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Slugs: Potential Novel Vectors of *Escherichia coli* O157

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Field and laboratory studies were performed to determine whether slugs could act as novel vectors for pathogen (e.g., *Escherichia coli* O157) transfer from animal feces to salad vegetables. *Escherichia coli* O157 was isolated from 0.21% of field slugs from an Aberdeenshire sheep farm. These isolates carried the verocytotoxin genes (*vt1* and *vt2*) and the attaching and effacing gene (*eae*), suggesting that they are potentially pathogenic to humans. Strain typing using multilocus variable number tandem repeats analysis showed that slug and sheep isolates were indistinguishable. Laboratory experiments using an *E. coli* mutant resistant to nalidixic acid showed that the ubiquitous slug species *Deroceras reticulatum* could carry viable *E. coli* on its external surface for up to 14 days. Slugs that had been fed *E. coli* shed viable bacteria in their feces with numbers showing a short but statistically significant linear log decline. Further, it was found that *E. coli* persisted for up to 3 weeks in excreted slug feces, and hence, we conclude that slugs have the potential to act as novel vectors of *E. coli* O157.

Infection caused by *Escherichia coli* O157 is an emerging zoonoses in many countries including the United Kingdom and the United States (39, 47). The incidence of human disease is relatively low in industrialized countries compared to other enteric pathogens such as *Campylobacter* and *Salmonella* spp. (4, 25, 35). However, the sequelae can be severe, with approximately 5 to 10% of patients exhibiting hemolytic-uremic syndrome with a mortality rate of 3 to 5% (28, 48). Scotland has the highest ratio of human infections in the United Kingdom with 4.1 cases per 100,000 in 2004 (25).

Farm ruminants are the major reservoirs of *E. coli* O157, which is carried asymptomatically and shed transiently in their feces (21, 38, 52). Both cattle and sheep can excrete high levels of this pathogen ($>10^4$ CFU/g), leading to gross contamination of the farm environment and pasture (34, 45, 49). *Escherichia coli* O157 has been reported to have long-term survival (in manure, soil, and pasture), and manure application can lead to the contamination of vegetable crops and the surrounding environment (11, 20, 22, 30, 32, 52).

Escherichia coli O157 has caused a number of outbreaks associated with meat and dairy products of bovine origin, and in recent years, other foodstuffs, including fruit and vegetables (cabbage, alfalfa sprouts, celery, coriander, cress sprouts, and lettuce) (6), have been implicated as vehicles for human infection. Such outbreaks are frequently traced back to growers where a potential local *E. coli* source is identified, e.g., cattle feedlots, deer feces, or other ruminant fecal sources (2, 7, 8, 15). One of the largest outbreaks worldwide occurred in Japan with >6,000 cases (29) where the vehicle was identified as radish sprouts.

Although ruminants are recognized as the major reservoir of *E. coli* O157, it has also been isolated from a number of other animals including wild birds and rodents (31). Rabbits have been implicated as the vector of *E. coli* O157 when visitors to a petting farm were infected (37). This demonstrates the potential of alternate sources of *E. coli* O157 where ruminants are not directly present. In terms of invertebrates carrying this pathogen, pathogenic serotypes of *E. coli* have been isolated from the darkling beetle in both adult and larva forms and from the lesser mealworm (*Alphitobus diaperinus*) in poultry farms where *Salmonella* and *Campylobacter* spp. were also present (1, 14, 18). Laboratory experiments have further confirmed lesser mealworms as potential vectors of *E. coli*, *Salmonella*, and *Campylobacter* (27, 46). *Escherichia coli* O157 has also been isolated from 2.1% of houseflies sampled at a cattle farm in Japan (17) and 2.2% of houseflies collected from a cattle farm in the United States. Laboratory research has shown that flies can transmit *E. coli* from an environmental source to food products, e.g., the transfer of *E. coli* to apples by fruit flies (*Drosophila melanogaster*) (19). These studies demonstrate the potential of invertebrates to act as vectors in the transmission of *E. coli* O157 (12).

Slugs are widespread agricultural pests and frequently contaminate leafy vegetables, some of which are grown for human consumption without prior cooking (36). Slugs continuously ingest bacteria from the soil and their environment (51) and therefore have the potential to become contaminated with *E. coli* O157, both internally and externally. In previous work on the yellow slug (*Limax flavus*) and great gray slug (*Limax maximus*), both species were shown to carry coliforms both on the surface and in their gastrointestinal tract. A significant proportion of these were *E. coli* (10, 55), which suggests that slugs could be potential carriers of *E. coli* O157. However, neither of these large slug species are usually associated with leafy salad crops (10, 55). In this study, we have focused our laboratory studies on the gray field slug (*Deroceras reticulatum*), which is a widely distributed agricultural and horticultural pest, and as such, it frequently contaminates lettuce and other salad vegetables (36). This invertebrate may therefore

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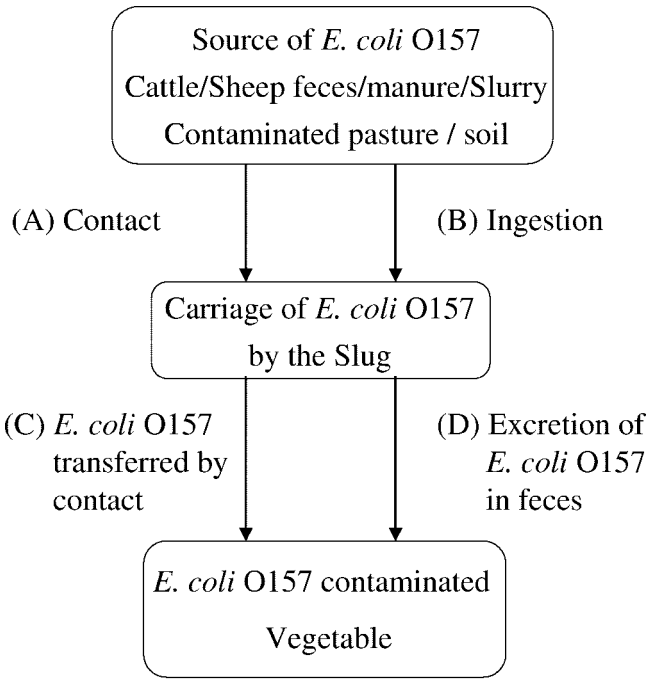

FIG. 1. Proposed transfer pathways of *E. coli* O157 from an environmental source to vegetable crops with slugs as the vector.

play a role in the transfer of *E. coli* O157 to fruit and vegetables, either by direct contact or contamination with feces (Fig. 1).

The aims of this study are to demonstrate that *E. coli* O157 and commensal *E. coli* can be found in slugs within the farming environment and that carriage and transfer of this pathogen to vegetable crops by slugs is feasible.

MATERIALS AND METHODS

Field survey. Slugs were collected during an 8-day period in July and a 3-day period in August 2004 from a farm in Aberdeenshire, Scotland, confirmed previously as having sheep shedding *E. coli* O157. During the study period, sheep feces were also collected and analyzed for continuing presence of *E. coli* O157 (50). The average number of slugs collected each day was 43 (23 to 98). The mean number of slugs pooled for enrichment was 15, with a variation of 2 to 26 depending on size and numbers available. On each sampling day, the slugs were pooled into groups of similar mass (mean ± standard deviation, 11.6 g ± 4.8 g) for the detection of *E. coli* O157. A total of 474 slugs were collected and pooled into 33 groups throughout the study period.

Isolation of *E. coli* O157. The slugs were homogenized in a sterile blender and enriched in a 10× volume of buffered peptone water (Oxoid CM509) supplemented with vancomycin (8 mg liter^{-1}) for 6 h at 42°C. This was followed by immunomagnetic separation (IMS) as described by Omisakin et al. (34). IMS beads were resuspended in 0.1 ml wash buffer and spread equally onto sorbitol MacConkey agar (SMAC, Oxoid CM813) supplemented with cefixime (0.05 mg liter^{-1}) and potassium tellurite (2.5 mg liter^{-1}) (CT-SMAC; Mast Diagnostics, Merseyside, United Kingdom) and on Harlequin SMAC BCIG agar (Lab M, IDG, Bury, United Kingdom) supplemented with cefixime and tellurite (Harlequin CT-BCIG). Both plates were incubated for 18 to 24 h at 37°C. Presumptive *E. coli* O157 colonies (non-sorbitol fermenting) were confirmed by latex agglutination (Oxoid DR620). Positive isolates were stored in a 13% glycerol solution and frozen at −20°C for further analysis.

Commensal *E. coli* carriage. A total of 40 slugs were separated into 2 equal groups and homogenized in a sterile blender, and 1 g from each was serially diluted in phosphate-buffered saline (PBS) and plated onto MacConkey agar. The plates were incubated for 24 h at 37°C, and presumptive target colonies were counted and confirmed by the detection of indole at 44°C from tryptone water (26).

Identification of *E. coli* O157 virulence genes. The *E. coli* O157 determinants of virulence (*vt1*, *vt2*, and *eae*) from isolates of both slug and sheep feces were identified using PCR (23). The PCR protocol was modified as follows: denaturing at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and then finally 4 min at 72°C for the VT1 primer. The PCR temperature sequence for VT2 and *eae* was the same except that the annealing temperature of 65°C was reduced to 55°C. PCR products were separated on a 1.4% agarose gel and visualized under UV.

Multilocus VNTR typing. The *E. coli* O157 strains from sheep feces with identical virulence profiles to those from slugs were subtyped by variable number tandem repeat (VNTR) typing (24). Here, PCR was used to amplify 7 loci that had previously identified nucleotide sequences which are repeated in tandem. The PCR temperature profile was 95°C for 4 min, followed by 30 cycles of 95°C for 30 s, an annealing temperature of 60°C for 60 s, 72°C for 60 s, and extension of 4 min at 72°C for all the primers except for primer 2, which required a lower annealing temperature of 55°C. Products from the seven primers were purified (QIAquick) and sequenced using dye-labeled dideoxy-labeled nucleotides. The number of times each set of base pairs is repeated was identified and used to fingerprint individual strains.

Nalidixic acid-resistant strains of commensal *E. coli*. Five strains (EC61, EC378, NCTC9001, ECB, and EC63) of non-O157 *E. coli* (all isolated previously from food except the NCTC strain, which was isolated from urine) were screened to produce nalidixic acid-resistant mutants (N^+) (5). Suspensions were prepared by adding 10-μl aliquots of each N^+ strain to 100 ml tryptic soy broth and incubated at 37°C for 24 h prior to centrifugation (5,000 × *g*), washing three times, and resuspension in 0.1 M PBS. All assays used MacConkey agar supplemented with 50 μl ml^{-1} nalidixic acid ($MacN^+$). Experimental samples were kept at 16°C, the average spring/autumn temperature when slugs are most active.

Carriage and transfer of *E. coli* from the external surface of the slug via direct contact. Two tests were performed where slugs were inoculated by placement into 90-mm petri dishes containing 5 ml of suspension (see above) of N^+ *E. coli.* The slugs were removed after 2 h and transferred to sterile dishes for 1 h to eliminate excess N^+ solution. Control batches of slugs were treated with sterile PBS.

In the first experiment, the inoculating suspension contained 6.0×10^9 CFU ml^{-1}, and 5 slugs were tested by wiping each slug directly over two $MacN^+$ plates. In the second experiment (5.8×10^9 CFU ml^{-1}), 5 slugs were swabbed with a cotton wool bud, previously dampened with PBS, and the cotton bud was spread over the $MacN^+$ plate. All plates were incubated for 24 h at 37°C, and colony numbers were recorded. This was repeated at 0 and 6 h and 1, 2, 3, 5, 7, 14, and 21 days. Each slug was transferred to a new sterile petri dish every 24 h to prevent recontamination.

Excretion of *E. coli* in slug feces. Slug food consisting of Ready Brek (an oat-based breakfast cereal), nonfat milk powder, and calcium carbonate (2:1:1) was hydrated with *E. coli* in PBS. In each of three experiments, five slugs were left exposed to food inoculated with the nalidixic acid mutant strain in separate 90-mm petri dishes containing filter paper moistened with 1.5 ml sterile PBS. One slug consumed no food and was excluded from further testing. After 24 h, feces were collected, weighed, serially diluted, and plated onto MacConkey agar, and the slugs were transferred to new sterile dishes with uninoculated food. This was repeated daily for 14 days. Controls were performed with uninoculated food.

Survival of *E. coli* in slug feces. Fresh feces were collected from slugs fed slug food (described above) for 48 h. The feces were divided into four equal portions of 0.41 g and each placed separately into 1.5-ml microcentrifuge tubes. Three of the tubes contained 65 μl N^+ *E. coli* in PBS (control was sterile PBS), and all tubes were mixed thoroughly. The three tubes of inoculated feces had an average initial *E. coli* concentration of 8.7 $\log_{10}$ CFU/g. At each sampling time (0 and 6 h and 1, 2, 3, 5, 7, 14, and 21 days), 0.04 g of slug feces was mixed with 360 μl PBS, serially diluted, and plated in duplicate onto $MacN^+$ agar.

RESULTS

Carriage of *E. coli* by slugs in the field. From the field sampling, 1/33 pooled samples (total of 474 slugs) tested positive for *E. coli* O157. Two colonies were confirmed by the latex agglutination test, and due to the low number of positive samples, it is plausible that both originated from the same slug. This equates to a field slug prevalence of 0.21%. The two pooled samples, tested for commensal *E. coli* carriage from

TABLE 1. Number of base pair tandem repeats at the loci isolated by each primer used from *E. coli* O157 isolates from slugs and sheep and a control (EDL 933)

Isolate	No. of repeats at locus no.:						
	1	2	3	4	5	6	7
Slug 1	ND[b]	1	5	ND	1	0	11
Slug 2	ND	1	5	ND	1	0	11
Sheep Feces 1	ND	1	5	ND	1	0	11
Sheep Feces 2	ND	1	5	ND	1	0	11
EDL 933[a]		3	9		1	0	11

[a] Reference strain.
[b] ND, no PCR product for this locus.

field slugs, were positive with values of 4.92 and 6.04 $\log_{10}$ CFU g^{-1}, respectively. Sheep feces collected from the same field on the same day had *E. coli* counts of 5.90 and 6.50 $\log_{10}$ CFU g^{-1}.

Molecular analyses (PCR and VNTR). Analysis of virulence factors by PCR showed that the *E. coli* O157 strain isolated from the slugs was positive for both verocytotoxin genes (*vt1* and *vt2*) and the attaching and effacing gene (*eae*). Slug and sheep isolates with the same virulence profiles (*vt1*, *vt2*, and *eae*) were analyzed by VNTR, and results are shown in Table 1. The slug and sheep isolates showed identical VNTR fingerprints. The time period between isolation of *E. coli* O157 from sheep feces and slug was 14 days. Interestingly, both sheep and slug isolates did not produce PCR products for VNTR primers 1 and 4, unlike three other *E. coli* O157 isolates from sheep (data not presented), including the reference EDL 933.

Carriage and transfer of *E. coli* from the external surface of the slug via contact. The transfer of *E. coli* from the external surface of the slug varied greatly between individuals and sampling times, with no obvious pattern except a general overall decline (Fig. 2). However, on comparison of the two different methods used (Fig. 2), a similar pattern is evident with a reduction in mean counts at 6 h followed by an increase at 24 h. In both experiments, the majority of *E. coli* isolates were shed within 48 h but were still detected (using the cotton wool bud swab method) at day 14 and by direct contact at day 7. It was found that wiping the slugs directly onto the agar produced higher counts than the cotton wool bud swab method during the first 24 h (Fig. 2).

***E. coli* concentration in slug feces after consumption of inoculated food.** The highest concentrations of *E. coli* were found in feces during the first 2 days following ingestion of inoculated food (Fig. 3). The majority of *E. coli* isolates were excreted during the first 48 h and showed a significant linear log reduction ($R^2 = 0.8879$, $P < 0.000005$) with a decimal reduction time of 0.34 days.

Survival of *E. coli* in feces. There was a small though insignificant increase in the *E. coli* concentration of inoculated slug feces (Fig. 4) during the first 2 days, from 8.7 to 9.0 $\log_{10}$ CFU g^{-1}. This was followed by a highly significant ($R^2 = 0.999$, $P < 0.0005$) log linear reduction from day 5, with a decimal reduction time of 4.15 days. *Escherichia coli* was still detected by direct plating at 21 days (the lack of results after day 21 was due to dehydration of the slug feces).

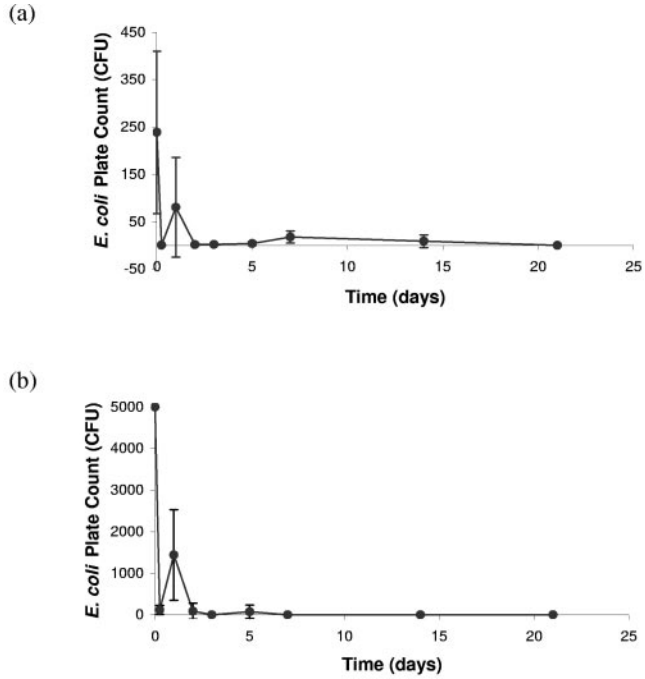

FIG. 2. Carriage and transfer of nalidixic acid-resistant *E. coli* from the external surface of the slug via contact and the reduction over time determined by swabbing the surface with a cotton wool bud (a) and wiping the external surface of the slug across an agar plate (b).

DISCUSSION

Fieldwork. This study is the first to confirm (by field sampling, PCR, and VNTR analysis) that slugs can be carriers of the same pathogenic *E. coli* O157 found locally in ruminant feces. This provides evidence to support transfer of *E. coli* O157 from ruminant feces to slugs (pathways A and B) (Fig. 1). Previous studies with slugs have shown no relationship between *E. coli* carriage and potential local sources (10) or failed to detect pathogens (41). The prevalence of *E. coli* O157 in slugs in the field studied here appears low at 0.21%, but slug populations of 300 m^{-2} have been recorded in arable fields (54), which equates to 1.44 positive slugs m^{-2} using the prevalence value reported here. This prevalence may have been higher with increased rainfall during the testing period where

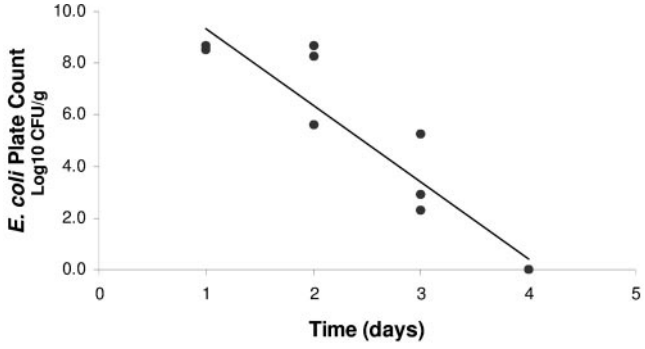

FIG. 3. Reduction with time of *E. coli* numbers in slug feces after consumption of inoculated food (detection limit, 1 CFU/g).

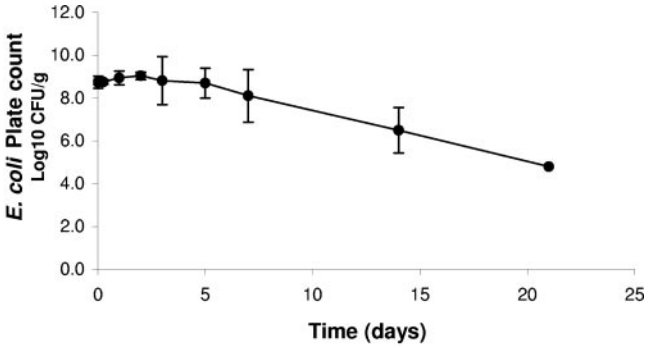

FIG. 4. Mean *E. coli* concentration in inoculated slug feces and its reduction over time.

E. coli O157 may have been distributed over a greater area, thus increasing the probability of slug contact. However, despite low rainfall (<1 mm per day), slugs tested here contained relatively high numbers of commensal *E. coli* isolates (approximately 1 log lower than the numbers recorded in sheep feces). The low prevalence of *E. coli* O157 in slugs is more likely due to low shedding rates of this pathogen in the ruminant reservoir where the concentrations in sheep fecal samples were $\leq$100 CFU g^{-1} (50). The prevalence of *E. coli* O157 in sheep here was estimated to be approximately 7% (50), which is similar to the 2003 Scottish Aberdeenshire value of 6.5% (33). In the present study, the pathogenic isolate of *E. coli* O157 found in the slugs was indistinguishable by VNTR from those carried by the sheep in the same field. Although the matching sheep isolate was recovered later than the isolate from the slug, it is likely that the strain was present in the sheep feces earlier but had not been sampled. Alternatively, it may have been sampled but the isolate not selected post-IMS for further analysis.

For contamination of vegetables by this vector to occur, the source of *E. coli* O157 (e.g., manure, feces, or runoff from pasture) and the vegetable site would need to be within the range of the slug. It has been reported previously (16) that this can be as much as 12 m per night for *D. reticulatum*. This distance may be extended in times of food deprivation (3) and during dispersal from areas of high population density (13). Laboratory experiments confirm that once a slug has become contaminated with *E. coli* via pathways A and B (contact and/or ingestion) (Fig. 1), it can persist for several days internally and externally, and therefore, there is sufficient time for an effective bacterial transfer (Fig. 1, pathways C and D).

Regardless of the sharp initial decline in *E. coli* numbers and high variation between individual slugs, the laboratory tests importantly demonstrate that external carriage of *E. coli* persists for relatively long time periods (Fig. 1, pathway A). Furthermore, *E. coli* can be readily transferred by contact (Fig. 1, pathway C). The results obtained at days 7 and 14 (Fig. 2) indicate sufficient time for pathogen transfer to a vegetable to take place.

The method of direct wiping of slugs to agar plates might be more realistic of how microorganisms transfer to vegetables. This method initially showed higher counts of *E. coli* than the swab method, probably because the swab failed to remove all bacteria present from the slug surface. Interestingly, there was a rise in counts at day 2 for both methods followed by a fall. It is unclear whether this is a real phenomenon, and further work would need to be done to verify this. The high variation between individuals may indicate a persistence of *E. coli* at different residual sites on the slug surface or simply slug-to-slug variation. Further work could be performed to determine the distribution of *E. coli* on the slug surface and to identify niches that *E. coli* preferentially colonizes.

The laboratory experiments demonstrate that slugs carry *E. coli* internally and excrete it in their feces following ingestion of contaminated food. This confirms pathways B and D (Fig. 1). It has also been reported that gastropods find mammalian feces an attractive food source (43), which together with the regular ingestion of potentially contaminated soil, demonstrates the potential of internal pathogen carriage. The majority of *E. coli* excretion by slugs occurred within the first 48 h, indicating direct passage through the intestines and agreeing with previous research (53). *Escherichia coli* excreted in the feces fell to below detection levels by day 4, and as slugs feed on average twice per day, there is sufficient time for them to travel to vegetables and contaminate the crop by excretion (Fig. 1, transfer pathway D). It has also been reported that other slug species (*Arion ater*) are stimulated to excrete while feeding (41), which would increase the likelihood of pathogen transfer to vegetables. The survival of *E. coli* in excreted slug feces was shown to persist for several days, where *E. coli* remained viable for >21 days in slug feces that was becoming dehydrated. A vegetable contaminated with slug feces, therefore, has the potential to harbor viable *E. coli* through harvest time and retail shelf life periods. The dehydration of feces during the later stages of the experiment also highlights the problem of applying laboratory data to the field.

The results of these experiments together with the field studies provide evidence that slugs have the potential to transfer *E. coli* O157 to vegetables via the pathways shown in Fig. 1. Care should be taken when considering the results of the laboratory studies, since commensal *E. coli* strains were used for ease of containment. It is likely, however, that toxigenic strains will have similar physiological characteristics. The likelihood of contaminated vegetables being ingested by the public will depend on the survival of *E. coli* on the crop. As it is advised that all fruit and vegetables be washed before consumption, research should investigate the removal of *E. coli* from the surface of vegetables at sites of both excretion and contact. Feces are more likely to be noticed by the consumer and the product discarded, whereas sites of contamination by contact would go unnoticed. Mucous deposited onto the vegetable by the slug may act as a beneficial substrate for bacterial growth and also afford protection against desiccation and sanitizing practices. Furthermore, it has been reported that *E. coli* will preferentially colonize cut edges on vegetables (40), e.g., in preparation of precut fruit and vegetables or at slug feeding sites. These sites will not only provide *E. coli* with some protection (from desiccation, washing, and sanitizing agents) but also offer entrance sites for internalization (42) and further reduce *E. coli* fatality by sanitary practices. The infective dose of *E. coli* O157 is thought to be low (9, 44); therefore, minimal survival in the vegetable has potential serious human health implications.

This study has found that slugs carry *E. coli* O157 in the

farming environment, which is indistinguishable by VNTR from *E. coli* O157 in sheep feces in the same field. The research demonstrates that *E. coli* in *D. reticulatum* has a relatively long external and internal survival time and also shows the ability of *E. coli* to persist at length in excreted slug feces. The potential for vegetable contamination is dependent on a number of factors which include slug control, slug population dynamics, weather, harvest, and postharvest practices. This study provides evidence that slugs can act as vectors of *E. coli* O157 from an environmental source to fruit or vegetables.

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