Thesis

Role of flies for spreading antimicrobial resistant bacteria and its resistance genes in farm environments

農場環境での薬剤耐性菌・耐性遺伝子の拡散における
ハエの役割

Department of Health and Environmental Sciences,
Doctoral course of Applied Veterinary Medicine,
Rakuno Gakuen University Graduate School

Akira Fukuda

Laboratory of Food Microbiology and Food Safety
Advising teacher: Prof. Ikuo Uchida

2017

ABBREVIATIONS

ARB: antimicrobial resistant bacteria

ARG: antimicrobial resistance gene

bp: base pairs

CFU: colony forming unit

CLSI: Clinical and Laboratory Standards Institute

DHL: deoxycholate-hydrogen sulfate-lactose

ESBL: extended-spectrum **β**-lactamase

JVARM: Japanese Veterinary Antimicrobial Resistance Monitoring

MIC: minimum inhibitory concentration

MLST: multilocus sequence typing

PBS: phosphate-buffered saline

PFGE: pulsed-field gel electrophoresis

pPCR: quantitative polymerase chain reaction

ST: sequence type

TCs: transconjugants

TSB: tryptic soy broth

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PREFACE

The advent of antimicrobials in the twentieth century dramatically improved treatment of bacterial infections and saved many human and animal lives in clinical settings [59, 113]. However, antimicrobial resistant bacteria (ARB) subsequently emerged and became widespread to such an extent that they are currently recognized as one of the most urgent threats to public health [112, 115]. At present, at least 700,000 deaths are attributable to ARB, and this number is estimated to reach 10 million people by 2050 [26, 48]. Regardless, the development and approval of novel antimicrobials has steadily decreased over the past three decades after peaking in the 1980s [46, 113]. Therefore, strategies to control infections without increasing the incidence of ARB and, thus, prolong the effectiveness of existing antimicrobials are gaining attention, including preventing the emergence and dissemination of ARB through the proper use of antimicrobials and improvement of hygiene [59, 71, 88].

ARB have emerged in human and veterinary clinical settings due to the use of antimicrobials, and they disseminate through various routes [11, 30, 36, 68, 81, 96]. Based on the Swann report, livestock-associated ARB can be transmitted to humans and in turn lead to the reduction in the efficacy of treatments for bacterial infections in human clinical settings [4]. Dissemination mechanisms and pathways of transmission of livestock-associated ARB to humans are mainly focused on food chain based, and they have been demonstrated in several studies [54, 86, 110]. Another important route of dissemination is through the environment, such as water, soil, air, wild animals, and insects [6, 66]. However, the origins of ARB found in the environment are uncertain [20, 74]. In some cases, insects have carried the crucial ARB that are resistant to antimicrobials important in human clinical settings [2, 32, 107]. Flies, in particular, can be found in the habitats of livestock and humans, and they move freely due to their strong ability to fly [41]. There are around 30,000 species of flies in the world, only a few of which are of human and/or veterinary concern [38, 51, 92]. Muscidae flies frequently inhabit livestock farms, where

different species have different food sources [5, 10]. For example, non-biting flies, such as house and false stable flies, eat feces, while the stable fly feeds on the blood of the livestock [51, 92]. Flies play a crucial role in the dissemination of livestock-associated ARB through the environments because the food they intake can contain livestock-associated ARB.

Several mechanisms of gaining antimicrobial resistance have been identified for bacteria: 1) chromosomal mutations that alter drug targets and/or change expression levels of several antimicrobial resistance-associated factors in bacteria, 2) acquisition of mobile elements containing antimicrobial resistance genes (ARGs), such as plasmids, transposons, integrons, and phages, and 3) changing the activities of bacteria, such as biofilm formation and the persisters [36, 37, 39, 104]. ARGs-containing mobile elements can transfer between bacteria across genera, and similar types of these elements have been are disseminated from several sources across the world [53, 81]. In particular, plasmids containing multiple ARGs that confer resistance to multiple drugs are important in the widespread development of gram-negative ARB [8]. When ARBs were categorized by different organizations, widely disseminated bacteria with plasmid-meditated antimicrobial resistance, such as carbapenem-resistant Enterobacteriaceae and drug-resistant *Neisseria gonorrhoeae,* were categorized into the highest threat level of microorganisms with antimicrobial resistance by the Center for Disease Control Prevention (CDC), USA, while carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baummanii* and carbapenem-resistant and/or extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae were categorized as priority pathogens for the research and development of new antibiotics by the World Health Organization [11, 114]. Therefore, when controlling development of ARB, preventing the formation and spread of ARGs is required.

In this thesis work, we attempted to clarify the roles that flies have in the spread of ARB and ARGs on farm environments. First, we clarified the role of flies as a mechanical vector in the transmission and spread of ARB originating from

farm-dwelling livestock (Chapter 1). Next, to study the role of flies in the spread of ARGs, we performed horizontal transfer of plasmid-mediated ARGs in the intestines of flies (Chapter 2). Finally, we investigated fly-mediated maintenance of ARB and ARGs on the farm environments (Chapter 3). The goal of this study was to assess the transmission and spread of ARB and ARGs by flies on a farm to prevent of the dissemination of livestock-associated ARB and ARGs, in future.

CHAPTER 1

The role of flies in spreading the extended-spectrum β-lactamase gene from cattle

1.1. Introduction

The emergence and spread of antimicrobial resistance has become a major global public health concern. A component of this problem is the spreading of the ESBL gene, which can confer resistance to third-generation cephalosporins [83]. Third-generation cephalosporins are clinically important antimicrobials in human and veterinary medicine. In recent years, the most prevalent type of ESBLs has been CTX-M [83]. In particular, CTX-M-15, a member of the CTX-M-1 group, is the most widely disseminated ESBL globally [57, 83].

Since the report of Swann *et al*. [4], the spreading of ARB and ARGs from food-producing animals to humans has been investigated. Nationwide surveillance studies have indicated that antimicrobial resistance have been harbored in food-producing animals [55]. However, the connection between antimicrobial resistance in bacterial isolates from food-producing animals and clinical isolates of humans is uncertain, because the ecology of these bacteria and their genes in the agricultural and urban environment is not well understood [1, 40, 90].

ARB are released into the environment via feces from food-producing animals and sanitary insects, including flies that feed on feces [1]. Flies move freely between habitats of food-producing animals and humans owing to their strong flight capabilities [28], possessing a great potential for dissemination of antimicrobial resistance from food-producing animals to humans [31]. Therefore, fly-mediated dissemination of antimicrobial resistance from food-producing animals to humans is drawing increasing attention.

The purpose of this Chapter was to clarify the role of the flies in the spread of ESBL-producing bacteria from food-producing animals to humans. We identified and characterized a third-generation cephalosporin resistant *E. coli* isolated from flies and cattle feces from a cattle barn.

1.2. Materials and Methods

1.2.1. Sample collection

All fly and fecal samples were collected from a cattle barn during August–October 2010 in Ebetsu city (Hokkaido prefecture, Japan). A total of 231 flies were collected using a sweep net. Flies were placed individually in sterile 50 mL Falcon tubes for laboratory processing. The fly species, including 91 houseflies (*Musca domestica*), 68 false stable flies (*Muscina stabulans*), and 72 stable flies (*Stomoxys calcitrans*), were morphologically identified by a stereomicroscope. A total of 93 samples of cattle feces were collected from the same cattle barn.

1.2.2. Bacterial isolation

The surface of flies were individually sterilized with sodium hypochlorite and ethanol as previously described [64]. The samples were then washed thrice with sterile distilled water and homogenized in PBS. The homogenized flies and cattle feces were inoculated into DHL agar medium (Nissui Pharmaceutical, Tokyo, Japan) or DHL agar medium supplemented with 2 μg/mL cefpodoxime (DHL-C; Daiichi-Sankyo, Tokyo, Japan). The isolation agar media were incubated at 37°C overnight. The isolate that was identified as *E. coli* by colony morphology and API20E tests (Sysmex, Kobe, Japan) was selected.

1.2.3. Antimicrobial susceptibility testing

We performed minimum inhibitory concentration (MIC) determinations using the broth microdilution method with an Eiken frozen plate (Eiken Chemistry, Tokyo, Japan) according to the CLSI guidelines [15]. The following antimicrobial agents were tested: ampicillin (Sigma-Aldrich, St. Louis, MO, USA), cefazolin (Sigma-Aldrich), cefpodoxime (Daiichi-Sankyo), streptomycin, kanamycin, gentamicin, tetracycline, chloramphenicol, nalidixic acid, ciprofloxacin, trimethoprim, fosfomycin, and colistin (Sigma-Aldrich). The resistance breakpoints

were defined for the antimicrobials in accordance with CLSI guidelines [15]. We obtained breakpoints for colistin, which were not defined by the CLSI guidelines, from a report on the JVARM system [55]. In this study, we defined the breakpoint for streptomycin as 64 μg/mL by taking into consideration the midpoint between the peaks of each MIC distribution. *E. coli* ATCC25922 was used as a quality-control strain. The cefpodoxime resistant isolates (MIC ≥8 μg/mL) were selected for further tests.

1.2.4. Characterization of resistance genes

The DNA from the cefpodoxime resistant isolates was extracted from cultures with an InstaGene Matrix (Bio-Rad Laboratories, Tokyo, Japan). The presence of genes encoding *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$, *bla*$_{ACC}$, *bla*$_{FOX}$, *bla*$_{MOX}$, *bla*$_{DHA}$, *bla*$_{CIT}$, and *bla*$_{EBC}$ was determined by multiplex PCR as previously described [22]. For the CTX-M-1 group, an additional PCR procedure was performed using external primers as previously described [70], and all amplicons were subsequently sequenced. The *tetA* and *aac(6′)-Ib-cr* genes were screened by PCR [77, 87].

1.2.5. Pulsed-field gel electrophoresis (PFGE), phylogenetic grouping, and sequence-type determination

The *bla*$_{CTX-M-1}$ group-harboring isolates were typed by PFGE analysis according to the Pulse Net CDC protocol [12]. Genomic DNA in each agarose plug was digested with *Xba*I (Takara Bio, Shiga, Japan). The PFGE procedure was performed using the CHEF-DRIII system (Bio-Rad Laboratories) under the following conditions: switch time, 2.2–54.2 seconds; running time, 18 hr; included angle, 120°; voltage, 6 V/cm; and temperature, 14°C. The PFGE profiles were analyzed using the BioNumerics program (Applied Maths, Sint-Martens-Latem, Belgium). Similarity and diversity were assessed by applying the Dice coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic means (UPGMA; position tolerance of 0.4% and optimization of 0.5%).

E. coli phylogenetic groupings (A, B1, B2, and D) were determined using a multiplex PCR method that was used for the *chuA* and *yjaA* genes and TspE4.C2 fragment, as previously described by Clermont *et al*. [14].

Multilocus sequence typing (MLST) was performed as previously described [95]. Gene amplification and sequencing were performed using the primers specified on the *E. coli* MLST Web site (http://mlst.ucc.ie/mlst/dbs/Ecoli). Allelic profile and ST were determined as per the scheme on the *E. coli* MLST Web site.

1.2.6. Transferability of the *bla*$_{\text{CTX-M-1}}$ group genes and plasmid characterization

Transferability was tested by performing previously described broth-mating assays with slight modifications [101]. In brief, the recipients used were rifampicin resistant *E. coli* K12 ML4909 strains, with a mating temperature set at 30°C. TCs were selected on Mueller–Hinton agar (OXOID, Cambridge, UK) supplemented with 50 µg/mL rifampicin (Sigma-Aldrich) and 32 µg/mL cefpodoxime. All *bla*$_{\text{CTX-M-1}}$ group-carrying plasmids were replicon typed by using the PCR-based replicon-typing method, as previously described [49]. The plasmid DNA was purified from the parental strains and TCs by using a modified alkaline lysis method [50], and the plasmid sizes were estimated using the BAC-Tracker supercoiled DNA ladder (Epicentre Biotechnologies, Madison, WI). Southern blot hybridization was subsequently performed. The plasmids were transferred using downward capillary transfer to Hybond-N + nylon membrane (GE Healthcare, Chalfont St Giles, UK), and the membrane was treated according to standard procedures. DNA probe labeling, hybridization, and detection were performed using the digoxigenin (DIG)-PCR and DIG Nucleic Acid Detection Kits (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. DNA probes were constructed with the β-lactamase gene (*bla*$_{\text{CTX-M-15}}$) and replicon FIB gene-purified PCR products.

1.3. Results

1.3.1. Isolation of *E. coli* from flies and cattle feces

By using DHL agar, *E. coli* was isolated from 42.9% (39/91) of houseflies, 67.6% (46/68) of false stable flies, and 66.7% (62/93) of cattle feces samples (Table 1). Cefpodoxime resistant *E. coli* isolated from DHL agar was isolated from only two houseflies (2.2%). By using DHL-C agar, cefpodoxime resistant *E. coli* was isolated from 12.1% (11/91) of houseflies, 10.3% (7/68) of false stable flies, and 7.5% (7/93) of cattle feces samples. No *E. coli* were obtained from the 72 stable flies. In total, 27 cefpodoxime resistant strains were isolated from 13 houseflies (14.3%; 2 from DHL and 11 from DHL-C), 7 false stable flies (10.3%), and 7 cattle feces samples (7.5%; Table 1).

Table 1. Isolation rate of *E. coli* from flies and cattle feces

Origin	Isolation rate (%)	
	DHL	DHL containing cefpodoxime
Housefly (*Musca domestica*)	39/91 (42.9%)	12/91 (13.2%)
False stable fly (*Muscina stabulans*)	46/68 (67.6%)	7/68 (10.3%)
Stable fly (*Stomoxys calcitrans*)	0/72 (0%)	0/72 (0%)
Cattle feces	62/93 (66.7%)	7/93 (7.5%)

1.3.2. Antimicrobial resistance

The proportions of antimicrobial-resistant *E. coli* obtained using DHL and DHL-C are shown in Table 2. Among *E. coli* isolates from houseflies and false stable flies grown in DHL agar, a high percentage of isolates with resistance to tetracycline (20.5% and 23.9%, respectively) and streptomycin (15.4% and 19.6%, respectively) were observed. Among *E. coli* isolates from cattle feces, however, isolates with resistance to tetracycline (45.2%), kanamycin (12.9%), and streptomycin (11.3%)

were observed. By using DHL-C agar, a high percentage of isolates with resistance to ampicillin, cefazolin, and cefpodoxime were observed among *E. coli* isolates from all origins. In addition, most of these isolates were resistant to streptomycin, tetracycline, and trimethoprim.

1.3.3. ARGs

Twenty-seven cefpodoxime resistant isolates were tested for the presence of ARGs. Twenty-two isolates from 11 houseflies, 5 false stable flies, and 6 cattle feces samples harbored the *bla*$_{\text{CTX-M-1}}$ group genes. Sequencing of the resulting PCR products demonstrated identity with *bla*$_{\text{CTX-M-15}}$ genes. All isolates harbored the *bla*$_{\text{CTX-M-15}}$ genes containing *bla*$_{\text{TEM}}$ and *tetA*. Three additional cefpodoxime resistant isolates (from one housefly and two false stable flies) harbored the *bla*$_{\text{CTX-M-2}}$ group genes. The β-lactamase genes were not detected in the remaining two cefpodoxime resistant isolates (from one housefly and one cattle feces sample). No *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{ACC}}$, *bla*$_{\text{FOX}}$, *bla*$_{\text{MOX}}$, *bla*$_{\text{DHA}}$, *bla*$_{\text{CIT}}$, *bla*$_{\text{EBC}}$, or *aac(6′)-Ib-cr* genes were detected in any of the cefpodoxime resistant isolates.

1.3.4. PFGE analysis of *bla*$_{\text{CTX-M-15}}$-carrying *E. coli*

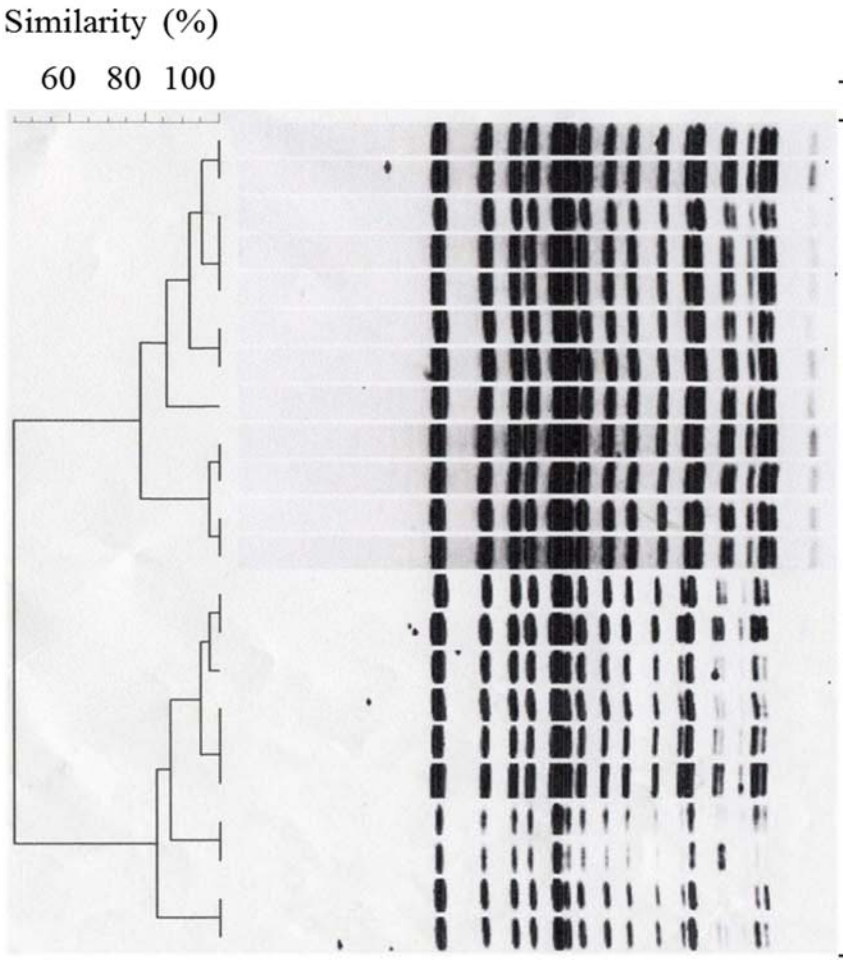
The genetic relationships, based on PFGE results, among the 22 *bla*$_{\text{CTX-M-15}}$-carrying isolates are shown in Figure 1. Analysis using the UPGMA resulted in the classification of the genes into two clusters with an 80% similarity level (Fig. 1). The first cluster contained 12 isolates derived from 4 houseflies, 4 false stable flies, and 4 cattle feces samples. The second cluster contained 10 isolates derived from 7 houseflies, 1 false stable fly, and 2 cattle feces samples.

Phylogenetic characterization revealed that all 22 isolates harbored the *bla*$_{\text{CTX-M-15}}$ genes belonging to group D. All 22 *bla*$_{\text{CTX-M-15}}$-harboring isolates were subjected to seven-locus MLST and exhibited the same combination of alleles across the seven sequenced loci, corresponding to an established ST, ST38.

Table 2. Antimicrobial-resistance rates of *E. coli* isolates

Antimicrobial agents	Break point (μg/mL)	Housefly		False stable fly		Cattle feces	
		DHL (n=39)	DHL containing cefpodoxime (n=12)	DHL (n=46)	DHL containing cefpodoxime (n=7)	DHL (n=62)	DHL containing cefpodoxime (n=12)
Ampicillin	32[a]	7.7%	100%	2.2%	100%	9.6%	100%
Cefazoline	4[a]	5.1%	91.7%	0%	100%	4.8%	100%
Cepodoxime	8[a]	5.1%	91.7%	0%	100%	0%	100%
Streptomycin	64[b]	15.4%	91.7%	19.6%	85.7%	11.3%	100%
Kanamycin	64[a]	2.6%	16.7%	0%	14.3%	12.9%	14.3%
Gentamicin	16[a]	0%	0%	2.2%	0%	0%	0%
Tetracycline	16[a]	20.5%	100%	23.9%	100%	45.2%	100%
Chloramphenicol	32[a]	0%	0%	0%	14.3%	1.6%	0%
Nalidixic acid	32[a]	0%	8.3%	2.2%	0%	0%	0%
Ciprofloxacin	4[a]	0%	0%	0%	0%	0%	0%
Trimethoprim	16[a]	5.1%	83.3%	0%	85.7%	1.6%	100%
Fosfomycin	256[a]	0%	0%	0%	0%	0%	0%
Colistin	16[c]	0%	0%	0%	0%	0%	0%

[a]The value was a CLSI breakpoint.
[b]The value was set as the midpoint between the peaks of each MIC distribution.
[c]The value was a JVARM breakpoint.



Strain	Origin	Antimicrobial resistance genes
101	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
133	Cattle feces	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
29	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
59	Stable fly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
30	Cattle feces	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
10	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
11	Cattle feces	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
134	Cattle feces	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
61	Stable fly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
100	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
58	Stable fly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
60	Stable fly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
123	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
143	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
142	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
135	Cattle feces	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
160	Cattle feces	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
144	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
80	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
125	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
78	Stable fly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*
114	Housefly	$bla_{\text{CTX-M-15}}$, bla_{TEM}, *tetA*

Fig. 1. PFGE analysis showing percent similarities of 22 isolates carrying $bla_{\text{CTX-M-15}}$ genes. DNA samples for PFGE analysis were digested with *Xba*I.

1.3.5. *bla*$_{\text{CTX-M-15}}$-carrying plasmid characterization

TCs derived from all 22 isolates harboring the *bla*$_{\text{CTX-M-15}}$ genes were stablished. Southern blot hybridization analysis revealed that the length of the plasmid harboring the *bla*$_{\text{CTX-M-15}}$ genes in all isolates and TCs of this study was approximately 120 kbp; further, all belonged to incompatibility group FIB (Fig. 2).

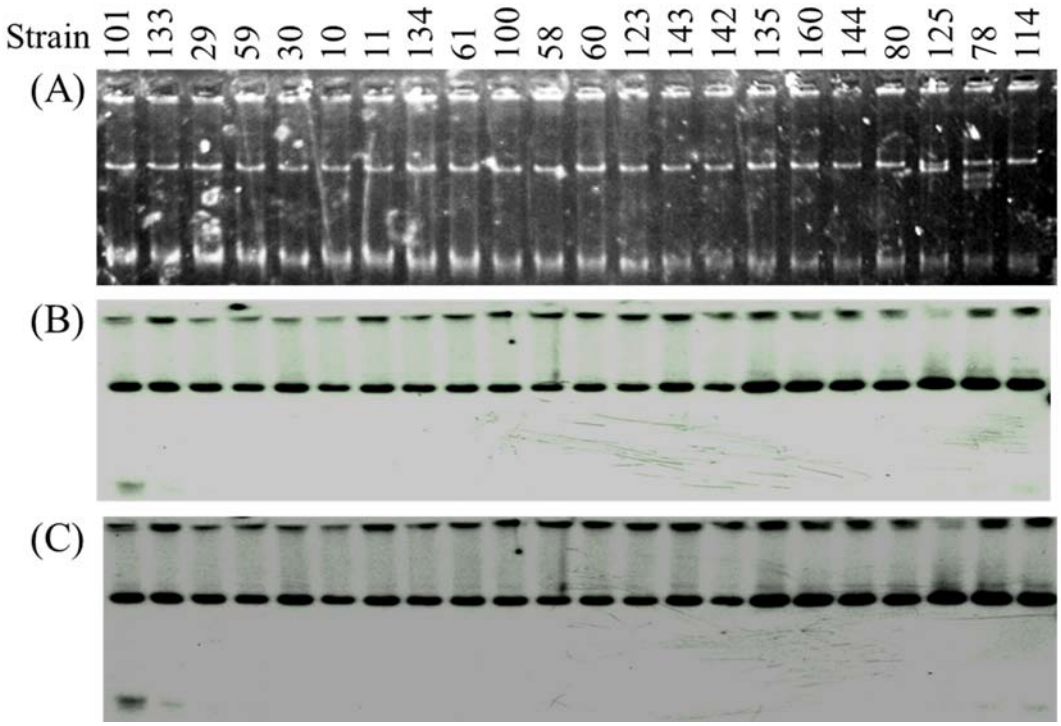


Fig. 2. Plasmid profiling and Southern blot hybridization of *bla*$_{\text{CTX-M-15}}$-carrying plasmids. (A) Plasmid DNA of parental strains. (B) Southern blot hybridization with a *bla*$_{\text{CTX-M-15}}$ probe. (C) Southern blot hybridization with replicon FIB probe.

1.4. Discussion

The current study using DHL-C showed that cefpodoxime resistant *E. coli* were isolated from 11 houseflies (12.1%), 7 false stable flies (10.3%), and 7 cattle feces (7.5%). By contrast, we were able to detect cefpodoxime resistant *E. coli* isolates from only two houseflies (2.2%) in non-selective DHL medium. These results suggest that cefpodoxime resistant isolates were not predominant in the cattle barn, despite their wide distribution.

E. coli was detected in housefly and false stable fly gut, but not in stable fly gut. Both houseflies and false stable flies feed on cattle feces which contained many *E. coli* from cattle, while stable flies are blood-feeding insects. An analysis of the results of PFGE and MLST in *bla*$_{\text{CTX-M-15}}$-harboring *E. coli* showed that houseflies and false stable flies carried several of the same clones that were detected in cattle feces. Chakrabati et al. reported that the prevalence of antimicrobial-resistant enterococci in houseflies decreased with increasing distance from the cattle feedlot [13]. These results suggest that the source of ESBL-producing *E. coli* in housefly and false stable fly gut was the cattle feces from the cattle barn.

In this Chapter, we observed closely related isolates harboring the *bla*$_{\text{CTX-M-15}}$-carrying incompatibility group FIB plasmids in houseflies, false stable flies, and cattle feces samples. This is an interesting finding, because ESBL-producing *E. coli* clones have rarely been reported to show clonal dissemination. Furthermore, the incompatibility group I1 plasmid has been the most commonly observed vector that is subject to clonal dissemination, especially in animals [99]. It has been reported that once ESBL-producing plasmids are acquired, clonal dissemination is another likely mechanism for the perpetuation of resistance [61]. These results suggest that the *bla*$_{\text{CTX-M-15}}$-carrying incompatibility group FIB plasmid had invaded the cattle barn and that clonal dissemination subsequently occurred between flies and cattle. However, further studies are required to clarify the invasion routes of the antimicrobial-resistant carrying plasmids.

The *bla*$_{\text{CTX-M-15}}$-carrying plasmids in *E. coli* isolated from flies were observed for the first time in this study. Furthermore, *bla*$_{\text{CTX-M-15}}$-carrying plasmids from cattle were observed for the first time in Japan. *bla*$_{\text{CTX-M-15}}$-carrying plasmids, particularly found in B2-O25b:H4-ST131 *E. coli*, have been spreading globally in humans [57, 79]. The *bla*$_{\text{CTX-M-15}}$ genes are commonly found in large plasmids that often carry other ARGs, including *bla*$_{\text{TEM-1}}$, *tetA*, *bla*$_{\text{OXA-1}}$, and *aac(6′)-lb-cr*, classifying most of them as members of incompatibility group F [18]. In this study, however, *bla*$_{\text{CTX-M-15}}$-harboring *E. coli* belong to phylogenetic group D and ST38, not B2-O25b:H4-ST131. In France, the *bla*$_{\text{CTX-M-15}}$-carrying plasmids from cattle were derived from non-ST131 *E. coli* isolates that were highly similar to those found in ST131 *E. coli* isolates in humans [65]. *bla*$_{\text{CTX-M-15}}$-carrying plasmids from human clinical isolates derived from ST131 *E. coli* have recently been reported in Japan [57, 69], although the molecular structure of the plasmids has not been examined. Therefore, the *bla*$_{\text{CTX-M-15}}$-carrying plasmids derived from non-ST131 *E. coli* in this study and those from ST131 *E. coli* isolates in humans were not comparable. In future studies, we will carefully monitor the ESBL-producing bacteria in food-producing animals, flies, and humans isolates.

In addition to cattle barns, flies are also observed in pig pens and hen houses [31, 62], and ESBL-producing bacteria have been observed in swine and poultry feces [60]. ESBL genes derived from swine and poultry feces may also be capable of transferring to the pathogenic bacteria in fly gut. Although most flies do not travel a distance greater than two miles, certain individual flies can travel as far as 20 miles [31]. It has been suggested that flies carry several bacterial pathogens of humans from hospitals into neighboring communities and vice versa [82]. Therefore, flies could transfer ARB from farms into the urban area. To prevent the transmission of ESBL-producing bacteria from food-producing animals to humans, pest control in the rearing environment of food-producing animals would be most effective. Multi-farm and urban area studies are now in progress in order to confirm this hypothesis [103].

In this Chapter, flies are vectors in the transmission of ESBL-producing bacteria from food-producing animals to humans. Ensuring good practices and hygiene around calving areas is important for reducing the dissemination of ESBL-producing bacteria.

1.5. Summary of Chapter 1

The spreading of ARB and ARGs from food-producing animals to humans has been a subject of increasing concern. To clarify the role of flies in spreading the ESBL gene from food-producing animals to humans, we isolated and characterized a third-generation cephalosporin resistant *E. coli* strain from flies and cattle feces from a cattle barn. Cephalosporin resistant strains were isolated from 14.3% (13/91) of houseflies, 10.3% (7/68) of false stable flies, and 7.5% (7/93) of cattle feces. Twenty-seven cephalosporin resistant strains were tested for the presence of ARG. Of the 27 samples, 22 isolates from 11 houseflies, 5 false stable flies, and 6 cattle feces samples harbored the *bla*$_{\text{CTX-M-15}}$ gene. All *bla*$_{\text{CTX-M-15}}$-harboring isolates belonged to phylogenetic group D and the ST38 clonal group. Analysis of PFGE showed that these isolates were divided into two clusters, indicating that flies carried several of the same clones that were detected in cattle feces. All *bla*$_{\text{CTX-M-15}}$ gene-harboring plasmids were transferable and were members of incompatibility group FIB. These results suggest that transferable plasmids encoding ESBL were prevalent among flies and cattle. As vectors, flies may play an important role in spreading ESBL-producing bacteria from food-producing animals to humans.

CHAPTER 2

Horizontal transfer of plasmid-mediated cephalosporin resistance genes in the intestine of houseflies (*Musca domestica*)

2.1. Introduction

Antimicrobials are used for treating and preventing infectious diseases in human and veterinary clinical settings. ARB have been selected and maintained under antimicrobial selective pressures [84, 98], and these conditions promoted the horizontal transfer of ARGs [85]. ARB derived from livestock have spread through foods and the environment to humans [105]. Insects, in particular, have played a crucial role in the dissemination of ARB through the environment because they inhabit the living environments of both humans and livestock [7, 28, 64].

Insects serve as a mechanical vector for ARB and pathogenic bacteria [28, 34, 42, 47, 103]. Moreover, the transfer of ARGs has been shown to occur in the intestine of insects [3, 42, 43, 79, 80], which poses a potential risk for the emergence of new ARB when ARGs are transferred to enteric bacteria in the intestine of insects. However, whether ARGs are transferred efficiently and stably in the insect intestine remains unclear. Previously in Chapter 1, we showed that closely related third-generation cephalosporin-resistant *E. coli* derived from houseflies and cattle feces were found in a farm [102]. If the intestine of houseflies is a site where transfer of ARGs occurs efficiently and stably, houseflies would pose a high risk, because of their strong flight ability [31], for the dissemination of farm-derived cephalosporin-resistant bacteria, a farm to humans by functioning as a biological vector.

Third-generation cephalosporin is one of the most important antimicrobials used in humans and animals because it can be used in a broad range of clinical treatments and it exerts few side effects. Resistance to third-generation cephalosporins mediated by CTX-M type of ESBL and CMY-2 type of AmpC β-lactamase has been commonly detected in humans and animals, and their resistance genes are often located on multidrug resistance plasmids [56, 60, 89, 99]. Therefore, the transfer of plasmids containing cephalosporin-resistance genes is a major risk for the occurrence of multidrug-resistant bacteria.

In this Chapter, to clarify whether cephalosporin-resistance genes are transferred efficiently and stably in the intestine of houseflies, we compared the transfer frequency measured in conjugation experiments conducted *in vivo* (in the housefly intestine) and *in vitro* by using *E. coli* harboring plasmid-mediated cephalosporin-resistance genes.

2.2. Materials and Methods

2.2.1. Strains of bacteria and houseflies

We used four *E. coli* strains harboring a third-generation cephalosporin-resistance gene (*bla*$_{\text{CTX-M}}$ or *bla*$_{\text{CMY-2}}$) on a plasmid as the donor strains (Table 3). *E. coli* ML4909 and *E. coli* DH5α, which exhibit chromosomal-encoded resistance to rifampicin, were used as the recipient strains [84, 101]. Conjugation experiments were conducted using eight combinations of these four donor strains and two recipient strains.

CSMA strain houseflies (*M. domestica*) were obtained from Sumika Life Tech, Ltd. (Osaka, Japan), and maintained through multiple generations in nets at 25°C on a 14-hr:10-hr light/dark cycle in an isolator. The houseflies were provided distilled water and a 1:1 mixture of skim milk and sugar. We used adult houseflies 8–15 days after emergence.

Table 3. Characterization of *E. coli* used in this study

Strain	Origin	Plasmid size[a] (kbp)	Resistance genes[a]	Replicon type[a]	Resistance phenotype	Reference
133	Cattle	**120**	***bla*$_{\text{CTX-M-15}}$**, *bla*$_{\text{TEM}}$, *tetA*	**FIB**, K/B	cefotaxime	[102]
TC7-1	Cattle	**50**, 70	***bla*$_{\text{CTX-M-2}}$**, *tetA*	**N**, FIA, FIB	cefotaxime, tetracycline	[84]
TC13-1	Cattle	**110**	***bla*$_{\text{CTX-M-14}}$**	**I1**	cefotaxime	[84]
TC7-9	Cattle	**100**	***bla*$_{\text{CMY-2}}$**	**I1**, FIB	cefotaxime	[84]

[a]Bold type indicates third-generation cephalosporin-resistance genes and their bearing plasmids

2.2.2. *In vivo* conjugation experiments

Approximately, 30 houseflies (8–15 days after emergence) were transferred to a cage (15 cm × 15 cm × 90 cm). A milk–sugar solution (MS) containing 1.56 g of skim milk and 1.67 g of sugar dissolved in 93 ml of sterilized distilled water was fed overnight to houseflies *ad libitum* [3]. The next day, the houseflies ingested the donor strain suspension for 6 hr *ad libitum* and then the recipient strain suspension

for 3 hr *ad libitum*. The suspensions were prepared as follows. Donor strain and recipient strain cultures were grown in 25 mL of TSB (Bacto™) at 37°C for 14–18 hr and then centrifuged for 15 min at 1,800×*g*. The supernatants were discarded, the pellets were resuspended in 5 mL of MS, and the suspensions were incubated for 1 hr at 37°C [79]. Houseflies received the suspensions in a sterile plate. After the houseflies had ingested the recipient strain suspension, skim milk, sugar, and sterilized distilled water were provided for 24 hr *ad libitum*. Next, the houseflies were surface sterilized with sodium hypochlorite and ethanol as described in Chapter 1 [64]. To prepare each sample, five sterilized houseflies were pooled and homogenized in 2.5 mL of PBS, and five samples in each *in vivo* conjugation experiment were quantitated by performing serial dilution and then plating onto DHL agar supplemented with 4 μg/mL cefotaxime (Sigma-Aldrich), 50 μg/mL rifampicin, and 4 μg/mL cefotaxime + 50 μg/mL rifampicin for the donor, recipient, and TCs, respectively [79, 102]. After incubation for 24 hr at 37°C, colonies were counted to determine the viable count (CFU/mL) of donors, recipients, and TCs. The transfer frequency was calculated as the ratio of TCs number to donor number (TCs/donor). The experiment was repeated thrice, and two putative TCs were randomly selected from each sample for further analysis.

2.2.3. *In vitro* conjugation experiments

The *in vitro* conjugation experiment was conducted using the broth-mating method [101]. Briefly, overnight cultures of the donor strain (100 μL) and the recipient strain (100 μL) were mixed with 1,800 μL of fresh TSB and incubated for 24 hr at 37°C. Next, the mixtures were quantified as described for the in *vivo* conjugation experiment. The experiment was repeated thrice, and two putative TCs were randomly selected from each experiment for further analysis.

2.2.4. Analysis of TCs

All putative TCs were tested for the possession of ARGs and plasmids as

donors by performing PCR amplification, PCR-based replicon typing, and plasmid profiling. The primers used in PCR were described previously [9, 56, 87, 116]. Plasmid profiling was conducted using a modified alkaline lysis method [50] and identified the plasmid size.

To detect the third-generation cephalosporin-resistance gene-encoding plasmid, we performed PFGE of whole bacterial DNA digested with S1 nuclease (S1-PFGE) and Southern blot hybridization as described in Chapter 1 previously [76, 84]. PFGE was performed by using the CHEF-DRIII system under the following conditions: switch time, 4–45 sec; run time, 17 hr; included angle, 120°; voltage, 6 V/cm; and temperature, 14°C. Lambda DNA ladders in InCert Agarose Gel Plugs (Lonza, Rockland, USA) were used as the size standard. DNA probes for the third-generation cephalosporin-resistance genes (*bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY-2}}$) were obtained using a PCR DIG Probe Synthesis Kit, according to the manufacturer's instructions [84]. We also performed Southern blotting with ARGs (*bla*$_{\text{TEM}}$ and *tetA*) and replicon typing (IncFIB, IncN, and IncI1) in the same manner [9, 56, 87].

All TCs were used as the donor to assess the secondary transfer of cephalosporin-resistance genes by using the broth-mating method, as described above. In this experiment, enrofloxacin-resistant *E. coli* DH5α was used as a recipient strain. Enrofloxacin-resistant *E. coli* DH5α was established in this study as follows. Briefly, the *E. coli* DH5α culture was inoculated on the Mueller-Hinton agar supplemented with 0.05 μg/mL enrofloxacin (Sigma-Aldrich), and then the colonies that grew on these plates were inoculated on higher concentration plates. This procedure was repeated up to a concentration of 4 μg/mL, and the colony that grew on the plate was used as enrofloxacin-resistant *E. coli* DH5α.

TCs isolated from the *in vivo* conjugation experiment were characterized using PFGE. PFGE analysis was performed according to the Pulse Net CDC protocol by using the restriction enzyme *Xba*I as described above Chapter 1 [12]. TCs that differed from the donor and recipient strains were identified using either API20E or API20NE (Sysmex) (identification rate, >80%). The strains whose API

identification rates were <80% were identified using their 16S rRNA gene sequence. The 16S rRNA gene was amplified as previously described [63]. The amplified product was purified using MBS®Spin PCRace (Invitek, Inc. CA, USA) and sequenced in both directions by using the primers used in the PCR; nucleotide sequences were determined by using the BigDye Terminator, version 3.1, Cycle Sequencing Kit with an automated DNA sequencer (ABI 3130; Applied Biosystems). The sequencing results were analyzed using BioEdit Sequence Alignment Editor software (http://mbio.ncsu.edu/bioedit/bioedit.html) and the Basic Local Alignment Search Tool.

2.3. Results

2.3.1. *In vivo* conjugations

TCs were isolated in only three out of eight tested combinations (Donor/Recipient: TC7-1/ML4909, TC7-1/DH5α, and TC7-9/DH5α). In the three combinations in which TCs were isolated, the *in vivo* transfer frequency (TCs/Donor) ranged from 2.0×10^{-4} to 5.7×10^{-5} (Table 4). In these three combinations, donors, recipients, and TCs were isolated from the intestine of houseflies in the concentration ranges of 4.0×10^4 to 6.7×10^5 CFU/mL, 7.9×10^3 to 6.1×10^4 CFU/mL, and 2.0×10^1 to 3.8×10^1 CFU/mL, respectively. TCs were isolated from more than half of the samples in each of the three combinations.

Table 4. TCs detected combinations in the *in vivo* conjugation experiment

		CFU count *in vivo* (CFU/mL)			Transfer frequency (TCs/Donor)	
Donor	Recipient	Donor	Recipient	TCs	*in vitro*	*in vivo*
TC7-1	ML4909	6.7×10^5	6.1×10^4	3.8×10^1	3.8×10^{-2}	5.7×10^{-5}
TC7-1	DH5α	4.0×10^4	1.6×10^4	2.1×10^1	1.3×10^{-4}	5.2×10^{-4}
TC7-9	DH5α	9.9×10^4	7.9×10^3	2.0×10^1	2.2×10^{-4}	2.0×10^{-4}

2.3.2. *In vitro* conjugations

When ML4909 and DH5α were used as recipients, transfer frequencies (TCs/Donor) ranged from 3.8×10^{-2} to 2.7×10^{-8} and from 1.3×10^{-4} to 7.5×10^{-9} *in vitro*, respectively (Table 5). The transfer frequency differed according to the recipient strains, and the donor strains TC7-1 and TC7-9 showed the highest transfer frequencies.

Table 5. *In vitro* transfer frequency

Recipient	TCs/Donor			
	133*	TC7-1*	TC13-1*	TC7-9*
ML4909	2.7×10^{-8}	3.8×10^{-2}	6.1×10^{-7}	6.7×10^{-5}
DH5α	7.5×10^{-9}	1.3×10^{-4}	1.1×10^{-8}	2.2×10^{-4}

*Donor strains

2.3.3. S1-PFGE and Southern blot hybridization

S1-PFGE and Southern blot hybridization results demonstrated the transfer of plasmids encoding third-generation cephalosporin-resistance genes (Fig. 3). In addition, the donor strain 133 and its TCs both possessed *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, and *tetA* on the 120-kbp plasmid, and the donor strain TC7-1 and its TCs both possessed *bla*$_{\text{CTX-M}}$ and *tetA* on the 40-kbp plasmid.

2.3.4. Characterization of TCs

PFGE profiles revealed that TCs from 5 out of the 28 samples from which TCs were isolated differed from the donor and the recipient. These TCs strains were identified as *E. coli* strains that differed from the donor and the recipient by API20E (from three samples), *Achromobacter* sp. (GenBank accession No. KM117218.1) by the 16S rRNA gene sequence (from one sample), and *Pseudomonas fluorescens* by API20NE (from one sample). The plasmid of TC7-1 was transferred to *E. coli* that differed from the donor and the recipient and to *Achromobacter* sp. The plasmid of TC7-9 was transferred to *P. fluorescens*. All TCs strains except *P. fluorescens* showed secondary transfer of cephalosporin-resistance genes to enrofloxacin-resistant *E. coli* DH5α. Secondary cephalosporin-resistance genes transfer frequencies (TCs/Donor) ranged from 9.4×10^{-7} to 7.7×10^{-8}.

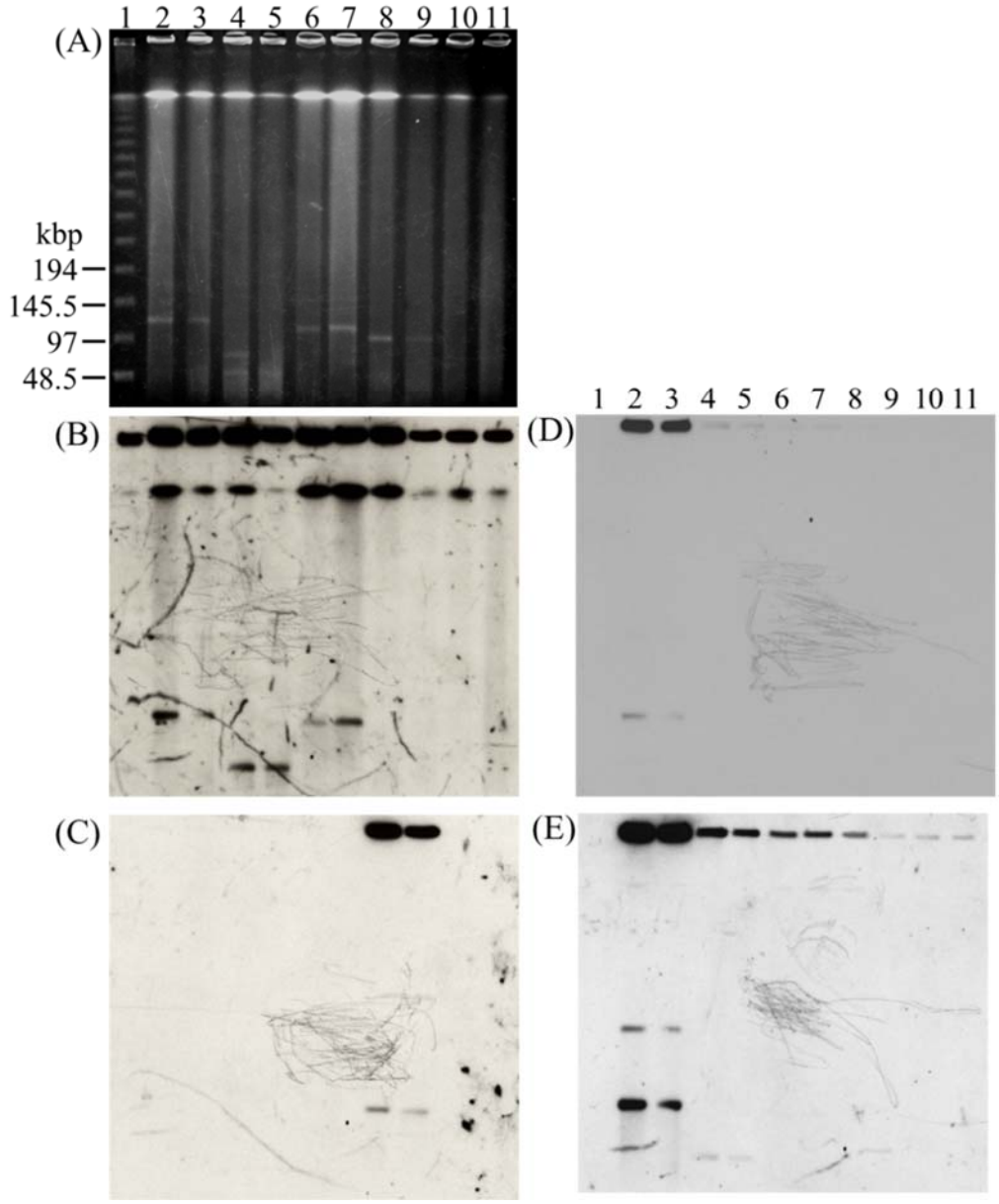


Fig. 3. S1-PFGE and Southern blot hybridization of third-generation cephalosporin-resistance genes. (A) PFGE analysis of representative strains digested with S1 nuclease. (B) Southern blot hybridization with *bla*$_{\text{CTX-M}}$ consequence probe. (C) Southern blot hybridization with *bla*$_{\text{CMY-2}}$ probe. (D) Southern blot hybridization with *bla*$_{\text{TEM}}$ probe. (E) Southern blot hybridization with *tetA* probe. Lane 1: 48.5 kb DNA ladder; lane 2: 133; lane 3: TCs from 133; lane 4: TC7-1; lane 5: TCs from TC7-1; lane 6: TC13-1; lane 7: TCs from TC13-1; lane 8: TC7-9; lane 9: TCs from TC7-9; lane 10: ML4909; lane 11: DH5α

2.4. Discussion

This study showed that plasmid-mediated horizontal transfer of third-generation cephalosporin-resistance genes occurred in the intestine of houseflies. We reported previously in Chapter 1 that plasmid-mediated third-generation cephalosporin-resistant *E. coli* was isolated from the intestine of houseflies [102]. Moreover, houseflies are involved in the dissemination of plasmids carrying ARGs [103]. Therefore, houseflies could be regarded as not only a mechanical vector for the dissemination of ARB but also a biological vector for the occurrence of new ARB through the horizontal transfer of plasmids carrying ARGs.

Third-generation cephalosporin-resistance genes were transferred to not only recipient strains but also enteric bacteria of houseflies, such as *E. coli* that differed from donors and recipients, *Achromobacter* sp., and *P. fluorescens*. Genus of *Achromobacter* and *Pseudomonas* usually existed in houseflies [28, 34]. These bacteria occasionally caused opportunistic infectious diseases in humans [45, 91]. Although identification of bacterial species based on the 16S rRNA gene sequence is sometimes incorrect [67], the housefly intestine has been reported to contain various types of bacteria, including pathogenic bacteria such as *E. coli* O157:H7, *Shigella* sp., *Proteus mirabilis*, and *Staphylococcus aureus* [28, 34, 47, 108]. Moreover, houseflies, acting as mechanical vectors, are involved in transmitting bacterial infections in human living environments [16]. These results suggest that third-generation cephalosporin-resistance genes are capable of transfer to pathogenic bacteria in the intestine of houseflies, and this would make difficult to treat infectious diseases by using antimicrobials.

The *in vivo* transfer frequency measured in this study was either at the same level as or at a lower level than that *in vitro*, or the transfer of cephalosporin-resistance genes did not occur *in vivo*. In the *in vitro* conjugation experiment, only specific bacteria were included, whereas in the *in vivo* conjugation experiment, the intestine of houseflies contained myriad enteric bacteria [28, 34, 108,

109] and contacts between donors and recipients were therefore restricted. In contrast, enteric bacteria readily came into contact with donor strains in the intestine of houseflies. Indeed, in this study, cephalosporin-resistance genes were transferred to enteric bacteria across genera. Previous studies showed interspecies transfer of ARGs, including enteric bacteria in the intestine [42, 43, 80, 85]. These results suggest that the potential diversity regarding interspecies transfer of ARGs was substantially increased in the intestine. Thus, the housefly intestine can be considered a suitable site for the transfer of ARGs.

In this study, the transfer of a multidrug resistance plasmid carrying cephalosporin- and tetracycline-resistance genes was observed in the intestine of houseflies in the absence of antimicrobial selective pressure. Antimicrobial activity, especially cephalosporin activity, in feces and soil has been shown to persist for a long period and contributes to the circulation of ARB [19, 93, 94, 95]. A previous study showed that the antimicrobial-resistance rates of *E. coli* derived from houseflies reflected that derived from livestock feces in the same locations [103]. Thus, houseflies are not directly treated with antimicrobials but are likely exposed to antimicrobials through feces and environmental residues as a result of feeding. Generally, the transfer of ARGs was increased under antimicrobial selective pressure [85], and multidrug resistance plasmids maintained and promoted the transfer of ARGs under the influence of various types of antimicrobials [63]. Moreover, even in the absence of selective pressure, ARB were maintained in the intestine of houseflies [109]. Houseflies would play a crucial role for maintaining ARB by some selective pressure, similar to antimicrobials, including a host innate immunity such as antimicrobial peptides [118]. According to the above results, it is suggested that the transfer of ARGs in the intestine of houseflies likely occurred more frequently than we observed.

In conclusion, houseflies act as a biological vector for the dissemination of ARB to elicit the occurrence of new ARB through plasmid-mediated horizontal transfer of ARGs in their intestine. Moreover, houseflies posed strong flight ability

to travel as much as nearly 10 km [31] and served as a mechanical vector for ARB in farm and urban areas [7, 64]. Consequently, reduction of the transmission of infections could be achieved by control of houseflies [16]. Thus, hygiene management, including the control of insects, is crucial for preventing the dissemination of ARB.

2.5. Summary of Chapter 2

Houseflies are a mechanical vector for various types of bacteria, including ARB. If the intestine of houseflies is a suitable site for the transfer of ARGs, houseflies could also serve as a biological vector for ARB. To clarify whether cephalosporin-resistance genes are transferred efficiently in the housefly intestine, we compared with conjugation experiments *in vivo* (in the intestine) and *in vitro* by using *E. coli* with eight combinations of four donor and two recipient strains harboring plasmid-mediated cephalosporin-resistance genes and chromosomal-encoded rifampicin-resistance genes, respectively. In the *in vivo* conjugation experiment, houseflies ingested donor strains for 6 hr and then recipient strains for 3 hr, and 24 hr later, the houseflies were surface sterilized and analyzed. *In vitro* conjugation experiments were conducted using the broth-mating method. In 3/8 combinations, the in *vitro* transfer frequency (TCs/Donor) was $\geq 1.3 \times 10^{-4}$; the *in vivo* transfer rates of cephalosporin-resistance genes ranged from 2.0×10^{-4} to 5.7×10^{-5}. Moreover, cephalosporin-resistance genes were transferred to other species of enteric bacteria of houseflies such as *Achromobacter* sp. and *P. fluorescens*. These results suggest that houseflies are not only a mechanical vector for ARB but also a biological vector for the occurrence of new ARB through the horizontal transfer of ARGs in their intestine.

CHAPTER 3

The role of flies in the maintenance of antimicrobial resistance in farm environments

3.1. Introduction

ARB are an emerging threat in human and veterinary clinical settings and are spread throughout the environment by water, soil, air, wild animals, and insects [111]. ARB are thought to circulate continually between clinical settings and the environment, contributing to the long-term maintenance of ARB [44]. Such maintenance of ARB in and around livestock farms could lead to ARB contamination of livestock products and consequently lead to the transmission of ARB from livestock to humans via the food chain.

Insects, including flies, normally inhabit the areas surrounding farms where they act as vectors for the dissemination of many microorganisms, including ARB [119]. We have proposed in Chapter 2 that flies not only act as a mechanical vector of ARB in the farm environment, but also as a biological vector, involved in the horizontal gene transfer of ARGs-harboring plasmids in their intestines [29, 101, 102]. Recently, Pava-Ripoll *et al*. have demonstrated that pathogenic bacteria ingested by adult flies are transmitted vertically to the next generation [78]. However, the dynamics of ARB maintenance and the carriage of the ARG-carrying plasmids over the fly life cycle remain unclear. In the absence of antimicrobial selective pressure, the growth rates of ARB are typically inferior relative to wild-type strains [17] and should therefore be eliminated by natural selection. However, other selective pressures may exist in the fly, such as host innate immunity through antimicrobial peptides, that would contribute to the maintenance of ARB [25]. If it is found that ARB and ARGs can persist throughout the life cycle of a fly, they would play an important role as a reservoir that maintain ARB in the farm environment.

In livestock-rearing environments, a great deal of attention is paid to the carriage of microorganisms between internally- and externally-housed livestock in order to control the spread of ARB and livestock-associated infectious diseases. However, this strategy has not been entirely successful because the transmission routes for many microorganisms have not yet been clarified [58, 106]. A previous

study suggested that application of fly-screens to broiler farms reduced the introduction of *Campylobacter* spp. into the internal housing area within chicken enclosures [35]. Based on this observation, we propose that a fly-mediated transmission route for inside of livestock barns might consist of maggots hatched from eggs laid by flies that originated in put outside of livestock barns. As maggots often contain microorganisms carried by their parental flies [78], livestock infection by these microorganisms in close contact with maggots is a distinct possibility.

To further investigate this possibility, the aim of this Chapter was to clarify the role of flies in maintaining ARB in the farm environment. We evaluated the fate of ingested antimicrobial-resistant *E. coli* and ARGs-carrying plasmids throughout the fly life cycle. In addition, to elucidate the possible transmission routes of ARB from flies to livestock, we orally administered maggots containing antimicrobial-resistant *E. coli* to chickens and quantified these bacteria over time in cecal samples.

3.2. Materials and Methods

3.2.1. Houseflies bacterial ingestion and rearing

CSMA housefly strain (*M. domestica*) was maintained as described above in Chapter 2, and used in this study [29]. *E. coli* TC7-1 strain used in this study [29], described below, and ARGs harbored by the ingested bacteria (*tetA* and *bla*$_{\text{CTX-M-2}}$) were not naturally carried by this housefly strain.

An overview of the experimental design is shown in Table 6. Approximately 100 adult houseflies (1 d after emergence) were transferred to a cage (15 × 15 × 90 cm) on Day 1. The houseflies were fed with suspensions of *E. coli* TC7-1 strain (resistant to cefazoline, cefotaxime, spectinomycin, and tetracycline) that carried a plasmid containing two ARGs (*tetA* and *bla*$_{\text{CTX-M-2}}$) twice a day, on Days 1 to 5, *ad libitum* [29]. To prepare the bacterial suspensions, cultures for ingestion were grown in 25 mL of TSB at 37°C for 14 to 18 hr and then centrifuged for 15 min at 1800×*g*. The supernatants were discarded and the pellets resuspended in 5 mL of a milk-sugar solution that had been previously sterilized at 115°C for 20 min. Suspensions were then incubated for 1 hr at 37°C. Houseflies were provided with suspensions on a sterile plate for ingestion. After the bacteria were ingested, houseflies were transferred to a new cage. Skim milk, sugar, and sterilized distilled water were provided for 24 hr *ad libitum* on Day 6. On Day 7, an oviposition substrate was placed in the cage to collect housefly eggs. The substrate contained a 1:7:14 (w/w/w) mixture of autoclaved rodent diet CE-2 (CLEA Japan, Inc., Tokyo, Japan), autoclaved bran (Oriental Yeast, Tokyo, Japan), and sterilized distilled water. Once the housefly eggs were laid, the oviposition substrate was recovered from the cages. The housefly eggs were surface-sterilized with sodium hypochlorite and ethanol, as described in above Chapters [64], and then transferred to a new oviposition substrate. The substrate, containing hatched maggots, was placed in an isolator at 25°C with a 14-hr:10-hr light/dark cycle until pupation (ca. 6–7 d). Next, the pupae were surface-sterilized as previously described and transferred to a Petri dish to allow the

F1 adult houseflies to emerge, avoiding cross-contamination with the substrate. The pupae in the Petri dish were placed in a new cage under the same conditions until the emergence of F1 adult houseflies (ca. 4–5 d). Houseflies were collected over several days for bacterial isolation and DNA extraction. Specifically, adult houseflies were collected on Day 0, before ingestion of bacteria and Day 7, after oviposition, eggs were collected on Day 7, maggots were collected on Days 10 and 12, pupae were collected on Days 14 and 16, and F1 adult houseflies were collected on Days 18 or 19. Maggots from Day 10 were surface-sterilized and stored at -80°C until needed for the experimental chicken model. Housefly rearing with bacterial ingestion was repeated three times. As a control for checking the involvement of bacterial ingestion in the development of a control group of houseflies under the same conditions, the development of houseflies reared without bacterial ingestion was conducted as for those with bacterial ingestion. A difference in the development of houseflies was not observed.

3.2.2. Bacterial isolation and DNA extraction

Surface-sterilized individual adult houseflies, maggots, pupae, F1 adult houseflies, and 10 pooled eggs were each homogenized in 0.5 mL of PBS. The persistence of the ingested bacteria was evaluated in three homogenized samples from each sampling day by plating the serial dilution of samples onto DHL agar supplemented with 4 μg/mL of cefotaxime [29]. After incubation for 24 hr at 37°C, the colonies were counted to determine viable counts (CFU/sample). In addition, the last distilled water rinse after surface-sterilization were plated on tryptic soy agar (Bact^{TM}) to confirm the complete removal of bacteria from each sample's surface. DNA was extracted from surface-sterilized samples using the ISOFECAL kit (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Three extracted DNA samples from each sampling day were used in a qPCR.

qPCR was performed to determine the copy number of *tetA* and 16S rRNA genes. To generate standard curves, the *tetA* and 16S rRNA genes of *E. coli* strain

TC7-1 were amplified using *tetA* gene-specific primers (F; 5′-GCGCCTTTCCTTTGGGTTCTC-3′ and R; 5′-CGTGATCGGGAGTATCTGGCTG-3′) that were designed according to the GenBank *tetA* sequence (GenBank accession number; KT950741), in addition to 16S rRNA gene universal primers [67]. The PCR products were cloned into a pTA2 vector and inserted into *E. coli* strain DH5α using a TArget Clone™ kit (TOYOBO, Osaka, Japan), according to the manufacturer's instructions. Transformants harboring the recombinant plasmid were selected and the plasmids extracted using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The concentrations of purified recombinant plasmid DNA in the samples were determined spectrophotometrically (BioSpectrometer; Eppendorf, Hamburg, Germany). qPCR was performed using SYBR® Premix Ex *Taq*™ II (Takara Bio) in 20 μL reactions containing 5 μL of template DNA and 0.4 μM of each primer [73, 109]. The thermal cycling conditions included a holding stage for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C, and 30 s at 50°C (for *tetA*) and 60°C for (16S rRNA) in a LightCycler 480 system (Roche).

Table 6. Experimental design of the housefly-rearing experiment

Fly life stage	**Day**	**Experimental process**
Adult housefly	1–5	・Ingestion of bacterial suspension
	6	・Transfer to a new cage
		・Feeding (skim milk, sugar, and sterilized water)
	7	・Placement of oviposition substrate in cages
		・Sample collection
Egg	7	・Surface sterilization and transfer of new oviposition substrate to a new cage
Maggot	10, 12	・Sample collection
Pupa	14, 16	・Sample collection
	16	・Surface sterilization and transfer to a Petri dish in a new cage
F1 adult housefly	18–19	・Sample collection

3.2.3. Experimental chicken model

Animal experimentation protocol was approved by President of Kitasato University through the judgment by Institutional Animal Care and Use Committee of Kitasato University (Approval no. 15-135) [75]. The chickens were reared in individual wire cages in an isolation building with a non-medicated layer ration and water supplied *ad libitum*.

Chickens (30 days-old; "Day 30") were assigned to three groups (n = 5 per group). On Days 30 and 32, the chickens were orally inoculated in the following groups; group 1 was a model for the fly-associated ARB transmission route and consisted of 10 pooled maggots containing *E. coli* strain TC7-1 (ca. 10^3 CFU/maggot), prepared as previously described; group 2 was a positive control consisting of a suspension of *E. coli* strain TC7-1 in saline (10^9 CFU/chicken); and group 3 was a negative control inoculated with saline.

Cecal droppings or contents were collected on Days 31, 32, 33, 36, 38, 40, 43, and 46. The samples were homogenized in PBS. ARB were quantified by performing serial dilution in PBS and plating onto DHL supplemented with 32 μg/mL of cefazolin and 32 μg/mL of spectinomycin (Sigma-Aldrich) for *E. coli* strain TC7-1. The colonies were counted after incubation for 24 hr at 37°C to determine the viable cell count [CFU/cecal sample (g)].

3.2.4. Data analysis

Data are presented as box-and-whisker plots, where the maximum and minimum values are displayed as whiskers. The interquartile ranges indicate quartile distribution [the distance between the third (75%) and first (25%) quartiles] and are displayed as boxes.

Statistical significance was determined using a Mann-Whitney U test, with the significance threshold set at $p < 0.05$.

3.3. Results

3.3.1. Persistence of the ingested ARB in houseflies throughout their life cycle

The ingested antimicrobial-resistant *E. coli* strain persisted in the houseflies throughout their life cycle, from adult houseflies, as they proceeded through oviposition and metamorphosis, to the next generation of adult houseflies. Adult houseflies (Day 7) harbored 10^2-10^5 CFU of the ingested *E. coli* per housefly (Fig. 4A). After oviposition, five of the nine pooled egg samples (Day 7) contained the ingested strain, with a mean CFU per sample of 2.1×10^1. On Days 10 and 12, this value increased to 1.7×10^3 and 8.9×10^3 CFU/maggot, respectively. After the maggots had pupated, the counts decreased to an average of 10^1 CFU/pupae (Days 14 and 16). Five of the nine newly-emerged F1 adult houseflies (Days 18 to 19) harbored 1.3×10^1 CFU/housefly. No bacteria were detected in any of the final distilled water rinse from the last step of the surface sterilization procedure.

3.3.2. Persistence of the ingested plasmid-encoded *tetA* gene in houseflies throughout their life cycle

The results of qPCR revealed that there were between 10^3 and 10^{10} copies of *tetA* gene per adult housefly (Fig. 4B). The *tetA* gene was detected in five of the nine pooled egg samples, with a mean number of 1.1×10^3 copies/sample. Later in the fly life cycle, the *tetA* gene copy number increased from 5.4×10^4 to 7.7×10^4 copies/maggots. Copy number was then maintained throughout pupation, albeit at somewhat lower levels of 1.4×10^3 to 8.2×10^3 copies/pupae on Days 14 and 16, respectively. Finally, the *tetA* gene was identified in five out of nine newly-emerged F1 adult houseflies, with an average of 2.1×10^3 copies/housefly.

To assess the potential persistence of *tetA* gene in houseflies throughout their life cycle, the ratios of *tetA* to 16S rRNA gene copies were determined (Fig. 4C). The mean copy number of 16S rRNA gene ranged from 10^5 to 10^9 copies/sample [adult houseflies, 3.0×10^8; eggs, 1.9×10^5; maggots, 1.7×10^8 (Day

10) and 7.2×10^8 (Day 12); pupae, 6.2×10^7 (Day 14) and 1.8×10^8 (Day 16); F1 adult houseflies, 1.2×10^8]. The mean ratio of *tetA* to 16S rRNA gene copies ranged from 4.2×10^{-5} to 8.5×10^{-3} per sample, except in adult houseflies (2.2×10^{-1} per sample).

3.3.3. Persistence of the ingested bacterial strain in the chicken model

After inoculation, *E. coli* strain TC7-1 was continuously detected in cecal samples from Days 31–46 in groups 1 and 2 (Fig. 5). The strain was not detected in samples from the negative control group (group 3). After inoculation with maggots harboring *E. coli* strain TC7-1 on Days 30 and 32, the mean concentration of bacteria in cecal droppings of the test chickens (group 1) first increased (1.6×10^5 CFU/g on Day 31, and 3.6×10^7 CFU/g on Day 33), and then gradually decreased (from 7.8×10^6 CFU/g on Day 36 to 1.3×10^6 CFU/g on Day 43) (Fig. 5A). On Day 40, *E. coli* strain TC7-1 was not detected in one of the five cecal droppings and on Day 43, it was not detected in two of the samples. The strain was detected in all cecal content samples on Day 46 (1.8×10^4 CFU/g). In group 2, the amount of *E. coli* strain TC7-1 in the cecal samples was 10^8 CFU/g following inoculations. This decreased gradually from 2.7×10^7 CFU/g on Day 36 to 4.0×10^7 CFU/g on Day 46 (Fig. 5B).

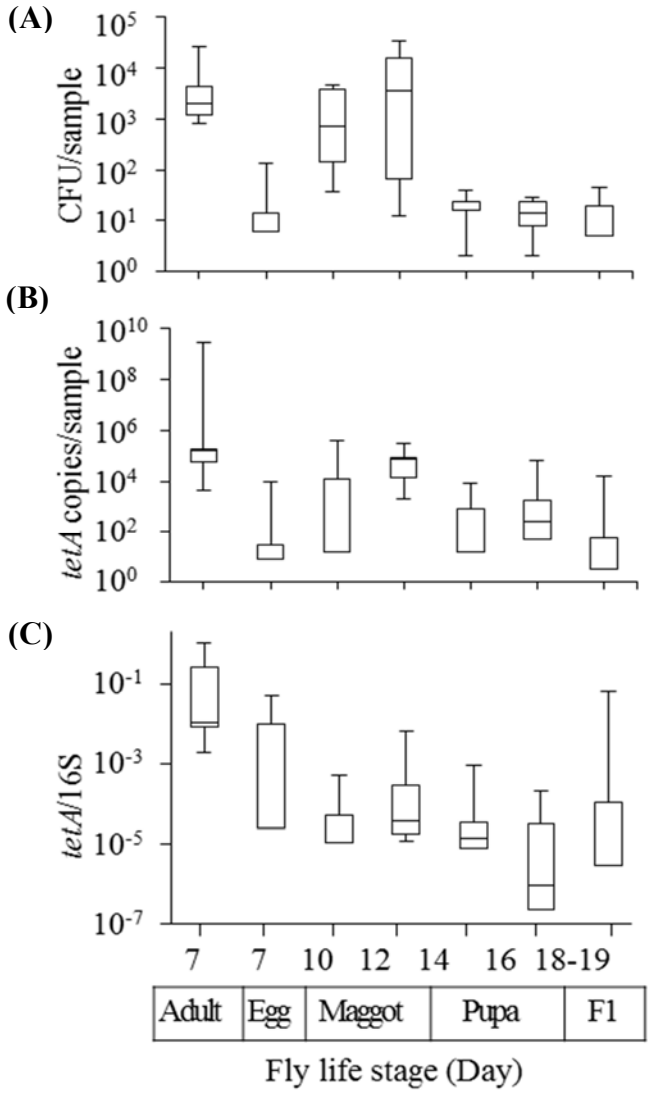


Fig. 4. Persistence of antimicrobial-resistant *E. coli* and ARGs in houseflies throughout their life cycle. (A) Persistence of ingested bacteria as assessed by bacterial isolation, plating, and colony counting. (B) Copy numbers of the *tetA* gene in fly samples, determined by qPCR. (C) The ratio of *tetA* to 16S rRNA gene copies in the samples, determined by qPCR.

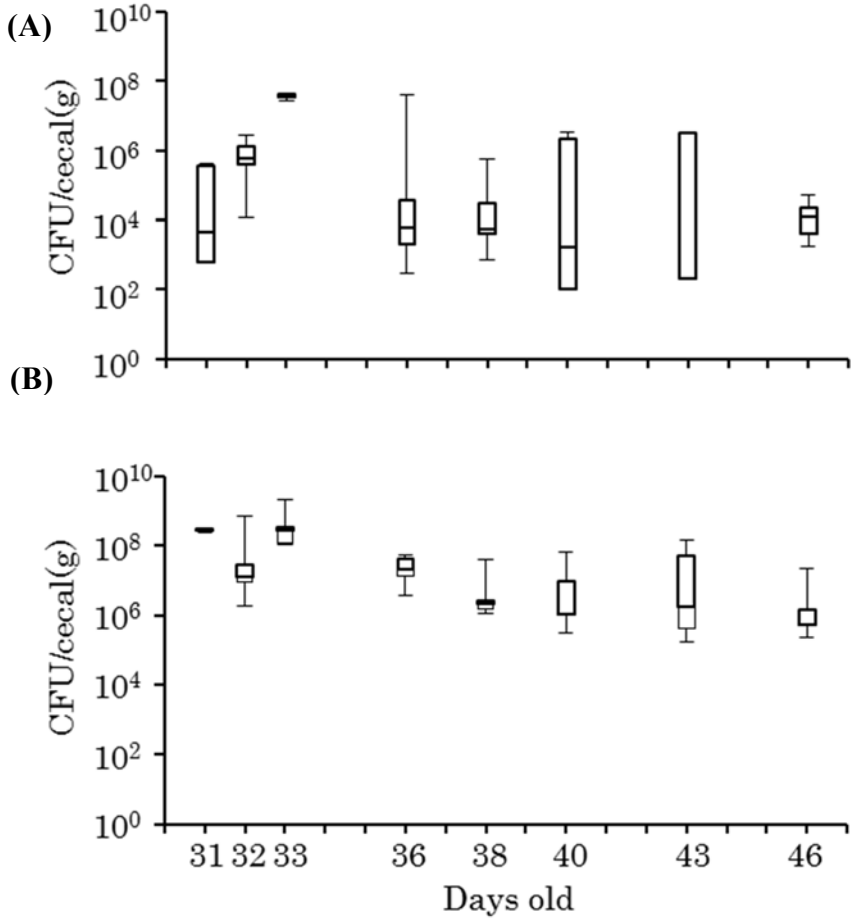


FIG. 5. Maintenance of antimicrobial-resistant *E. coli* strain TC7-1 in the intestine of orally-inoculated chickens. (A) Group 1, chickens inoculated with *E. coli* TC7-1–infected maggots (10 maggots/chicken, ca. 10^3 CFU/maggot). (B) Group 2, chickens inoculated with an *E. coli* strain TC7-1 cell suspension (10^9 CFU/chicken). n = 5 per group.

3.4. Discussion

Our study has revealed that antimicrobial-resistant *E. coli* can be vertically transmitted from adult houseflies to the next generation, despite previous studies reporting that the microbiota of the housefly intestine changes at different developmental stages [100, 108, 119]. The fly intestinal microbiota can be affected by various factors during the life cycle, including behavior and biological defense mechanisms [52, 117]. The persistence of ARB in our study suggests that ARB are selected and persisted in flies throughout their life cycle, though there is not selective pressure by antimicrobials. In addition, as flies can travel more than 10 km due to their strong flight capability [72], they could act as a vector to widely disseminate ARB [103]. This is compounded by the persistence of ARB throughout the life cycle of the fly.

In this study, the transmission of ingested bacteria to housefly eggs and subsequent stages of the fly life cycle was observed despite surface-sterilization of eggs and pupae before hatching or emergence, respectively. We found no bacteria in the final distilled water rinse after surface-sterilization, suggesting that ingested bacteria had contaminated the internal chorion of the egg. In a previous study, the transmission of ingested bacteria from adult houseflies to eggs has been demonstrated by directly plating surface-sterilized eggs. However, the presence of ingested bacteria in surface-sterilized eggs was not evaluated by histology or transmission electron microscopy [78] and some bacteria, including ARB, may be transmitted to the progeny due to strong adhesion to the egg surface.

A relatively consistent maintenance of ARGs (as assessed by the *tetA*/16S rRNA gene ratio) was detected in the microbiota of houseflies during a single generation in our study. Horizontal gene transfer of plasmids carrying ARGs between genera in the housefly intestine, including pathogenic and environmental bacteria, is therefore a potential risk that could contribute to the emergence of new ARB [29]. This would facilitate the maintenance of antimicrobial-resistance

plasmids in the environment [27]. However, antimicrobial-resistance plasmids generally impose a fitness cost in the absence of antimicrobial selective pressure [17, 33]. Despite this, no significant difference in the viability of ARB carrying antimicrobial-resistance plasmids and antimicrobial susceptible bacteria in the fly intestine has been observed [109]. The constant presence of ARGs and their antimicrobial-resistance in the housefly intestine may arise from a selective pressure in the fly.

Here, chickens were infected with maggots that harbored antimicrobial-resistant *E. coli* that persisted throughout the fly life cycle. This is supported by another study demonstrating that ARB were easily maintained in a broiler flock upon ARB infection [97]. ARB derived from livestock could therefore enter and circulate in the farm environment through transmission between inside and outside of livestock barns via flies and other insects [103, 119]. This suggests that vector control in farms is vital to decrease the risk of dissemination and maintenance of harmful microorganisms, including ARB. Additionally, we found that chickens carried ARB until they were 46 days old after infection at 31 days-old. Broiler chickens are usually shipped at approximately 50 days of age [97]. Therefore, the persistence of ARB in chicken intestines could lead to the presence of residual ARB on retail meat and subsequent transmission into the human through food chain [21]. In addition, antimicrobial-resistance plasmids could potentially be transferred to enteric bacteria residing in the chicken intestine [24], raising the possibility of ARG dissemination across genera. To prevent the spread of ARB from livestock to humans via food, our study suggests that ARB and vector control in the farm environment is essential [23].

In conclusion of this Chapter, flies can act as both a vector for the transmission of ARB and as a reservoir throughout their life cycle. Our data also suggest that they are a vector for ARB transmission between inside an outside of livestock barns. On farms, especially in the poultry industry, livestock are bred in an all-in/all-out replacement system. Therefore, it is important to inhibit the influx of

microorganisms and flies from the outside of livestock barn environment during the replacement period. Hygiene management, including the control of insects, may prevent the maintenance and circulation of ARB in the farm environment.

3.5. Summary of Chapter 3

Flies play an important role as vectors in the transmission of ARB and are hypothesized to transfer ARB between inside and outside of livestock barns. The aim of this study was to understand the role that flies may play in the maintenance of ARB in the farm environment. We first evaluated the fate of ingested antimicrobial-resistant *E. coli* harboring a plasmid containing ARGs throughout the housefly (*M. domestica*) life cycle, from adult to the subsequent F1 generation. Antimicrobial-resistant *E. coli* was isolated from different life cycle stages and ARG carriage quantified. The ingested *E. coli* persisted throughout the fly life cycle, and ARG carriage was maintained at a constant level in the housefly microbiota. To clarify the transmission of ARB from flies to livestock, 30 days-old chickens were inoculated with maggots containing antimicrobial-resistant *E. coli*. Based on the quantification of bacteria isolated from cecal samples, antimicrobial-resistant *E. coli* persisted in these chickens for at least 16 days. These results suggest that flies act as a reservoir of ARB through their life cycle and may therefore be involved in the maintenance and circulation of ARB in the farm environment.

CONCLUSION

First, we clarified the origin of ARB with respect to the vector in the farm (Chapter 1). *E. coli* was detected in non-binding flies that feed on feces, i.e., houseflies and false stable flies, but not in stable flies that feed on blood. Non-binding flies were found to carry several of the same ESBL-producing cephalosporin-resistant *E. coli* clones detected in cattle feces. The results of this study suggest that non-binding flies are mechanical vectors for transmitting ARB from food-producing animals to the environment and humans.

Next, we developed the role of flies as vectors in the spread of ARB and ARGs (Chapter 2). Plasmid-mediated horizontal gene transfer of ARGs was observed using antimicrobial-resistant *E. coli* in the intestine of houseflies. In addition, plasmid-mediated ARGs were transferred to other species of enteric bacteria in houseflies, occasionally causing opportunistic infectious diseases in humans. Our study suggests that flies are not only a mechanical vector for ARB, but also act as a biological vector for the dissemination of ARB and ARGs and, thus, elicit the occurrence of new ARB through the acquisition of ARGs in the intestines of flies.

Finally, we demonstrated that flies play a crucial role in the maintenance of ARB on farm environments (Chapter 3). Throughout the fly life cycle, antimicrobial-resistant *E. coli* strains were vertically transmitted from adult houseflies to the next generation, and ARG carriage was consistently maintained within the housefly microbiota. Thirty-one-day-old chickens were infected with maggots harboring an antimicrobial-resistant *E. coli* strain that persisted throughout the fly life cycle, where carriage continued until the chickens were at least 46 days old (typical age when the chickens are shipped). These results suggest that flies act as a reservoir of ARB and ARGs throughout their life cycles, and they may be involved in the maintenance of ARB on farm environments.

In conclusion, we determined that flies act as both a biological vector for the

transmission of ARB and as a reservoir for the maintenance of ARB throughout their life cycle. This thesis demonstrates that the insects, especially flies, play an important role in the spread of ARB and ARGs in farm environments. We expect that our study will aid further research on the routes of spread of livestock-associated ARB and ARGs and help in preventing the maintenance and circulation of ARB and ARGs in farm environments.

ACKNOWLEDGEMENT

I would like to acknowledge Professor Yutaka Tamura and Professor Ikuo Uchida (Rakuno Gakuen Univ.) as my advising teacher. They taught me a lot of things not only scientific techniques but also the fun in researching. Great appreciation is extended for Dr. Ikuo Uchida, Dr. Masaru Usui, Dr. Katsuro Hagiwara, Dr. Yasukazu Muramatsu for reviewing my manuscript. I also would like to express my appreciation to the following members who supported me: Dr. Masaru Usui, Dr. Toyotaka Sato, and Dr. Torahiko Okubo, and Dr. Tomomi Sato (Rakuno Gakuen Univ.) for general advice in experiment techniques; Dr. Hitoshi Sasaki (Rakuno Gakuen Univ.) for morphologically identical techniques in flies and handling advices to houseflies; Mr. Tomohiro Iwasa (Rakuno Gakuen Univ.) for his help in experiments in Chapter 1; Sumika Life Tech, Ltd., for providing us with the CSMA strain houseflies in Chapter 2 and 3; Dr. Masashi Okamura, Dr. Hu Dong-Liang, and members of their laboratory (Kitasato Univ.) for technical advices and supports in the experiment of chicken model in Chapter 3. Special thanks to all the students and staff in Laboratory of Food Microbiology and Food Safety, Rakuno Gakuen Univ. Finally, I would like to extend my indebtedness to family for their understanding and support throughout my study.

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RESEARCH ACHIVEMENTS

Publication relating this thesis

Fukuda, A., Usui, M., Okamura, M., Dong-Liang, H. and Tamura, Y. 2018. The role of flies in the maintenance of antimicrobial resistance in farm environments. *Microb. Drug Resist*. in press.

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.

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Publication of other studies

Fukuda, A., Sato T., Shinagawa M., Takahashi S., Asai Tetsuo., Yokota SI., Usui M. and Tamura Y. 2018. High prevalence of *mcr-1*, *mcr-3*, and *mcr-5* in *Escherichia coli* derived from diseased pigs in Japan. *Int. J. Antimicrob. Agents*. **51**: 163–164.

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ABSTRACT IN JAPANESE（和文要旨）

抗菌薬は細菌性感染症の治療のためにヒト医療及び獣医療において広く用いられ、多くの命を救ってきた。しかし、薬剤耐性菌の出現・拡散に伴い、薬剤耐性菌は世界的な公衆衛生学上の問題となっている。一方で、新規抗菌薬の開発は停滞している。このため、既存抗菌薬の有用性を保ち、薬剤耐性菌の出現・拡散を制御するため、抗菌薬の適切な使用と衛生管理の徹底などが行われている。家畜由来薬剤耐性菌は様々な経路を介して伝播・拡散することが考えられているが、ヒトへの食品を介した伝播経路に関する研究が主であった。これに加え、環境を介した伝播・拡散経路も重要な因子であることが指摘されている。また、薬剤耐性菌は薬剤耐性遺伝子の受け渡しにより、新たな薬剤耐性菌を生み出すことがあり、薬剤耐性遺伝子の伝播・拡散についても重要視される。

本研究では、家畜由来薬剤耐性菌の環境を介した伝播・拡散経路を解明するため、衛生昆虫（特にハエ）に着目し、農場環境中における薬剤耐性菌の伝播･拡散におけるハエの役割を明らかにすることを目標とした。まず、第 1 章では、農場内に存在するハエの薬剤耐性菌の保有状況と保有薬剤耐性菌の由来を明らかにするため、農場においてハエ及び家畜糞便を採取し、薬剤耐性菌を分離し、菌性状及びその近縁性を解析した。次に第 2 章では、薬剤耐性遺伝子の拡散におけるハエの役割を明らかにするため、実験室内において、ハエ腸管内における薬剤耐性遺伝子の水平伝達試験を行った。最後に第 3 章では、薬剤耐性菌の維持におけるハエの役割を明らかにするため、ハエの発育環における薬剤耐性菌・耐性遺伝子の検出と鶏への薬剤耐性菌保有ウジの投与試験を行った。

第 1 章の農場における野外調査により、農場で捕まえたハエが保有する薬剤耐性菌の由来を明らかにした。薬剤耐性菌の指標細菌である大腸菌は、糞食性のハエ（イエバエとオオイエバエ）から分離されたが、吸血性のハエ（サシバエ）からは分離されなかった。また、糞食性のハエからは、ウシ糞便から分離された菌株と近縁な extended-spectrum beta-lactamase 産生セファロ

スポリン耐性大腸菌が複数分離された。本研究において、糞食性のハエが家畜由来 extended-spectrum beta-lactamase 産生セファロスポリン耐性大腸菌を保有していることを初めて報告し、家畜由来薬剤耐性菌の環境中のベクターとなっていることを示した。

第 2 章の実験室内におけるハエへの薬剤耐性大腸菌を用いた感染実験では、薬剤耐性菌・耐性遺伝子の拡散におけるハエの役割をより明確にした。イエバエ腸管内において薬剤耐性大腸菌が保有するプラスミド性の薬剤耐性遺伝子の水平伝達を確認した。また、水平伝達はイエバエ腸管内の異なる菌種の細菌へも伝達し、これらの菌種は、ヒト医療において日和見感染症の原因細菌となるものも含まれていた。今回の結果からハエは薬剤耐性菌を運ぶ機械的ベクターであるのみならず、腸管内において薬剤耐性遺伝子の水平伝達により、新たな薬剤耐性菌を発生させ得る生物学的ベクターとなる可能性を証明した。

第 3 章では、農場環境における薬剤耐性菌維持におけるハエの役割を明らかにした。まず、薬剤耐性大腸菌は成虫のイエバエから次世代まで発育環を通し維持され、薬剤耐性遺伝子もハエの腸内細菌叢内で一定量維持され続けた。その後、薬剤耐性大腸菌を保有したウジを 30 日齢の鶏へ経口投与したところ、46 日齢（出荷日齢付近）まで維持され続けた。これらの結果はハエが発育環を通し薬剤耐性・耐性遺伝子のレゼルボアとなり、農場環境において薬剤耐性菌が維持されることに関与していることを示した。

以上のことから、ハエは生物学的ベクターとして薬剤耐性菌を伝播すると共に、レゼルボアとして薬剤耐性菌を発育環で維持することを明らかにした。これまで、薬剤耐性菌を含む病原体の衛生昆虫からの報告はあるが、衛生昆虫が多く存在する畜舎環境におけるハエを介した拡散経路に着目した研究は今回が初めてである。本研究の結果は畜舎環境における薬剤耐性菌の拡散・維持における衛生昆虫の役割の重要性を明らかにし、微生物の制御において昆虫の防除を含む衛生環境の改善の重要性を示した。したがって、本研究はこれまでの薬剤耐性菌に対して新規抗菌薬での対応に加え、伝播経路を遮断するという新たな視点での対策の有用な知見を提供することができた。