

1 For: *Applied and Environmental Microbiology*

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3 MINIREVIEW

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7 **Insects represent a link between food animal farms and the urban environment for**
8 **antibiotic resistance traits**

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29 Antibiotic resistant bacterial infections result in higher patient mortality rates, prolonged
30 hospitalization, and increased healthcare costs. Extensive use of antibiotics as growth promoters
31 in the animal industry represents great pressure for evolution and selection of antibiotic resistant
32 bacteria on farms. Despite growing evidence showing that antibiotic use and bacterial resistance
33 in food animals correlate with resistance in human pathogens, the proof for direct transmission of
34 antibiotic resistance is difficult to provide. In this review, we make a case that insects commonly
35 associated with food animals likely represent a direct and important link between animal farms
36 and urban communities for antibiotic resistance traits. Houseflies and cockroaches have been
37 shown to carry multi-drug resistant clonal lineages of bacteria identical to those found in animal
38 manure. Furthermore, several studies demonstrated proliferation of bacteria and horizontal
39 transfer of resistance genes in the insect digestive tract as well as transmission of resistant
40 bacteria by insects to new substrates. We propose that insect management should be an integral
41 part of pre- and post-harvest food safety strategies to minimize spread of zoonotic pathogens and
42 antibiotic resistance traits from animal farms. Furthermore, the insect link between the
43 agricultural and urban environment presents an additional argument for adopting prudent use of
44 antibiotics in the food animal industry.

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47 Antibiotic resistance has become a serious global public health problem; reduced
48 effectiveness of antibiotics results in higher patient mortality rates, prolonged hospitalization,
49 and increased healthcare costs (1-4). The annual cost to the U.S. health care system from
50 antibiotic resistant infections is estimated between \$21 and \$34 billion which includes more than
51 8 million additional hospital days (5). In a recent report entitled “Antibiotic resistance threats in
52 the United States” published by Centers for Disease Control and Prevention (6), it is estimated
53 that 2 million people become infected with bacteria that are resistant to antibiotics and at least
54 23,000 people die as a direct result of these infections in the United States each year. This
55 problem has been recognized in the clinical community and efforts for more prudent use of
56 antibiotics are under way (7, 8).

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58 **ANTIBIOTICS AND ANIMAL AGRICULTURE**

59 Antibiotic producing and antibiotic resistant bacteria are commonly found in various soil
60 environments (9). However, extensive use of antibiotics, especially as growth promoters, in the
61 animal industry has resulted in great pressure for evolution and selection of antibiotic resistant
62 bacteria in the food animal environment (10-15). As a result, food animals and animal production
63 environments have become reservoirs for antibiotic resistant strains that are released to the
64 environment in animal feces and then potentially spread to surrounding habitats (16-23). Despite
65 a growing body of evidence that antibiotic use in animals correlates with resistance in human
66 pathogens (24-30), direct proof for movement of antibiotic resistance traits between the
67 agricultural and urban environment is difficult to provide. Nonetheless, all countries in the
68 European Union adopted the precautionary principle and banned the use of all antibiotics as
69 growth promoters in animal agriculture in 2006 (31). Although the U.S. Food and Drug

70 Administration has recently taken the first step to reduce the use of medically important
71 antibiotics for enhancing animal growth (32), with one notable exception (ban on use of
72 fluoroquinolones in poultry in 2005) (33), no such policy has been implemented in the United
73 States yet, partly because of the argument made by the food animal industry pointing to the lack
74 of data that demonstrate a direct connection between animal farms and public health for
75 antibiotic resistant strains.

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77 **ANTIBIOTIC RESISTANCE AND INSECTS**

78 While insects are a numerous and diverse group in many environments, their potential to
79 play a role in the ecology of antibiotic resistance traits has not been recognized, with a few
80 exceptions. Tian et al. (34) used a metagenomic approach to screen for antibiotic resistance in
81 bacteria from the gut of honeybees (*Apis mellifera* L.) and showed an accumulation of mobile
82 genes coding for resistance to tetracycline and oxytetracycline which were closely related to genes
83 from human pathogenic strains. Allen et al. (35) reported several antibiotic resistance
84 determinants from the midgut bacteria of the gypsy moth larvae (*Lymantria dispar* L.) and Lowe
85 and Romney (36) authored a highly publicized but rather limited study where they isolated
86 vancomycin resistant *Enterococcus faecium* (VRE) and methicillin resistant *Staphylococcus*
87 *aureus* (MRSA) from five human bedbugs (*Cimex lectularius* L.) in Vancouver, Canada.
88 Antibiotic resistant enterococci were also isolated from stored-product beetles collected from a
89 feed mill, a grain storage silo, and a retail store (37). A few other studies showed the Mexican
90 fruit flies (*Anastrepha ludens* L.) from laboratory-reared colonies (38), the oil fly (*Helaeomyia*
91 *petrolei* L.) larvae from asphalt seeps (39), and cockroaches (*Periplaneta americana* L. and

92 *Blattella germanica* L.) from food-handling facilities, households, and a hospital (40, 41) as
93 carriers of antibiotic resistance traits.

94 **Livestock insects and food-borne pathogens**

95 With continuing urban expansion into agriculturally-zoned areas, the concern in the
96 public health community about insect pests, such as flies and cockroaches, associated with
97 animal productions, has increased because of the capacity of these insects to spread zoonotic
98 food-borne pathogens (reviewed in 42, 43). For example, in Japan, houseflies (*Musca domestica*
99 L.) were implicated in transmission of *Escherichia coli* O157:H7 from reservoir animals to other
100 animals and humans (44). Houseflies and blow flies collected from dumpsters of urban
101 restaurants were shown to carry *Cronobacter* spp., *Salmonella* spp., and *Listeria monocytogenes*
102 (45). Alam and Zurek (46) reported *E. coli* O157:H7 from the digestive tract of houseflies
103 collected in a cattle feedlot from feed bunks and cattle-feed storage and suggested that houseflies
104 in cattle farms play a role in the dissemination of this food-borne pathogen. In the same study,
105 they also showed that 95% of houseflies sampled were positive for fecal coliforms in their gut in
106 the level ranging from 3.0×10^1 to 3.0×10^6 CFU/fly. The large number of fecal coliforms in
107 houseflies indicates a potential to harbor other zoonotic pathogens. In a subsequent study, calves
108 were individually exposed for 48 h to houseflies that were orally inoculated with a mixture of
109 four strains of nalidixic acid-resistant *E. coli* O157:H7 (*Nal^REcO157*) (47). Rectal sampling of
110 fresh cattle feces showed the presence of *Nal^REcO157* strains until the end of the study (11 days
111 after fly exposure), with a concentration as high as 10^6 CFU/g, demonstrating the capability of
112 houseflies not only to carry this pathogen but actually transmit *E. coli* O157:H7 to the cattle
113 digestive tract through contamination of feed and water and/or direct contact with animals (47).

114 **Livestock insects as carriers of antibiotic resistance traits**

115 **a) Food animal environment.** Many antibiotics used as growth promoters are poorly
116 absorbed in the animal digestive tract and are therefore released to the environment in animal
117 feces (19, 20, 22). At the same time, organic waste in and around animal productions provides an
118 excellent habitat for the development of insects such as houseflies and stable flies (*Stomoxys*
119 *calcitrans* L.). In addition, some animal facilities (e.g. confined swine productions) provide a
120 new and ideal habitat for insects that are typically considered urban pests, particularly German
121 cockroaches (*Blattella germanica* L.) (48). As a consequence, the likelihood that the livestock
122 insect pests acquire and carry bacteria with antibiotic resistance traits is high (Table 1). Insects
123 such as houseflies and German cockroaches have a great potential to disseminate fecal bacteria
124 because of their developmental habitat, unrestricted movement, mode of feeding, strong
125 attraction to human food, and synanthropic nature (42, 43).

126 The first report on the potential of flies to acquire antibiotic resistant *Escherichia coli*
127 from food animals (swine and cattle) was published in 1990 by Marshall et al. (49). The
128 Australian bush fly (*Musca vetustissima*) was reported as a carrier of multi-drug resistant
129 *Salmonella* sp. and *Shigella* sp. on a cattle farm and in urban areas in Australia (50). Literak et al.
130 (51) found that houseflies from two swine operations in the Czech Republic carried *E. coli* with
131 the same antibiotic resistance patterns and genotypic profiles as those from swine manure. The
132 same group isolated *E. coli* with the same antibiotic resistance phenotypes and genetic
133 backgrounds from both, flies and manure, from a dairy farm (52). Usui et al. (53) sampled flies
134 (houseflies and false stable flies) and cattle feces from a cattle farm in Japan and found 14.3%
135 (13/91) of houseflies, 10.3% (7/68) of false stable flies and 7.5% (7/93) of cattle feces were
136 positive for a third-generation cephalosporin resistant strains of *E. coli* that contained
137 transferrable plasmids encoding the *bla*_{CTX-M-15} gene. Pulsed-field gel electrophoresis (PFGE)-

138 based genotypic analysis indicated that the flies carried the same *E. coli* clones that were
139 detected in cattle feces. Extended-spectrum beta-lactamase (ESBL)-producing *E. coli* were also
140 isolated from houseflies and blowflies from two poultry farms in Netherlands, and the genetic
141 background of these isolates was identical to that of ESBL-producing *E. coli* isolates from the
142 chicken manure (54). In a study from poultry farms in the U.S., houseflies collected at and near
143 confined chicken operations carried antibiotic resistant enterococci that matched genotypically
144 and phenotypically those from poultry litter (55).

145 Our research team has focused on the association of insects and antibiotic resistant
146 enterococci in several studies. We compared enterococci from houseflies, German cockroaches,
147 and pig feces from two commercial swine operations in Kansas and North Carolina (56).
148 Enterococci were detected in the majority (>89%) of all samples and multi-drug (mainly
149 tetracycline and erythromycin) resistant enterococci were common from all three sources.
150 Genotypic PFGE analysis of selected *Enterococcus faecalis* and *E. faecium* isolates
151 demonstrated that cockroaches and houseflies shared the same enterococcal clones that were
152 detected in the swine manure, indicating that insects acquired enterococci from swine manure
153 (56). The above studies demonstrate that insects on farms commonly carry the same clonal
154 lineages of multi-drug resistant bacteria that are found in animal feces.

155 **b) Urban environment.** Previous studies using fly traps and multilocus DNA
156 fingerprinting reported random dispersal (up to 125 km) of houseflies from poultry and cattle
157 farms (57, 58). We screened the digestive tract of houseflies collected at five fast-food
158 restaurants in a town in northeastern Kansas and found that antibiotic resistant enterococci were
159 common (59). The majority (97%) of flies were positive for enterococci with a mean CFU of 10^3
160 per fly. *Enterococcus faecalis* was found as the most abundant species (88.2%) harboring

161 resistance to tetracycline (66.3% of isolates), erythromycin (23.8%), streptomycin (11.6%),
162 ciprofloxacin (9.9%), and kanamycin (8.3%). In addition, the conjugative transposon Tn916 and
163 members of the Tn916/Tn1545 family that are frequently involved in the horizontal transfer of
164 antibiotic resistance traits during bacterial conjugation were common and detected in 30.2% and
165 34.6% of the identified isolates, respectively (59). Our subsequent study showed that ready-to-
166 eat food from the same restaurants was commonly contaminated with antibiotic resistant
167 enterococci (60). Overall concentration of enterococci throughout the year averaged $\sim 10^3$ CFU/g
168 with greater prevalence during the summer than the winter. The higher prevalence of
169 enterococcal contamination among food samples in summer correlated with housefly activity.
170 Enterococci from summer samples were resistant to tetracycline (22.8% of isolates),
171 erythromycin (22.1%), and kanamycin (13.0%) (60). These studies implied that food served in
172 restaurants is commonly contaminated with antibiotic resistant enterococci and that houseflies
173 may play a role in this contamination.

174 Most recently, we assessed the prevalence of enterococci in houseflies collected from
175 four municipal wastewater treatment facilities (WWTF) as these sites are another potential
176 source of antibiotic resistant strains. Interestingly, the highest prevalence of multi-drug resistant
177 enterococci was detected from a WWTF (sludge and associated houseflies) that processed the
178 waste from a nearby sausage factory, pointing again to animal agriculture as a source of these
179 bacteria (61). Genotypic analysis (PFGE) revealed the same clones of *E. faecalis* present in the
180 waste and the housefly digestive tract. Doud et al. (61) also collected houseflies from the
181 residential environment (restaurant, apartment complex, mobile homes) close (0.7 – 2.0 km) to
182 one of the WWTF and found similar antibiotic resistance profiles in *E. faecalis* and *E. faecium*

183 although in lower prevalence and with no clonal matches to enterococci isolated directly from
184 the WWTF environment.

185 **Bacterial proliferation in the insect digestive tract and transmission of bacteria by insects**

186 Bacterial proliferation and transfer during insect feeding has been demonstrated
187 previously in houseflies for *E. coli* (62, 63). We used a GFP-labeled *E. faecalis* OG1RF:pMV158
188 to track the fate of this bacterium in the digestive tract of houseflies and to assess the vector
189 potential of this insect for *E. faecalis* (64). Analysis of viable fluorescing cells within various gut
190 components over several time points revealed the highest bacterial count in the midgut in first
191 few hours (1-4 h) after feeding and that declined gradually; while the CFU peaked in the fly
192 foregut (crop) after 48 h and remained high until the end (96 h) of the experiment. This
193 suggested that *E. faecalis* was digested in the midgut but proliferated in the crop (64). Bacterial
194 proliferation in the housefly crop and digestion in the midgut have also been reported for
195 *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (65, 66). This is important because the
196 content of the crop, including associated bacteria, is typically released on a food source by
197 housefly regurgitation during feeding (42, 67). Both drinking water and feed (flaked corn)
198 sampled at the end of the assay were contaminated by fluorescing *E. faecalis*, demonstrating that
199 the flies disseminated *E. faecalis* to their surroundings (64). Furthermore, we also directly
200 assessed the ability of houseflies to contaminate ready-to-eat food with enterococci under
201 laboratory conditions (68). Within 30 minutes, exposure of as few as five flies collected from a
202 cattle feedlot resulted in an average of $\sim 10^3$ CFU/g of enterococcal deposit on the food (beef
203 patty from a hamburger) (68). These studies further support the notion that houseflies can act not
204 only as a mechanical but also as a bioenhanced vector for bacteria, and have great potential to
205 contaminate substrates by microbes during feeding and by defecation.

206 Livestock insects and horizontal transfer of antibiotic resistance traits

207 In addition to bacterial proliferation in the digestive tract of houseflies, the potential for
208 horizontal transfer of genes coding for toxins and antibiotic resistance among bacteria was also
209 evaluated. Petridis et al. (69) observed relatively frequent (10^{-3} to 10^{-2} transconjugant per donor)
210 transfer of genes for chloramphenicol resistance and the Shiga-toxin among strains of *E. coli* in
211 both the midgut and crop of houseflies after 1 h of post-feeding. Our study showed that the
212 tetracycline resistance gene (*tetM*) on a pheromone-responsive plasmid pCF10 was frequently
213 transferred between *E. faecalis* strains in the housefly mouthparts and digestive tract (70). The
214 transfer occurred within 24 h after exposure with a transconjugant/donor rate from 8.6×10^{-5} to
215 4.5×10^1 . The implications of these studies are significant to public and animal health as they
216 point to the ability of bacteria to actively share toxins and antibiotic resistance genes within the
217 housefly gut beyond what is consumed initially by the fly and beyond simple bacterial
218 proliferation.

219

220 CONCLUSIONS

221 The above studies demonstrate: a) the association of multi-drug resistant bacterial strains
222 of food animal origin with flies and cockroaches; b) bacterial proliferation and horizontal
223 transfer of antibiotic resistance genes in the insect digestive tract; and c) potential of these insects
224 to transmit multi-drug resistant bacteria from food animals to the urban environment. We
225 propose that integrated pest management should be incorporated into pre- and post-harvest food
226 safety programs to minimize spread of antibiotic resistant bacterial strains. In addition, the insect
227 link between agricultural and urban environments presents another reason for implementation of
228 prudent use of antibiotics in the food animal industry.

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TABLE 1 Insects with antibiotic resistant bacteria from food animal productions and surrounding urban environments

Insects	Bacteria	Antibiotic resistance*	Environments	References
Cockroaches (Dictyoptera)				
German cockroach (<i>Blattella germanica</i>)	<i>Enterococcus faecalis</i> <i>Enterococcus faecium</i> <i>Enterococcus hirae</i> <i>Enterococcus casseliflavus</i>	AMP, CHL, CIP, ERY, KAN, STR, TET	Swine farms	56
Flies (Diptera)				
House fly (<i>Musca domestica</i>)	<i>Enterococcus faecalis</i> <i>Enterococcus faecium</i> <i>Enterococcus casseliflavus</i>	CIP, ERY, KAN, STR, TET	Fast-food restaurants	59
House fly (<i>Musca domestica</i>) Blow fly (<i>Lucilia</i> spp.) Bottle fly (<i>Phaenicia</i> spp.)	<i>Enterococcus faecalis</i> <i>Enterococcus faecium</i> <i>Staphylococcus</i> spp.	CLN, ERY, PEN, SYN, TET	Poultry farms	55
House fly (<i>Musca domestica</i>)	<i>Enterococcus faecalis</i> <i>Enterococcus faecium</i> <i>Enterococcus hirae</i> <i>Enterococcus casseliflavus</i>	AMP, CHL, CIP, ERY, KAN, STR, TET	Swine farms	56
House fly (<i>Musca domestica</i>)	<i>Enterococcus faecalis</i> <i>Enterococcus faecium</i>	DOX, ERY, GEN, STR, TET	Wastewater treatment facilities	61
House fly (<i>Musca domestica</i>)	<i>Escherichia coli</i> O157:H7	AMP, CER, CTE, GEN, NEO, OXY, SPC, SXT	Cattle farm	46
House fly (<i>Musca domestica</i>)	<i>Escherichia coli</i>	AMP, STR, SUL, TET	Swine farms	51
House fly (<i>Musca domestica</i>)	<i>Escherichia coli</i>	AMP, AMX, CHL, CEP, CIP, GEN, NAL, SUL, STR, SXT, TET	Dairy cattle farm	52
Stable fly (<i>Stomoxys calcitrans</i>)		CIP, GEN, NAL, SUL, STR, SXT, TET		
House fly (<i>Musca domestica</i>)	<i>Escherichia coli</i>	AMP, CED, CEZ, STR, TET, TRM	Cattle farm	53
False stable fly (<i>Muscina stabulans</i>)				
House fly (<i>Musca domestica</i>)	<i>Escherichia coli</i>	CAZ, CEF	Poultry farms	54
Blow fly (<i>Lucilia</i> spp.)				
Australian bush fly (<i>Musca vetustissima</i>)	<i>Escherichia coli</i> <i>Salmonella</i> sp. <i>Shigella</i> sp.	AMX, CLR, ROX	Cattle farm Urban area Outdoor eateries	50

* AMP, ampicillin; AMX, amoxicillin; CAZ, ceftazidime; CED, cefpodoxime; CEF, cefotaxime; CEP, cephalotin; CER, ceftiofur; CEZ, cefazolin; CHL, chloramphenicol; CIP, ciprofloxacin; CLN, clindamycin; CLR, cefaclor; CTE, chlortetracycline; DOX, doxycycline; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NEO, neomycin; OXY, oxytetracycline; PEN, penicillin; ROX, roxythromycin; SPC, spectinomycin; STR, streptomycin; SUL, sulfonamides; SXT, sulfamethoxazole/trimethoprim; SYN, quinupristin-dalfopristin; TET, tetracycline; TRM, trimethoprim.