

HOUSEFLIES: NOT SIMPLE MECHANICAL VECTORS OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7

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Abstract. An epidemic of enterohemorrhagic colitis caused by *Escherichia coli* O157:H7 (EHEC-O157) occurred in a nursery school in a rural area of Japan in September 1996. The EHEC-O157 were isolated both from patients and houseflies collected at the school. The flies were suspected to be mechanical vectors of the pathogen. Feeding experiments of EHEC-O157 to houseflies showed that the ingested bacteria were harbored in the intestine of flies and continued to be excreted at least for 3 days after feeding. Scanning electron microscopy showed that a large number of EHEC-O157 adhered to the surface of the housefly mouthparts and actively proliferated in the minute spaces of the labellum. Food masses containing EHEC-O157 in the fly intestine were completely surrounded by a peritrophic membrane during digestion and discharged rapidly. The persistence of bacteria in the intestine and feces is mainly a result of proliferation in the mouthparts and accumulation in the crop. Our results strongly suggest that houseflies are not simple mechanical vectors of EHEC. The epidemiologic potential of houseflies to disseminate EHEC-O157 may be greater than initially suspected.

The housefly (*Musca domestica vicina*) and flies in general are considered to be mechanical vectors of many kinds of pathogens such as bacteria,^{1,2} protozoa,³ viruses,⁴ and helminth eggs.^{5,6} In recent outbreaks of enterohemorrhagic colitis in nursery schools in Japan, the epidemiologic survey isolated enterohemorrhagic *Escherichia coli* O157:H7 (EHEC-O157) from both houseflies collected in the school and from patients. The DNA patterns and the type of verotoxin were identical in EHEC-O157 isolated from both flies and patients.^{7,8} This result strongly indicated that houseflies in nursery schools disseminated EHEC-O157 to foods, drinks, plates, and utensils, although direct evidence of the transmission has not been clarified. The infective dose for EHEC-O157 is considered to be very low, similar to that of *Shigella flexneri* or *S. dysenteriae*.^{9,10} There are many qualitative reports of microbial flora in flies, but there are no reports of the number of bacteria that are harbored in the intestine or crop or how long the potential for dissemination of bacteria persists. We investigated the vector potential of houseflies for EHEC-O157 by feeding the flies with bacteria and monitoring them for several days after feeding. We also used scanning electron microscopy to determine whether EHEC-O157 adhered to the surface of housefly mouthparts and transmission electron microscopy to determine whether bacteria proliferated and persisted in the alimentary canal of flies.

MATERIALS AND METHODS

Experimental feeding of EHEC-O157 to houseflies. Houseflies maintained in our insectary were used. Two strains of EHEC-O157 (verotoxin 1- or verotoxin 1- and 2-producing strains) were grown in trypticase soy broth (Becton Dickinson, Cockeysville, MD). The concentration of bacteria was approximately 10⁹ colony-forming units (CFU)/ml. Adult (6–8-day old), female flies were allowed to feed on 2 strains of EHEC-O157 for 30 min in a safety cabinet in a laboratory with a P3 level of physical containment. After feeding, flies were anesthetized with CO₂ and 8–10 flies were dissected to remove the alimentary canal and

crop (Figure 1). Each sample was placed into a 1.6-ml tube, homogenized with a plastic pestle in phosphate-buffered saline (PBS) (Dulbecco's), and centrifuged at 2,000 rpm for 2 min to produce a clear supernatant.

Estimation of the number of bacteria in alimentary canals. The number of EHEC-O157 in each organ was estimated by plating aliquots of serially diluted supernatant of the organs homogenated on a EHEC-O157-specific Chromoagar plate (Chromoagar, Inc., Paris, France). Colonies of EHEC-O157 appear clear green on this plate. We also reconfirmed the colonies from the Chromoagar plate by replating them on Rainbow Agar O157 (Biolog, Inc., Hayward, CA). Intestine or crop samples from control flies never showed EHEC-O157-positive results on the Chromoagar plate. An estimate of feeding volume was obtained by feeding eosin dye-supplemented trypticase soy broth to the flies and measuring the optical density at 514 nm of the supernatant of a midgut homogenate with a photometer.

Dissemination potential of houseflies. Flies fed EHEC-O157 were anesthetized with CO₂ and enclosed inside individual petri dishes with Rainbow Agar O157 for 2 hr to examine the potential for dissemination of EHEC-O157. Within the petri dishes, the flies walked upon and tasted the surface of the agar plate, and excreted freely. After removing the flies from the petri dishes, the plates were incubated at 37°C overnight and EHEC-O157 colonies were counted.

Preparation of flies for scanning electron microscopy. Individual fly heads were fixed with the mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS, pH 7.2, for 30 min and washed with 0.1 M PBS. After dehydration through an ascending acetone series, the specimens were dried by critical point drying with a liquid CO₂ dryer (HCP-2 type; Hitachi, Tokyo, Japan), coated with osmium with a model NL-OPC 80A osmium plasma coater (Nippon Laser and Electronic Laboratory, Nagoya City, Japan), and examined with a scanning electron microscope (Model S-5000; Hitachi). For scanning immunoelectron microscopy, fly heads were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M PBS, pH 7.2, for 30 min and washed with 0.1 M PBS. The specimens were then washed with 0.1

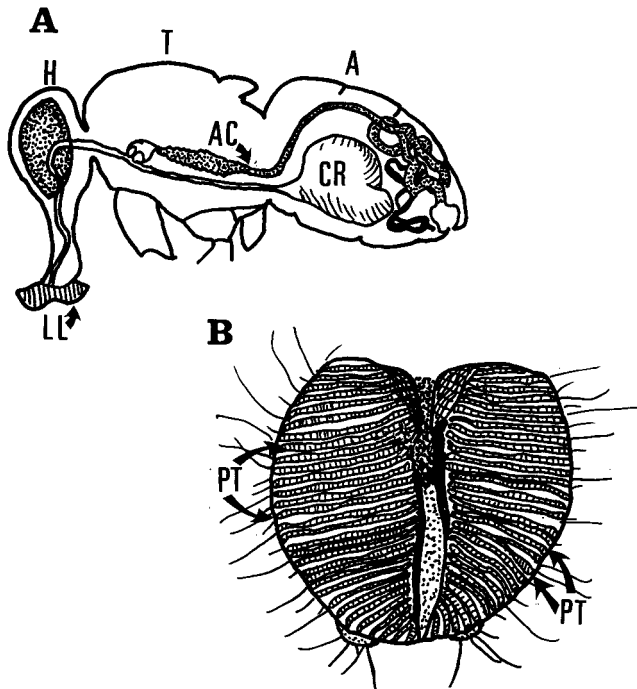


FIGURE 1. Internal anatomy of the housefly *Musca domestica* *vinica*. A, digestive system. CR = crop; AC = alimentary canal; LL = labellum; H = head; T = thorax; A = abdomen. B, detailed structure of the labellum. PT = pseudotrachea.

M PBS, incubated with 1% bovine serum albumin in 0.1 M PBS for 30 min to avoid nonspecific binding, and washed again with 0.1 M PBS. The specimens were then incubated with rabbit anti-*E. coli* H7 polyclonal antibody at a dilution of 1:100 in 0.1 M PBS for 60 min. The antibody (produced by Dr. K. Tamura, National Institute of Infectious Diseases, Tokyo, Japan) has been found to react specifically with H7 antigen. The specimens were then washed with 0.1 M PBS and incubated with colloidal gold-labeled anti-rabbit immunoglobulin (diameter of colloidal gold particles = 15 nm) at a dilution of 1:50 in 0.1 M PBS for 30 min. After a final washing with 0.1 M PBS, they were prepared in the same manner as for scanning electron microscopy.

Preparation of flies for transmission electron microscopy. Alimentary canals removed from flies that fed on EHEC-O157 were fixed with the mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min, washed with the same buffer, and then postfixed in 1% of osmium tetroxide in 0.1 M cacodylate buffer for 1 hr. After dehydration through an ascending ethanol series, they were embedded in Epon 812 resin (TAAB, Berkshire, United Kingdom). Ultrathin sections were cut using a diamond knife on an ultramicrotome (8800; LKB, Stockholm-Bromma, Sweden), mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-7000; Hitachi).

RESULTS

Harboring of EHEC-O157 in alimentary canals.

Counts of bacteria in alimentary canals of flies dissected

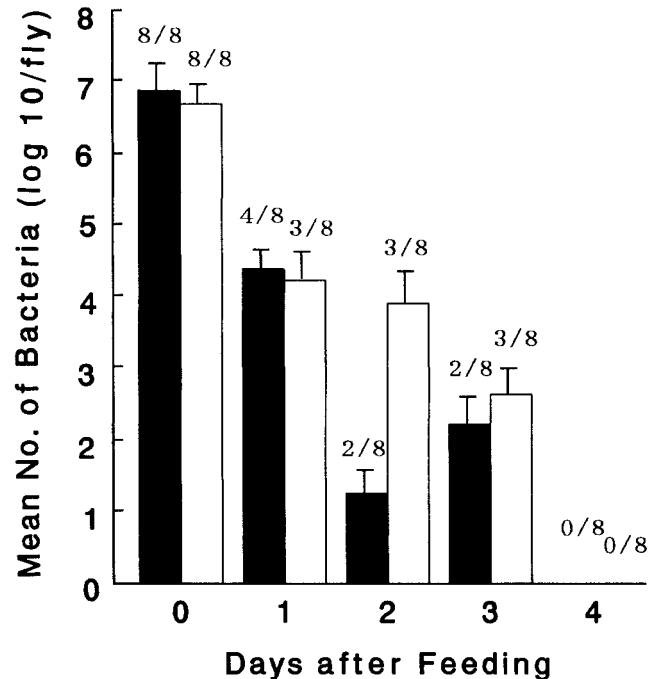


FIGURE 2. Estimated number of enterohemorrhagic *Escherichia coli* (EHEC)-O157 in fly intestine after initial feeding. Numbers above the columns are no. of positive flies/no. tested. Closed columns indicate verotoxin 2-producing EHEC-O157 and open columns indicate both verotoxin 1- and 2-producing EHEC-O157. Bars show the mean \pm SD.

immediately after feeding showed that 10^6 – 10^7 bacteria per fly were present in the alimentary canal of all flies tested (Figure 2). This number coincided with the estimated volume (2.6–4.1 μ l/female fly) of ingested medium per female fly, which was measured by feeding eosin-supplemented medium to the flies. After 24 hr, the number of bacteria in the intestine rapidly decreased, but approximately 30% of the flies continued to harbor several hundred bacteria in their intestines 3 days after feeding (Figure 2). No EHEC-O157 was detected in the alimentary canals of the flies 4 days after feeding. The crop also contained a large number of EHEC-O157 just after feeding and continued to harbor the bacteria 4 days after feeding as in the alimentary canal. The presence of EHEC-O157 in the crop may be related to frequent regurgitation of the contents of the midgut and crop to the labellum, although houseflies did not ingest protein-rich medium (trypticase soy broth) supplemented with eosin into crop directly.

Bacterial mass in the peritrophic membrane in alimentary canals of flies. Transmission electron microscopy of the midgut of the housefly showed that the ingested bacteria were localized inside a space surrounded by the peritrophic membrane (PM) and never contacted microvilli of midgut epithelial cells of flies directly (Figure 3). No bacteria were observed outside the PM of the alimentary canal (Figure 3). The indigestible material within the PM seems to be excreted rapidly because thousands of EHEC-O157 were detected in the excreta of the flies 1 and 3 hr after feeding, and female flies fed on eosin-supplemented medium stained filter paper with red excreta for 2 hr after feeding.

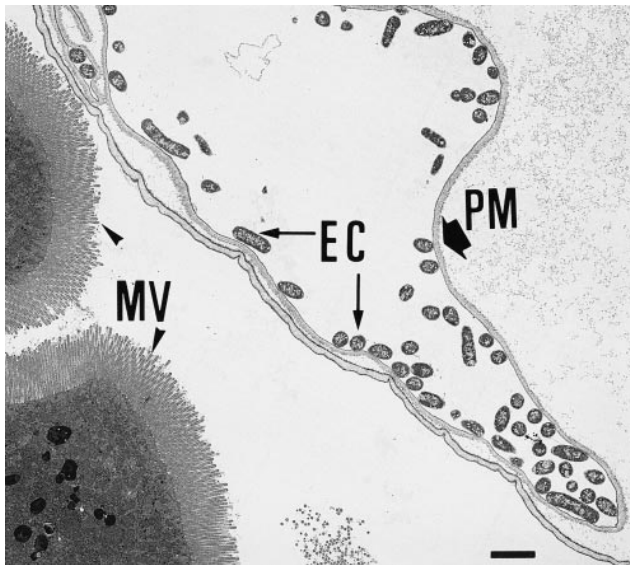


FIGURE 3. Enterohemorrhagic *Escherichia coli* (EHEC)-O157 completely surrounded by a peritrophic membrane in the lumen of the fly intestine. PM = peritrophic membrane; MV = microvilli; EC = enterohemorrhagic *E. coli*. Bar = 2 μ m.

Proliferation of EHEC-O157 in the mouthparts of flies.

Scanning electron microscopy of the mouthparts of control flies showed that a small number of cocci adhered to the surface of mouthparts and the tips of the legs. When the flies were fed EHEC-O157, a large number of these bacteria adhered to the inner and outer surfaces of the labellum (Figure 1) 24 hr after feeding (Figure 4a). In preliminary experiments, bacilli were examined by scanning immunoelectron microscopy using specific antibodies to H7 or O157 antigen of *E. coli*. Immunogold particles were bound to the bacterial cell wall by antibody to O157 and to flagella by antibody to H7 (Figure 4b). Interestingly, proliferating bacteria were also observed in the minute space of the labellum of the flies (Figure 4c). Active proliferation of EHEC-O157 in the mouthparts was observed in the flies 24 hr after feeding. It is interesting that the morphology of the bacteria drastically changed within the pseudotracheae of the labellum. The binding intensity of immunogold particles to the bacteria clearly changed with the corresponding ultrastructural changes, although the exact mechanism is unknown. Three days after feeding, individual bacteria could not be seen on the surface of the mouthparts because a mesh-like structure completely packed the minute spaces of the pseudotrachea (Figure 4d). However, it was not clear whether the morphologic changes in the bacteria were related to the number of bacteria decreasing and/or the removal of bacteria off the surface of fly mouthparts. It is significant that the formation of the mesh-like structure coincides with the drastic decrease in the number of EHEC-O157 in the alimentary canals of the flies.

Dissemination potential of flies in EHEC-O157. Flies placed into petri dishes containing Rainbow Agar O157 for 2 hr were able to disseminate several hundred colonies of EHEC-O157. Each colony appeared to be derived from more than 1 bacterium on this agar plate, since the number of bacteria directly disseminated by the excreta should be great-

er than several hundreds or thousands, particularly in flies immediately after feeding. Our results clearly showed that flies have potential to disseminate the bacteria on the plate at least for 3 days after feeding (Figure 5). Two days after feeding, 80% of the flies disseminated approximately 100 colonies of EHEC-O157 on the agar plate. In this experiment, we observed 2 types of colonies of EHEC-O157, a small colony and a large colony. It is plausible that the large colony was derived from excreta and the small colony from contact with the labellum. In fact, we observed on filter paper large, red dots derived from excreted fluid and small dots derived from the labellum of female flies fed on eosin-supplemented medium. Our data clearly show that EHEC-O157 ingested by houseflies was viable in the excreta of the flies, and that the flies are able to disseminate EHEC-O157 from the mouthparts and crop for a number of days after feeding.

DISCUSSION

Dysentery due to infection with *Shigella*, *Vibrio*, and *Salmonella* is an important health problem in humans. Some epidemiologists have pointed out that *Shigella* infections could be transmitted by flies under poor sanitary conditions.¹¹ Fly control measures have markedly diminished the prevalence of *Shigella*, diarrhea, and mortality due to the diarrheal disease compared with non-controlled areas.² It has been recently shown that *Helicobacter pylori*, a major pathogenic factor in gastroduodenal disease, is disseminated by houseflies.¹² Viable *H. pylori* could be isolated from gut and excreta for as long as 30 hr after ingestion of the bacteria. Houseflies frequently come in contact with human food and excrement under poor sanitary environments in developing countries. The relationship between flies and dung or similar situations may continue in some rural areas of Japan because the larval habitat of houseflies, i.e., dung in cowsheds and compost, may be located closely to residential areas. In most insects, food material in the alimentary canal is surrounded by the peritrophic membrane,¹³ which is an acellular membrane secreted by epithelial cells. The food mass is digested within the membrane.

It is important to understand why flies continue to excrete viable bacteria for at least 3 days, although the indigestible mass in the PM passes rapidly through their intestines. The presence of EHEC-O157 in the crop is most likely related to continuation of bacterial excretion. Another reason bacterial excretion persists for a number of days might be related to proliferation of EHEC-O157 on the mouthparts of the fly. The labellum of flies is usually shut and kept moist by repeated regurgitation and frequent tasting of liquid nutrients. The labellum seems to provide an adequate environment for proliferation of EHEC-O157 and other bacteria.

It is not known how the number of EHEC-O157 in the alimentary canal of the flies declines, although the morphologic changes seen in EHEC-O157 in the mouthparts of the flies may be related to this phenomenon. The ultrastructural changes in the bacteria may also be related to a physiological change in the bacteria to a so-called viable but non-culturable form.¹⁴ It is interesting that the changes in morphology of EHEC-O157 3 days after feeding coincided with the drastic decrease in the number of the bacteria in the alimentary canal. Our data clearly show that EHEC-O157 was actively

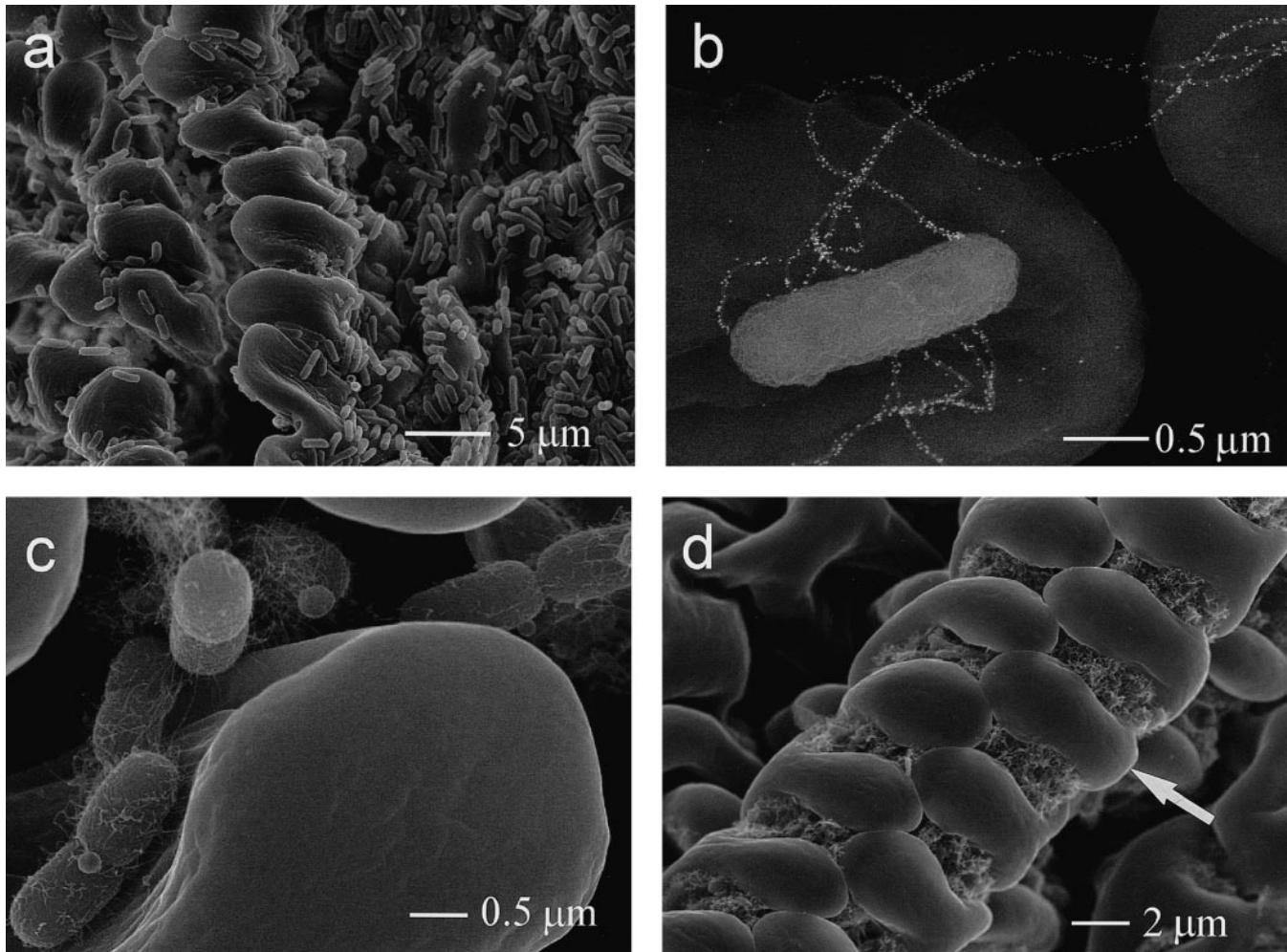


FIGURE 4. Scanning electron micrograph of the mouthparts of the flies ingesting enterohemorrhagic *Escherichia coli* (EHEC)-O157. **a**, EHEC-O157 adhering to the surface of fly mouthparts 24 hr after feeding. **b**, immunogold particles bound to the flagella of EHEC-O157 on the mouthparts 2 hr after feeding. **c**, actively proliferating EHEC-O157 in the minute space of pseudotrachea of mouthparts 24 hr after feeding. **d**, pseudotrachea (**arrow**) packed with mesh-like structures probably derived from EHEC-O157 (3 days after feeding).

proliferating in the mouthparts of houseflies, and that they are disseminated on agar plates at least for 3 days after ingestion.

Another important point is how many bacteria are present on the surfaces of bovine feces or in water contaminated with cattle feces. Recent study showed that heifers shed EHEC-O157 in the feces for 1–16 weeks at levels ranging from 2×10^2 to 8.7×10^4 CFU/g.¹⁵ When bovine feces was mixed with EHEC-O157 and the mixture was kept at 22°C or 37°C, they survived for 42–59 days after low and high inocula.¹⁶ This means that EHEC-O157 can persist in the environment on dairy farms. Widespread contamination of cattle feeds with *E. coli* and the ability of *E. coli* to replicate in feeds suggest that feeds are a potentially important factor in the ecology of organisms that can be transmitted from feces to the mouths of cattle,¹⁷ although the vehicle for the bacteria was not determined. In our experiments, the concentration of EHEC-O157 fed on by houseflies was clearly high compared with that in bovine feces, cattle feeds, and contaminated water. However, it should be noted that the infective dose of EHEC-O157 was considered low (< 100 bacteria),^{9,10} and bacteria, including *E. coli*, easily replicate

in various environment such as milk, filthy water, wounded fruits, boiled vegetables, and meats. When foods contain adequate moisture and nutrients and are kept at adequate temperatures for proliferation of bacteria, EHEC-O157 multiplies quickly, even though the number of EHEC-O157 on foods disseminated by flies is low.

Mechanical transmission by flies or cockroaches refers simply to dissemination of pathogens by contact with contaminated legs or mouthparts and by the excreta or regurgitated fluid within a short time after exposure to pathogens. The present data and other reports^{1,2} strongly suggest that houseflies are not simple mechanical vectors of EHEC-O157. For this type of transmission, a new technical term, bioenhanced transmission, should be used, particularly in the case of houseflies disseminating EHEC-O157.

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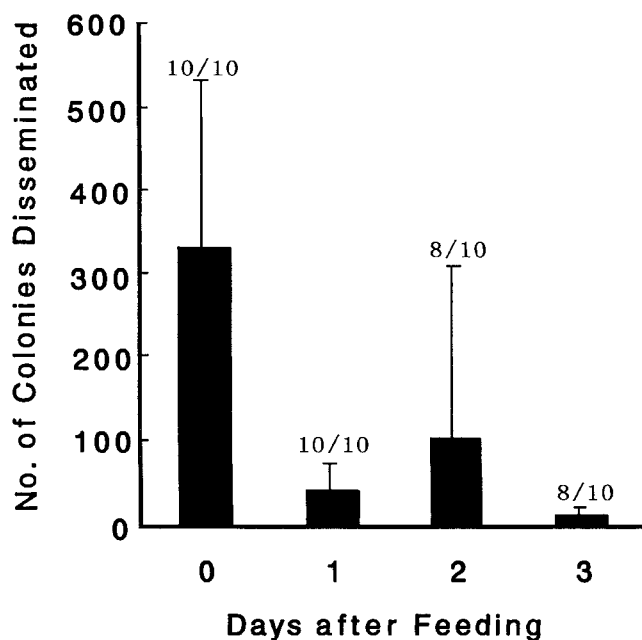


FIGURE 5. Number of enterohemorrhagic *Escherichia coli* (verotoxin 2-producing strain) colonies disseminated by houseflies after contact with agar plates for 2 hr. Numbers above the columns are no. of positive flies/no. tested. Bars show the mean \pm SD.

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