Vectorial capacity of larvae, pupae and adult of housefly (*Musca domestica*) for *Escherichia coli* O157:H7 and the possibility of transmission from source to human

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Abstract: *Enterobacteriaceae* and enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 were detected in the body of the larvae and pupae of housefly, *Musca domestica* (Linnaeus), reared on an artificial diet containing EHEC. However, the detection rates of *Enterobacteriaceae* and EHEC O157:H7 in the body of housefly at each developmental stage tended to be reduced during metamorphosis. *Enterobacteriaceae* was detected in 32.6% of the newly-emerged adult houseflies, while no EHEC O157:H7 was detected in them. Moreover, the adult houseflies carried EHEC O157:H7 from sources to foods, and the majority of the foods were contaminated with EHEC O157:H7 for 24 h exposure in the transmission experiment. These results suggested that EHEC O157:H7 would be exclusively transmitted by adhesion to their external surface, feeding, flight and excretion of the adult houseflies. Judging from epidemiological characterization: polymerase chain reaction analysis (*stx1*, *stx2*, *eaeA* and *hlyA* genes), plasmid profiling (90-kbp plasmid), reversed-passive latex agglutination analysis (verotoxin-producing ability) and pulsed-field gel electrophoresis, EHEC O157:H7 strains from houseflies and those from patients were very similar. Therefore, EHECs found in houseflies possibly cause diseases against humans. Collectively, the houseflies as a trespasser on the distribution routes of human food may play an important role as a vector for pathogens such as EHEC O157:H7.

Key words: enterohemorrhagic *Escherichia coli* O157:H7 (EHEC O157:H7), housefly, vector, metamorphosis, transmission, bacterial pollution

INTRODUCTION

In 1996, an infectious disease caused by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 sporadically broke out all over Japan (IASR, 1998), and the causative foods or drinks and the route of infection are still unknown. However, EHEC O157:H7 was isolated from the body (Iwasa et al., 1999; Moriya et al., 1999; Buma et al., 1999a) and excreta (Buma et al., 1999b) of adult houseflies collected at a cattle farm.

These reports suggested that the housefly, *Musca domestica* (Linnaeus), possibly maintains EHEC O157:H7 and expands the infectious disease by transmission of it. In the case of Moriya et al., EHEC O157:H7 strains were isolated from houseflies captured inside and outside a nursery school, where the infection with enterohemorrhagic colitis broke out in October of the same year, in Saga Prefecture, Japan. EHEC O157:H7 strains were isolated from the school staff and families of the children, and from houseflies that

were captured in and out of school buildings for the period of three weeks, and verotoxin (VT) types of isolates from houseflies and humans were identical. Thereafter, the houseflies carrying EHEC O157:H7 found at cattle farms and slaughterhouses throughout Japan were summarized by Agui (2001).

It has been well known that the housefly is a vector of various pathogenic bacteria related to conservative infectious disease like *Salmonella* and *Shigella* (Greenberg, 1964; Bidawid and Edeson, 1978; Levine and Levine, 1991). The types of vectors such as housefly are usually classified as mechanical or biological based on the way of transmitting pathogenic bacteria. As for the mechanical vector, Kobayashi et al. (1999) reported that EHEC O157:H7 ingested by the adult housefly proliferated on the mouthparts and was excreted from the digestive tract. Moreover, Sasaki et al. (2000) reported that, after feeding EHEC O157:H7, an adult housefly drops 1.8×10^4 colony-forming units (cfu) of EHEC per 3 h which persist in the body of the housefly for at least 4 days. Houseflies breed on the dung of animals, and around garbage dumps or unsanitary building. EHEC colitis is held in the digestive tract of domestic animals like cattle and sheep, and was isolated from their dung (Chapman et al., 1994). Therefore, the transmission of bacteria from larvae to pupae or adult housefly in the sources must be considered.

There are several reports on the increase and the preservation of pathogenic bacteria as *Salmonella enteritidis*, *Salmonella paratyphi* B, *Salmonella typhi*, *Shigella flexneri* or other intestinal bacteria in the body of larvae or pupae of the housefly (Ostrolenk and Welch, 1942; Greenberg, 1959; Takeuchi et al., 1966). Those reports revealed that *S. enteritidis* and *S. paratyphi* B survive through the larval and pupal stages, while it is unknown whether other pathogenic bacteria including EHEC O157:H7 ingested in the larval stage are maintained through the pupal stage or

not.

In this report, we investigated the possibility of transmission of EHEC O157 from larvae to pupae or adult in the housefly. The transmission of EHEC from food to food by houseflies was also examined. Moreover, we examined the difference between some EHEC O157:H7 strains isolated from adult houseflies collected at a farm field and those from patients, by PCR detection (*stx1*, *stx2*, *eaeA* and *hlyA* genes), plasmid profiling, reversed-passive latex agglutination (RPLA) analysis, pulsed-field gel electrophoresis (PFGE) and antibiotics susceptibility test.

MATERIALS AND METHODS

Tested insect

The housefly, *Musca domestica* (Linnaeus) used in this study was from an insecticides-susceptible colony in Takatsuki, Japan. The later stages of 2nd and 3rd instar larvae, 4-day-old pupae and newly-emerged and 6–10-day-old adults were reared in the laboratory at 25°C, 60–70% RH and a photoperiod of 16:8 (L:D) h.

Bacterial strains

EHEC O157:H7 strains used in this study were isolated from the excreta and body of houseflies (*M. domestica*) collected at a cattle farm in Tokushima Prefecture, in 1997 (F-1: Buma et al., 1999b) and 1998 (F-6: Buma et al., 1999a). Twenty strains of EHEC O157:H7 from patients used were isolated from an outbreak in Sakai City of Osaka Prefecture in 1997 (H-S), Oku-cho of Okayama Prefecture in 1997 (H-O), Tokushima Prefecture in 1997 (6 strains, H-T1, -T2, -T3, -T4, -T5, -T6), and in 1998 (12 strains, H-T7, -T8, -T9, -T10, -T11, -T12, -T13, -T14, -T15, -T16, -T17 and -T18).

Survival of EHEC O157:H7 during the metamorphosis of housefly

Two EHEC strains used in this experiment were more similar strains between

those from houseflies and from patients, by epidemiological analysis. EHEC O157:H7 isolated from the body of houseflies and from patients in Oku-cho was cultured in Nutrient Broth (Eiken Chemical Co., Ltd., Tokyo, Japan) for 18 h at 37°C, harvested and suspended in sterilized saline. One ml of the bacterial suspension ($\approx 10^7$ cfu/ml) was added to 100 g of artificial diet composed of insect diet 22.0% (Oriental Yeast Co., Ltd., Tokyo, Japan), mush 22.0% (Oriental Yeast), dry yeast 1.0% (Oriental Yeast) and ion-exchanged water 55.0% in a 130 ϕ ×60 mm polyethylene cup (Mineron Kasei Co., Ltd., Osaka, Japan), and they were mixed using a sterilized stick. The newly-hatched housefly larvae were continually reared on an artificial diet with EHEC till pupation and were removed at different developmental stages using sterilized forceps.

Isolation of EHEC was carried out from the bodies of later stages of 2nd and 3rd instar larvae, 4-day-old pupa from which puparium was removed and newly-emerged adult houseflies which were anesthetized with 100% CO₂. Tested insects were individually washed several times with sterilized saline and dipped in 70% ethyl alcohol for 30 min. The surface sterilized larva, 4-day-old pupa, and adult were ground in a sterilized eppendorf tube using a sterilized glass stick and were mixed with 0.2 ml sterilized saline. After centrifugation at 3,000×*g* for 2 min at 25°C, the supernatant was plated onto CHROMagarO157 (CHROMagar Microbiology, Paris, France) and incubated for 24 h at 37°C. The growing colonies were examined for the serotype with O157 antigens (Denka Seiken Co., Ltd., Tokyo, Japan) and identified using rapid identification kits (Api20E; bioMerieux, Lyon, France). The experiment was repeated twice.

Transmission of EHEC O157:H7 from sources to food by the houseflies

Rice, minced meat, banana and radish sprouts were purchased from a retail shop

in Tokushima City. Prior to the experiment, the rice was cooked in an electric rice cooker and the banana was sliced into pieces about 5-mm thick using a sterilized knife. Radish sprouts were washed with distilled water several times and cut to about 2-cm lengths. The EHEC O157:H7 strain from the body of the housefly was incubated for 18 h at 37°C, harvested and suspended in sterilized saline, and 0.05 ml of the bacterial suspension ($\approx 10^8$ cfu/ml) was spread on 10 g of each food using a sterilized syringe.

Two 90×150 mm sterilized plates (Asahi Techno Glass Co., Ltd., Tokyo, Japan), one containing food contaminated with EHEC O157:H7 and another containing non-contaminated food, were placed 30 cm apart in a 250×350×220 mm plastic box. Two sterilized plates, one containing 20 ml of distilled water and the other containing 10 anesthetized houseflies, were placed in the center of the plastic box. The top of the plastic box was covered with 80 mesh nylon gauze, and the food was exposed to the houseflies for 6 and 24 h at 25°C.

After exposure, the foods were removed from the plastic box after the box was filled with CO₂ gas until all houseflies were anesthetized. The foods were placed in a test tube and homogenized in 10 ml of sterile saline that contained 0.2% of Tween80. The mixture was centrifuged at 1,300×*g* for 10 min at 25°C, and 0.1 ml of the supernatant was plated on CHROMagarO157. After incubation for 18 h at 37°C, the growing colonies were examined for the serotype with O157 antigens (Denka Seiken). In the case where EHEC O157:H7 was not detected in the food, 1 ml of supernatant was added to 5 ml of mEC-n broth (EC broth (Eiken) containing novobiocin sodium salt (20 mg/liter E. Merck, Darmstadt, Germany), and incubated for 18 h at 37°C. The enriched culture was inoculated directly onto CHROMagarO157 using a loop and incubated under the previously described conditions. The serotypes of growing colonies were

examined with O157 antigens (Denka Seiken).

Total DNA extraction

The total DNA of EHEC O157:H7 was prepared as follows. The bacterial cells cultured in nutrient broth (100 ml) at 37°C for 18 h were harvested by centrifugation at $8,000\times g$ for 5 min at 4°C and suspended in 10 ml of TE buffer (50 mM Tris, 50 mM EDTA, pH 8.0). After centrifugation, the pellet was suspended in 4 ml of TE buffer containing 15% of sucrose and kept at 37°C for 30 min, and 4 ml of TE buffer containing 1% of sarkosyl was then added. The crude DNA was purified by CsCl-ethidium bromide density-gradient centrifugation at $280,000\times g$ for 16 h at 18°C and dialyzed overnight against TE buffer (Harris et al., 1975). The purified total DNA was examined by the PCR and PFGE analyses described below.

PCR analysis

The structure genes of verotoxin (*stx1*, *stx2*), intimin (*eaeA*) and hemolysin (*hlyA*), were detected by the PCR method (Paton and Paton, 1998). The *stx1* and *stx2* genes were detected with the O157 PCR Typing Set (Takara, Kyoto, Japan) as previously described (Buma et al., 1999b). The specific primers of the *eaeA* gene and *hlyA* gene were designed with reference to published sequence data (Schmidt et al., 1995; Yu and Kaper, 1992): *eaeA*, 5'-GGCTTC-GTCACAGTTGCAGG-3' and 3'-CTTGTC-CGCTTTGGCTTCCG-5'; *hlyA*, 5'-GGGTA-TCGAACTGACAACTC-3' and 3'-TCTC-GCCTGATAGTGTGG-5'. The sizes of amplified DNA fragments are 932 bp for *eaeA* and 1017 bp for *hlyA*.

PFGE analysis

PFGE analysis was done by the method reported previously (Izumiya et al., 1997). Bacterial cells harvested from culture were embedded in low-melting-temperature agarose (Bio-Rad Laboratories, Richmond, Calif., USA). After appropriate preparations were made according to the

manufacturer's manual, the DNAs in each plug were digested with 40 U of *XbaI* (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C overnight. PFGE was performed with a 1% agarose gel by using a CHEF Mapper apparatus (Bio-Rad Laboratories) in $0.5\times$ TBE (Tris-borate-EDTA) buffer at 14°C at 200 V. For separation of a whole genome, a linearly ramped switching time from 4 to 8 s was applied for 12 h and then a linearly ramped switching time from 8 to 50 s was applied for 10 h. After PFGE, the gels were stained and observed as described above.

Plasmid profiling

Purification of plasmid DNA of EHEC O157:H7 was performed by the alkaline detergent method (Birnboim and Doly, 1979). Plasmid DNA was separated and detected by agarose gel electrophoresis, stained with ethidium bromide solution and detected by ultraviolet illumination (Sambrook et al., 1989).

Verotoxin-production of EHEC O157:H7

Verotoxin-production of EHEC O157:H7 was detected using the VTEC-RPLA test (Denka Seiken) (Kai et al., 1997). Tested EHEC O157:H7 strains were cultured at 37°C for 16 h in EC broth (Eiken) and transferred into a mini-tube by a sterilized syringe and centrifuged at $4,000\times g$ for 30 min at 4°C. The supernatant was mixed with the reagents of the kit and incubated at room temperature for 16 h. The existence of VT was judged by visual inspection.

Test for antibiotic susceptibility

Antibiotic susceptibility of the isolated EHEC O157:H7 was examined by a disk diffusion method with antimicrobial disks (Eiken). Tested agents were ampicillin, chloramphenicol, erythromycin, tetracycline, kanamycin, cefazolin, colistin, ciprofloxacin, lincomycin, sulfamethoxazole/trimethoprim and fosfomycin which is recommended to treat patients infected with EHEC O157:H7. The antibiotic sus-

ceptibility of the bacteria was judged according to the method recommended by National Committee for Clinical Laboratory Standards (1997).

RESULTS

Survival of EHEC O157:H7 during the metamorphosis of housefly

The detection rate of *Enterobacteriaceae* and EHEC in the body at different developmental stages of housefly is shown in Table 1.

Enterobacteriaceae was detected in 100% (20/20) of the later stage of 2nd instar larvae, in 100% (20/20) of the later stage

of 3rd instar larvae, in 97.8% (132/135) of the 4-day-old pupae and in 32.6% (46/141) of the newly-emerged adults, and it tended to be reduced in the process of development to the adult housefly, when no bacteria was detected from the surface of tested insects. The detection of two EHEC strains at each developmental stage showed a similar tendency. EHEC O157:H7 derived from patients was detected in 90% (9/10) of the later stage of 2nd instar larvae, in 60% (6/10) of the later stage of 3rd instar larvae and 11.9% (10/84) of 4-day-old pupae. EHEC O157:H7 derived from the houseflies was detected in 100% (10/10) of the later stage of

Table 1. The detection rate of *Enterobacteriaceae* and EHEC O157:H7 isolated from the housefly at different developmental stages.

Life stage of housefly	<i>Enterobacteriaceae</i>	Source of EHEC O157:H7	
		Housefly	Patient
larva ¹⁾	20/20 (100.0)	10/10 (100.0)	9/10 (90.0)
larva ²⁾	20/20 (100.0)	7/10 (70.0)	6/10 (60.0)
pupa ³⁾	132/135 (97.8)	9/51 (17.6)	10/84 (11.9)
adult ⁴⁾	46/141 (32.6)	0/41 (0.0)	0/100 (0.0)

¹⁾ the later stage of 2nd instar, ²⁾ the later stage of 3rd instar, ³⁾ 4-day-old pupa, ⁴⁾ immediately after emergence.

The numbers in parentheses are detection rate expressed in percent.

Fig. 1. The numbers of *Enterobacteriaceae* and EHEC O157:H7 isolated from the houseflies at different developmental stages.

¹⁾ the later stage of 2nd instar, ²⁾ the later stage of 3rd instar, , *Enterobacteriaceae*; , EHEC O157:H7 from patients, ; EHEC O157:H7 from houseflies.

2nd instar larvae, in 70% (7/10) of the later stage of 3rd instar larvae and 17.6% (9/51) of 4-day-old pupae. Both strains were not detected from the newly-emerged adults.

The quantitative prevalence of the isolated bacteria in the body at different developmental stages of housefly is shown in Fig. 1. The number of detected *Enterobacteriaceae* was on average 2.1×10^8 cfu per 2nd instar larva, on average 1.2×10^8 cfu per 3rd instar larva, on average 2.6×10^3 cfu per pupa and on average 1.6×10^3 cfu per adult. The number of EHEC O157:H7 derived from houseflies was detected

Table 2. Identification of isolated bacteria from the houseflies.

Source of isolates	Species
larva ¹⁾	<i>Escherichia coli</i> O157:H7
	<i>Enterobacter</i> sp.
	<i>Providencia rettgeri</i>
	<i>Providencia stuartii</i>
	<i>Proteus mirabilis</i>
	<i>Proteus vulgaris</i>
pupa ²⁾	<i>Escherichia coli</i> O157:H7
	<i>Enterobacter cloacae</i>
	<i>Klebsiella pneumoniae</i>
	<i>Providencia rettgeri</i>
	<i>Proteus vulgaris</i>
	Gram-negative rods
adult ³⁾	<i>Escherichia coli</i>
	<i>Providencia rettgeri</i>
	<i>Providencia stuartii</i>
	Gram-negative rods
artificial diet	<i>Escherichia coli</i> O157:H7
	<i>Providencia stuartii</i>
	Gram-negative rods

¹⁾ the later stage of 2nd instar, ²⁾ 4-day-old pupa, ³⁾ immediately after emergence.

on average 8.4×10^7 cfu per 2nd instar larva, on average 1.6×10^4 cfu per 3rd instar larva and on average 1.2×10 cfu per pupa. In the case of EHEC O157:H7 derived from patients, on average 4.1×10^6 cfu per 2nd instar larva, on average 6.8×10^4 cfu per 3rd instar larva and on average 2.0×10 cfu per pupa were detected. In the later stage of 3rd instars, larvae of housefly stopped feeding and excreted the contents of the digestive tract, and the detection and number of EHEC O157:H7 decreased. Moreover, those were continuously decreased during metamorphosis. The detection of the strain derived from houseflies in 4-day-old pupae was the same as the strain from patients, while the number of EHEC O157:H7 derived from patients has a tendency to survive longer than that from houseflies in the pupal stage.

Isolated bacteria from the digestive tract of the larvae, pupae and the newly-emerged adults was identified (Table 2). In the larval stage, *Enterobacteriaceae*: EHEC O157:H7, *Enterobacter* sp., *Providencia stuartii*, *Providencia rettgeri*, *Proteus vulgaris* and *Proteus mirabilis* were detected. *Enterobacter cloacae*, *Klebsiella pneumoniae*, *P. rettgeri*, *P. vulgaris* and EHEC O157:H7 were detected in the pupal stage. *E. coli*, *P. stuartii* and *P. rettgeri* were also isolated from the newly-emerged adults. These species of isolated bacteria are known as opportunistic pathogens in the human. *P. rettgeri*, which was a common species for larvae, pupae and newly-emerged adults, was also isolated from the artificial diet.

Table 3. The number of EHEC O157:H7 transmitted by the houseflies.

Tested foods	n	No. of EHEC O157:H7 in the tested food after exposure (cfu/g) ¹⁾	
		6 h	24 h
rice	6	—	$(7.2 \pm 11.6) \times 10^1$
minced meat	3	+	$(2.4 \pm 2.8) \times 10^5$
radish sprouts	3	+	+
banana	6	—	$(1.7 \pm 2.0) \times 10^3$

¹⁾ Mean $\pm$ SD obtained by the determination of three-six samples.

+, detectable; —, not detectable.

EHEC O157:H7 pollution of various foods by the housefly

The number of EHEC O157:H7 detected from the foods is shown in Table 3. Three fly groups composed of ten flies in each group carried EHEC O157:H7 to the exposed foods for 6 h with a transmission rate of 17% (3/18). After 24 h exposure, the majority of the foods was found to be contaminated with EHEC O157:H7; however, there was a difference among the numbers of EHEC O157:H7 detected from each of the foods. The rice, which partially dried during the exposure time, had on average 7.2×10 cfu per 1 g EHEC O157:H7 detected. One gram of minced meat had on average 2.4×10^5 cfu of EHEC O157:H7 detected in comparatively moist areas. In the case of radish sprouts, the number of EHEC O157:H7 was not counted, because other aerobic bacteria in it outnumbered EHEC O157:H7. The number of EHEC O157:H7 detected from banana was on average 1.7×10^3 cfu per 1 g.

Molecular analysis of isolates

The result of epidemiological analysis of EHEC O157:H7 is shown in Table 4. In EHEC O157:H7 isolated from the houseflies and patients in Sakai City in Osaka Prefecture and Oku-cho in Okayama Prefecture, the genes of the virulence factor

(*stx1*, *stx2*, *eaeA* and *hlyA*), verotoxin (VT1, VT2)-producing ability and large plasmid (90-kbp and/or smaller than 90-kbp) were detected. On the other hand, the isolates from patients in Tokushima Prefecture and the isolates from houseflies differed in the detection rate of virulence factors. All the isolates from patients possessed the *stx1*, *stx2* and *eaeA*; however, 17% (3/18) of the isolates did not possess the *hlyA* gene, and VT1- and VT2-producing abilities were not confirmed for 39% (7/18) and 6% (1/18) of the isolates, respectively.

As for the PFGE profiles of EHEC O157:H7 strains, their typical DNA patterns were indicated in Fig. 2. Usually, the DNA patterns of EHEC in PFGE profiling are classified on the basis of the differences of the characteristic areas: <100 kbp, 100–200 kbp and >350 kbp. Many variations were observed in the DNA patterns of the eighteen strains from patients in Tokushima Prefecture. The two strains from patients (H-T5 and H-T12) resembled the strains from houseflies at the area <90 kbp, while the strains from houseflies differed from the strains from patients in Tokushima in the existence of DNA bands at the areas 100 kbp and 190–200 kbp. The DNA profiles of the two strains from patients (H-S, H-O) were very similar, and the existence of 80 kbp DNA band in the

Table 4. PCR assay, RPLA and prasmid profiling of EHEC O157:H7 from houseflies and patients.

Origin	No. of tested strain	PCR				RPLA		Large plasmid
		<i>stx1</i>	<i>stx2</i>	<i>eaeA</i>	<i>hlyA</i>	VT1	VT2	
fly ¹⁾	1	+	+	+	+	+	+	+
fly ²⁾	1	+	+	+	+	+	+	+
patient (Sakai)	1	+	+	+	+	+	+	+
patient (Oku)	1	+	+	+	+	+	+	+
patient ³⁾	7	+	+	+	+	+	+	+
patient	1	+	+	+	+	+	+	—
patient	6	+	+	+	+	—	+	+
patient	1	+	+	+	+	+	—	+
patient	2	+	+	+	—	+	+	—
patient	1	+	+	+	—	—	+	—

¹⁾ strain isolated from excreta of housefly in 1997 in Tokushima, ²⁾ strain isolated from body of housefly in 1998 in Tokushima, ³⁾ strains from patients in 1997 or 1998 in Tokushima.

Fig. 2. The typical DNA patterns of EHEC O157:H7 from houseflies and patients by PFGE.

Lanes: 1, EHEC O157:H7 from excreta of housefly (F-1); 2, EHEC O157:H7 from body of housefly (F-6); 3, EHEC O157:H7 from Sakai City (H-S); 4, EHEC O157:H7 from Oku-cho (H-O); 5, 6, 7, 8, 9, 10, 11 and 12, EHEC O157:H7 from Tokushima city (H-T3, -T5, -T7, -T8, -T9, -T10, -T12, -T14); M, marker (λ DNA digested with *Hind*III).

Table 5. Antibiotic resistance patterns of EHEC O157:H7 from houseflies and patients by disk diffusion method.

Antibiotics	Sources of EHEC O157:H7			
	Houseflies	Patients		
		Tokushima	Sakai	Oku
ABPC ¹⁾ , EM ²⁾ , LM ³⁾ , ST ⁴⁾ , TC ⁵⁾		1		
ABPC, EM, LM, TC		2		
ABPC, EM, LM	1	0	1	
EM, LM, TC		2		
EM, LM	1	12		1
CL ⁶⁾ , LM		1		
Total	2	18	1	1

¹⁾ ampicillin, ²⁾ erythromycin, ³⁾ lincomycin, ⁴⁾ sulfamethoxazole/trimethoprim, ⁵⁾ tetracyclin, ⁶⁾ colistine.

profiles was confirmed. Four strains including two strains from houseflies showed a close resemblance in the DNA profiles, and the strain from houseflies and from patients in Oku-cho in Okayama Prefecture were identical. The PFGE profiles implied that housefly strains have their origin in common with the strains from

human. In conclusion, EHEC O157:H7 isolated from houseflies was possible pathogens for humans.

Antibiotic susceptibility of EHEC O157:H7 isolated from houseflies and patients

The antibiotic susceptibility of EHEC O157:H7 isolated from houseflies and

from patients by the disk diffusion method is shown in Table 5. Tested EHECs were resistant to two to five antibiotics, the majority of them had resistance to erythromycin and lincomycin. EHEC strains from patients had a tendency to be resistant to more agents than the strains from houseflies. Chloramphenicol, kanamycin, cefazolin, ciprofloxacin, and fosfomycin were effective against them.

DISCUSSION

Usually, during larval and pupal metamorphosis, the tissues of larvae disintegrate and develop into adult structure (Kettle, 1995), and antibacterial proteases produced in the digestive tract play an essential role in disintegration of the gut tissue of housefly larvae (Nakajima et al., 1997). As the result of our experiment, *Enterobacteriaceae* decreased during larval and pupal metamorphosis as previously reported (Greenberg, 1959; Takeuchi et al., 1966). The disintegration of larval tissues, the decline in pH value in the digestive tract and other various biochemical and physical processes would lead to the decrease of *Enterobacteriaceae* in the pupal body. When we observed that ptilinum of pupal, wing and polyneustic lobes were formed in the cuticle two days after pupation, the digestive tract organs of housefly larvae partially remained in the puparium. The reconstruction of the digestive tract of pupae was a later event than that of the surface. Therefore, those processes have an influence on the bacterial population in digestive tract of houseflies through the process of pupal development as described above; however, the influence is not enough to cause bacterial extinction and a few *Enterobacteriaceae* survived.

Detection of *Enterobacteriaceae* from all stages of housefly suggested that these bacterial species compose intestinal flora of the digestive tract of housefly. Zurek et al. (2000) reported the diversity of the bacterial community in the intestinal tract of housefly larvae collected from natural en-

vironments and confirmed that the development of housefly was dependent on the presence of the bacteria. Accordingly, a complex bacterial community harbored in the digestive tract likely supports the development of housefly at each stage.

As for the EHEC O157:H7 isolated from houseflies and patients, the detection rate and the number during metamorphosis decreased as other *Enterobacteriaceae*. At 4-day-old pupal stage, the strains from patients tended to survive more than the strains from houseflies. It was suggested that EHEC O157:H7 isolated from patients had higher acid tolerance than the strain from houseflies (Maeda et al., 2002) and was resistant against antibiotics. However, EHEC O157:H7 isolated from houseflies and patients were not detected in newly-emerged adults. Therefore, it was indicated that the larvae and pupae of housefly are not very important as a vector of EHEC O157:H7 in environment.

The adult housefly maintained EHEC O157:H7 for 3–4 days, excreted 6.5 drops per min (Sasaki et al., 2000), and the number of bacteria excreted by adult for 3 h was $>10^4$ cfu per fly (Sasaki et al., 2000; Buma et al., 2000). Kobayashi et al. (2002) reported that bacterial inoculation onto food by the houseflies would directly depend on the number of EHEC O157:H7 ingested: the houseflies transmitted $<10^2$ cfu/sample after the feeding 10^6 cfu/ml of EHEC O157:H7, and 10^5 cfu/sample was carried in the case of feeding 10^9 cfu/ml for 30 min of exposure time. From the test food contaminated with EHEC O157:H7, about 10^5 cfu/g of bacteria were transmitted to other foods during 6 h of exposure time by houseflies. Judging from the difference in the number of EHEC O157:H7 in each food, the feeding and excretion of houseflies were the most active in the case of minced meat. Such food rich in protein was the most palatable food for housefly in all of the tested foods, and especially female houseflies with mature eggs prefer this food (Kobayashi et al., 2002). Furthermore, minced meat rich in

protein, lipid or other nutrients would be suitable for growing EHEC O157:H7, while carbohydrates like rice or vegetables are not. This result showed that the feeding and excretion of adult houseflies possibly play a role as a mechanical vector for EHEC O157:H7 from sources to food.

According to Agui (2001), the existence of houseflies carrying EHEC was recognized throughout Japan, and 7.2% of houseflies held VT genes as a result of PCR analysis. In addition to PCR analysis, PFGE profiling is an effective means in epidemiological study. The PFGE profiles in our study implied that housefly strains caught in Tokushima have their origin in common with the strains from patients of Oku-cho in Okayama. Though the adult housefly can fly as far as about 30 km from sources (Schoof and Siverly., 1954), Oku-cho in Okayama is too far from Tokushima for housefly to fly. Therefore, it is necessary to consider carefully that the spread of EHEC O157:H7 may be possible through the various ways in the distribution routes of human food besides the housefly.

From our experimental results and the reports by Sasaki et al. (2000) and Kobayashi et al. (1999, 2002), the spread of EHECs by housefly was mainly brought about through feeding, flight and excretion of adult houseflies. The vectorial capacity of adult houseflies for EHEC O157:H7 would be the same as for *Shigella* (Levine and Levine, 1991) and *Salmonella* (Greenberg, 1964).

Recently, there has been an epidemic of disease caused by various kinds of pathogenic bacteria worldwide. Although EHEC strains including EHEC O157:H7 have been isolated from various sites of the dairy farm and environment (Rahn et al., 1997), not all of them that maintain viability in the natural environment have respiration activity, enzymatic activity and proliferation under the growth conditions thought to be suitable (Tani and Nasu, 1999). Therefore, the existence of EHECs in houseflies cannot be bypassed

even if the isolates may be few. There is a possibility that houseflies always possess many pathogenic bacteria (Hawley et al., 1951; Greenberg, 1971; Grubel et al., 1997; Buma et al., 2000), and transmit diseases from sources to foods or drinks consumed by humans. Analysis of the transmission route of gastrointestinal infection caused by pathogens and undertaking proper control would be necessary to reduce the risk of pathogenic bacteria including EHEC O157:H7 carried by houseflies from the reservoir in breeding areas.

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