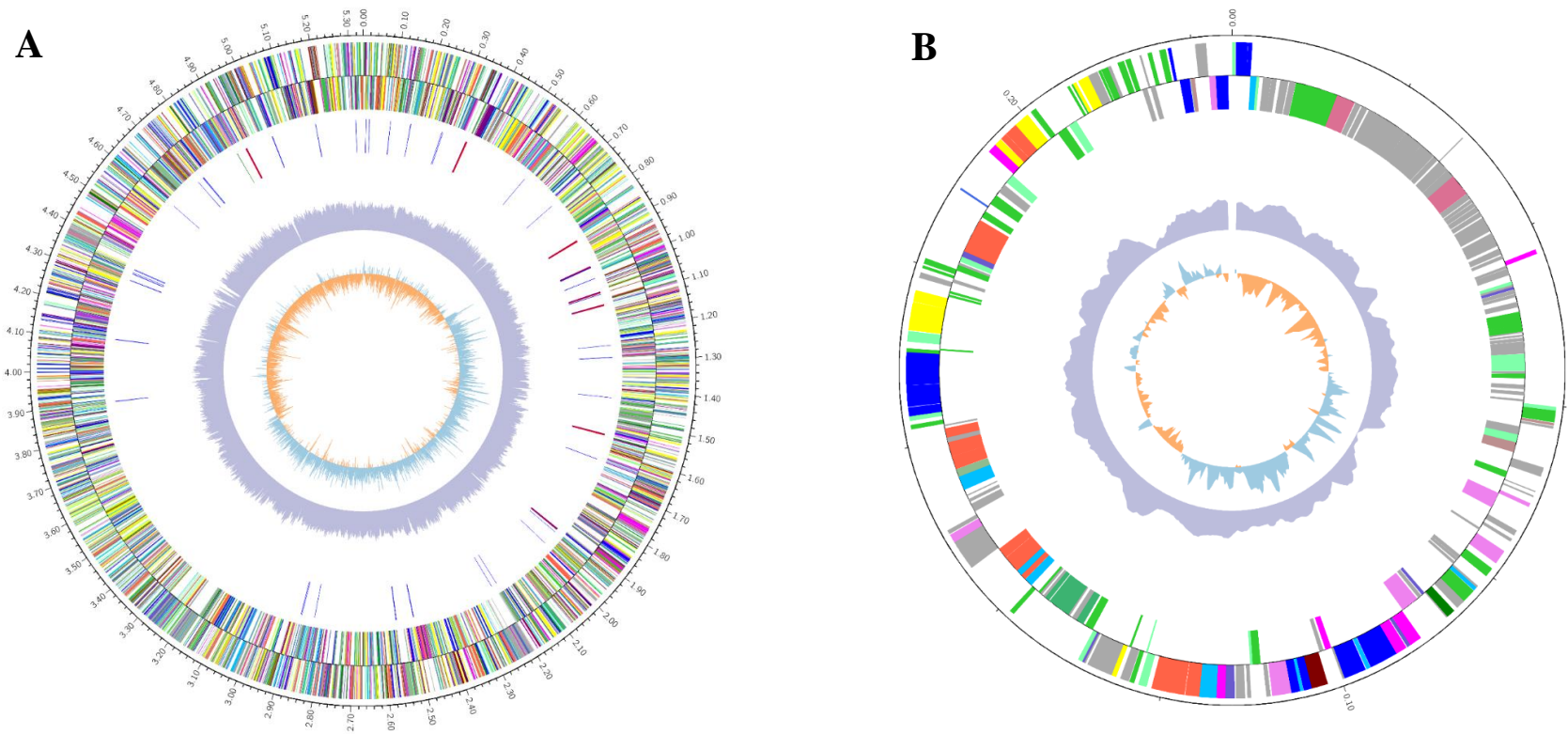


Figure 4.3. *K. pneumoniae* 8701 comprehensive genome circular maps of chromosome (A) and plasmid (B). The map was created using circos 0.69. The two inner rings display the GC skew and GC content from inside to outside. The inside outer ring in the chromosome displays *rRNA* genes (red), *tRNA* genes (blue), and *sRNA* genes (green). The two outer rings display distribution of genes based on COG classification on negative and positive strands on inner and outer rings, respectively.

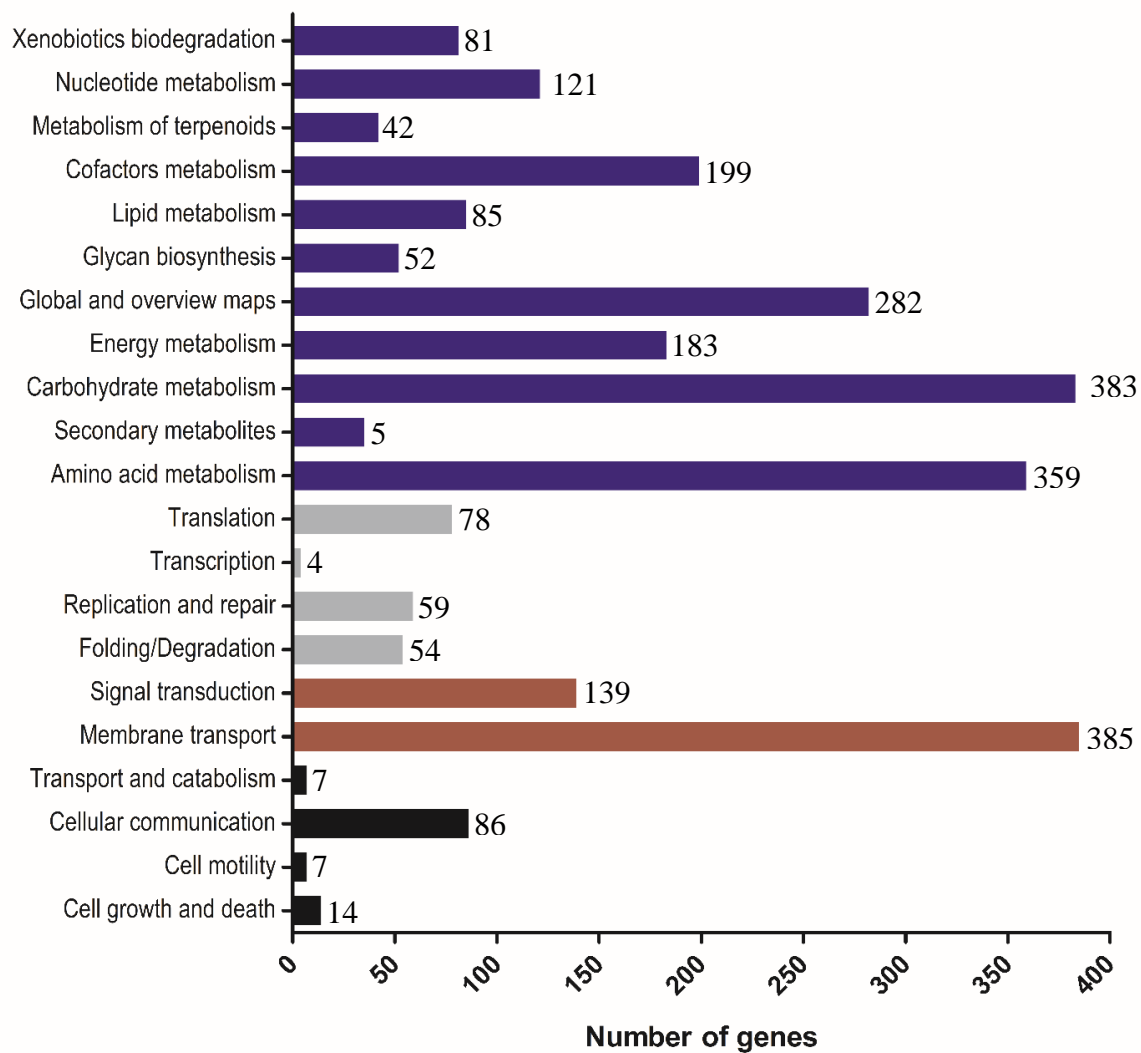


Figure 4.4. KEGG classification of chromosomal genes in Kp8701. Genes were classified into four main categories: metabolism (navy), genetic information processing (gray), environmental information processing (brown), and cell community (black).

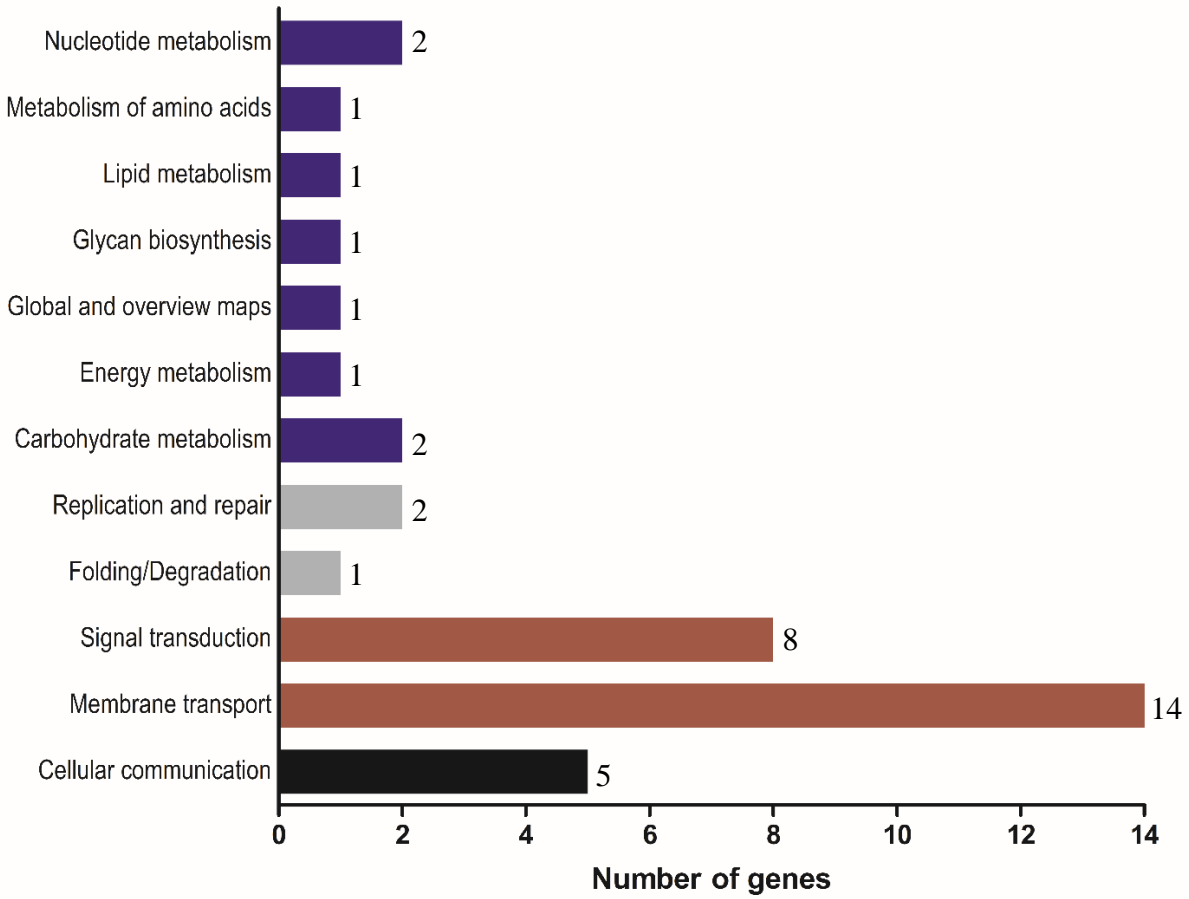


Figure 4.5. KEGG classification of plasmid genes in Kp8701. Genes were classified into four main categories: metabolism (navy), genetic information processing (gray), environmental information processing (brown), and cell community (black).

Chapter 5

Overall Conclusion

5.1 Rationale and hypothesis

Antimicrobial resistance is one of the most serious public health challenges of our time. In the United States, at least 2.8 million people are infected with antimicrobial-resistant bacteria, and more than 35,000 people die as a result (Hoelzer *et al.*, 2017; U.S. Centers for Disease Control and Prevention, 2019). The environment plays a major role in the spread of antimicrobial resistance (D'Costa *et al.*, 2006). However, studies on the environmental link to spread of antimicrobial resistance are lacking. Filth flies are the most common and widespread flies in the world. They have well adapted to live in close association with humans and animals, and they are often found in abundance in areas of human activities and animal husbandry. Flies are attracted to microbe-rich organic materials for reproductive purposes (Onwugamba *et al.*, 2018). As a result, they can acquire bacteria including antimicrobial-resistant bacteria from animal wastes and act as a potential vector of bacterial infections (Khamesipour *et al.*, 2018). The research conducted for this dissertation aimed to address the role of flies as a sentinel candidate for surveillance of antimicrobial resistance by analyzing the relationship between flies, their environment, and the antimicrobial resistance shared between them.

5.2 Findings

Flies were collected from six locations in Auburn, Alabama for three sets of analyses. Additionally, fecal samples were collected from sympatric animals in four animal husbandries at the same time of fly trapping.

The first set of flies (n = 221) was used for isolation of *Escherichia coli* and *Klebsiella pneumoniae*. Antimicrobial-resistant *E. coli* and *K. pneumoniae* were identified in 30 (13.6%) and 4 (1.8%) flies, respectively. Most of the isolates, 16/30 of *E. coli* and 3/4 of *K. pneumoniae*, were classified as MDR. Moreover, three of the MDR *E. coli* and one *K. pneumoniae* isolates were phenotypically extended-spectrum beta-lactamase (ESBL) positive. In particular, two ESBL-producing *E. coli* isolates were recovered from different flies that were trapped at a dog kennel. The *E. coli* isolates were found to belong to the same sequence type (ST68) and serotype (O25:H6). Moreover, those isolates were found to harbor the same virulence and antimicrobial resistance genes identified by whole genome sequencing, indicating that the isolates are closely related, and flies might have acquired them from the same source.

Microbiota and antimicrobial resistance genes as well as mobile genetic elements (ARG/MGE) detected in flies from the second set (n = 55 flies) were compared to those present in fecal samples of sympatric animals (n = 20). Flies and fecal microbiota shared 98.0% of their bacterial phyla. Moreover, nine potentially pathogenic bacterial genera that showed significant relative abundance (>0.1% of total genera) were found in flies and feces. Microbiota of flies were similar to the microbiota of the feces of their sympatric animals than those of the feces from the three other locations studied. However, the SourceTracker analysis indicated a variable contribution of fecal microbiota to fly microbiota except flies collected inside the poultry house shared 64.5% of the bacteria found in poultry feces. Comparison of ARG/MGE compositions between flies and fecal samples was performed to assess the potential acquisition of those genes by flies from feces. The qPCR array amplified a total of 276 genes in feces (237 ARGs and 39 MGEs) and 216 genes in flies (182 ARGs and 34 MGEs) in all 12 categories of tested antimicrobials. In total, 198 genes were shared between flies and feces, with 91.7% of the ARGs

and MGEs in flies (198/216). In spite of the large proportions of ARG/MGE shared between flies and feces, there was variability in shared ARG/MGE at a given location. Only poultry and dairy flies were found to share most genes with chicken feces (88%) and dairy feces (88.5%), respectively.

A total of 255 flies in the third set were trapped at a trash facility in Auburn, AL, and used for isolation of a total of 485 colistin-resistant bacteria, the majority of which were naturally resistant bacteria such as *Providencia*, *Serratia*, and *Proteus*. A colistin-resistant *K. pneumoniae* strain (Kp8701) was subjected for whole genome sequencing. *In silico* MLST, O- and K-antigens classify the isolate as ST784, O1, and KL146. The genome contains various virulence determinants involved in adhesion, iron acquisition and, most importantly, the regulator gene *magA*, which contributes to *K. pneumoniae* virulence by hypermucoviscosity phenotype. The genome does not harbor any known genes or mutations conferring resistance to colistin.

5.3 Significance

The findings in this work indicate that flies can be an important environmental link to spread of antimicrobial-resistant bacteria. In addition, based on general similarities in microbiota and ARG/MGE between flies and animals as well as the high prevalence of ARG/MGE in flies, it can be concluded that flies can be an important epidemiological tool in the surveillance of antimicrobial resistance and a potential sentinel for a one-health approach to antimicrobial resistance in different environments. However, detailed studies using optimized isolation protocols and epidemiological designs in addition to fly artificial challenge models with bacterial communities and acquired antimicrobial resistance genes will be necessary to clearly demonstrate a sentinel role for flies in documenting antimicrobial resistance. Finally, flies can

play a major role in spreading colistin-resistant bacteria, especially those with clinical importance including naturally resistant organisms such as *Providencia*, *Proteus*, and *Serratia*, which certainly results in health threats for humans and animals.

5.4 Future directions

Flies are able to travel long distances unrestrictedly. Therefore, this study did not fully demonstrate the possibility of flies as sentinels for antimicrobial resistance. Long-term studies on more geospatially distinct populations, more controlled experiments to mimic the environment, and a fly challenge model are needed to fully evaluate the possibility of flies as sentinels for antimicrobial resistance. Finally, investigating the bacterial clonal spread from animal feces to flies can be performed by isolating bacteria from feces and flies and studying isolated bacteria epidemiologically.

References

- D'Costa, V., McGrain, K., Hughes, D., and Write, G. (2006) Sampling the antibiotic resistome. *Science* **311**: 374–377.
- Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., and Coukell, A. (2017) Antimicrobial drug use in food-producing animals and associated human health risks: What, and how strong, is the evidence? *BMC Vet Res* **13**: 211.
- Khamesipour, F., Lankarani, K., Honarvar, B., and Kwenti, T. (2018) A systematic review of human pathogens carried by the housefly (*Musca domestica L.*). *BMC Public Health* **18**: 1049.
- Onwugamba, FC., Fitzgerald, JR., Rochon, K., Guardabassi, L., Alabi, A., Kühne, S., et al. (2018) The role of 'filth flies' in the spread of antimicrobial resistance. *Travel Med Infect Dis* **2018**: 8–17.
- U.S. Centers for Disease Control and Prevention (2019) Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA, USA.

List of publications

10. Barua S, Hoque M, Kelly P, Poudel A, Adekanmbi F, **Kalalah A**, Yang Y, Wang C. First report of *Rickettsia felis* in mosquitoes, USA. *Emerging Microbes & Infections*. 2020; 9 (1): 1008-1010.
9. **Kalalah A**, Poudel A, Gong J, Chen L, Yang Y, Hathcock T, Wang C. Complete genome sequence of a colistin-resistant *Klebsiella pneumoniae* isolate from houseflies (*Musca domestica*) in a trash disposal truck in the United States. *Microbiology Resource Announcements*. 2020; 9 (22): e00257-20.
8. Ngbede E, Poudel A, **Kalalah A**, Yang Y, Adekanmbi F, Adikwu A, Adamu A, Mamfe L, Terwase S, Useh N, Kwaga J, Adah M, Kelly P, Butaye P, Wang C. Identification of mobile colistin resistance genes (*mcr-1.1*, *mcr-5*, *mcr-8.1*) in *Enterobacteriaceae* and *Alcaligenes faecalis* of human and animal origins, Nigeria. *International Journal of Antimicrobial Agents*. 2020. Accepted manuscript.
7. Poudel A, Kang Y, Mandal R, **Kalalah A**, Butaye P, Hathcock T, Kelly P, Walz P, Macklin K, Cattley R, Price S, Adekanmbi F, Zhang L, Kitchens S, Kaltenboeck B, Wang C. Comparison of microbiota, antimicrobial resistance genes and mobile genetic elements in flies and the feces of sympatric animals. *FEMS Microbiology Ecology*. 2020; 1;96 (4). pii: fiae027.
6. Tonga Z, Silo-Suh L, **Kalalah A**, Dawson P, Chin B, Suh SJ. Efficient affinity-tagging of M13 phage capsid protein IX for immobilization of protein III-displayed oligopeptide probes on abiotic platforms. *Applied Microbiology and Biotechnology*. 2020; 104 (3): 1201-1209.
5. Izenour K, Zikeli S, **Kalalah A**, Ditchkoff SD, Starkey LA, Wang C, Zohdy S. Diverse *Bartonella* spp. detected in white-tailed deer and associated keds in the southeastern United States. *Journal of Wildlife Diseases*. 2020; doi: 10.7589/2019-08-196.
4. Poudel A, Hathcock T, Butaye P, Kang Y, Price S, Macklin K, Walz P, Cattley R, **Kalalah A**, Adekanmbi F, Wang C. Multidrug-resistant *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus* spp. in houseflies and blowflies from farms and their environmental settings. *International Journal of Environmental Research & Public Health*. 2019; 16 (19): 3583.
3. Huang K, Kelly P, Zhang J, Yang Y, Liu W, **Kalalah A**, Wang C. Molecular detection of *Bartonella* spp. in China and St. Kitts. *Canadian Journal of Infectious Diseases and Medical Microbiology*. 2019; 3209013.
2. Hathcock T, Liles M, Kang Y, White A, Newton J, **Kalalah A**, Waits DS, Wang C. Complete genome sequence of *Paralagenidium karlingii* strain 1391. *Journal of Bacteriology and Mycology*. 2018; 5 (4):1074.
1. Hathcock T, Mendoza L, Liles M, Kang Y, Newton J, Bond A, **Kalalah A**, Waits DS, Wang C. Draft genome sequence of the mammalian pathogen *Lagenidium giganteum* strain MTLA-03. *Journal of Bacteriology and Mycology*. 2018; 5(4):1073.