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ORIGINAL ARTICLE

Prevalence of *Escherichia coli* O157 and O157:H7-infecting bacteriophages in feedlot cattle feces

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Abstract

Aim: To estimate the distribution and prevalence of both *Escherichia coli* O157 and O157:H7-infecting bacteriophages within a 50 000 head commercial beef feedlot.

Methods and Results: *Escherichia coli* O157 was detected in ~27% of the individual samples, distributed across seven of the 10 pens screened. In a simple initial screen to detect O157:H7-infecting phages, none were detected in any pen or individual sample. In contrast, after a series of enrichment procedures O157:H7-infecting phages were detected in every pen and in the majority of the samples from most pens; virulent bacteriophages active against *E. coli* O157:H7 were detected post-enrichment from 39/60 (65%) of the feedlot samples, and 58/60 (~97%) contained phage that infected *E. coli* B or O157:H7.

Conclusions: The data we present here indicates that we may be grossly underestimating the prevalence of O157:H7-infecting phages in livestock if we simply screen samples and that enrichment screening is required to truly determine the presence of phages in these ecosystems.

Significance and Impact of the Study: Our data suggest that O157:H7-infecting phages may play a role in the ecology and transient colonization of cattle by *E. coli* O157:H7. Further, this and previous data suggest that before starting *in vivo* pathogen eradication studies using phage or any other regime, test animals should be enrichment screened for phage to avoid erroneous results.

Introduction

Escherichia coli O157:H7, one of today's leading public health concerns, is a foodborne pathogen that lives commensally in the guts of ruminant animals such as cattle and sheep (Rasmussen *et al.* 1993; Grauke *et al.* 2002). Cattle are a major reservoir leading to human exposure; the levels of *E. coli* O157:H7 detected in cattle feces have varied widely from study to study depending on the detection method used, the geographic location, the soil moisture content in the pen, cattle density, and the time of year sampling was undertaken, further complicated by the transient nature of O157:H7 gut colonization (Elder *et al.* 2000; Smith *et al.* 2001; Khaitsa *et al.* 2003; Van Baale *et al.* 2004). Upon slaughter, this pathogen can be transferred to meat products destined for human consumption (Elder *et al.* 2000; Barkocy-Gallagher *et al.* 2003). In ground beef, the U.S. Food Safety and Inspection Service enforce a 'zero tolerance' policy that requires the destruction of the entire batch of adulterated meat, at great cost. Additionally, manure used as fertilizer or water runs off from feedlots or dairies can taint crops or water supplies (Nataro and Kaper 1998; Brabban *et al.* 2004; CDC 2006).

Human illnesses from the contamination of food with Enterohemorrhagic *E. coli* lead to direct and indirect costs of 400–700 million dollars/year (Frenzen *et al.* 2005). It has been suggested that preharvest intervention methods could largely eliminate this pathogen from the gastrointestinal tracts of ruminants, thereby greatly reducing

human exposure, and as such be key in both disease prevention and reducing the costs to the U.S. economy (Callaway *et al.* 2003, 2004). Bacteriophage therapy is one 'old' idea undergoing a renaissance that may have a major role to play in near-future foodborne pathogen control.

Bacteriophages (phages) have long been recognized as important components of the gastrointestinal microflora (Klieve and Bauchop 1988; Swain *et al.* 1996; Brussow and Kutter 2005; Sulakvelidze and Barrow 2005). Recent *in vitro* data from a number of researchers have shown that virulent phages have great potential to selectively eliminate *E. coli* O157:H7 from its host environment; in contrast, *in vivo* results have been inconsistent (Kudva *et al.* 1999; Bach *et al.* 2003; Tanji *et al.* 2005; Raya *et al.* 2006; Sheng *et al.* 2006). Further, it has been suggested that naturally resident O157:H7-infecting phages may play a role in preventing gut colonization by *E. coli* O157:H7 in sheep (Raya *et al.* 2006). As a prelude to implementing such phage-based preharvest technologies and to further clarify the role that native phages play in the ecology of *E. coli* O157:H7 in food animals, we have examined the distribution and diversity of phages in feedlot cattle. In the current study we present data on the prevalence of *E. coli* O157 and O157:H7-infecting phages in a major commercial feedlot.

Materials and methods

Bacterial strains, growth and maintenance

The majority of the bacterial strains and phage types used in this study were drawn from our own culture collections (College Station, TX, and Evergreen, Olympia, WA, USA), including *E. coli* B (Neidhardt and Umbarger 1996), or isolated during this study. *Escherichia coli* O157:H7 NCTC 12900 (ATCC 700728), a strain in which the *stx* toxin region has been deleted rendering it nontoxigenic (Biosafety level 1), was obtained from the American Type Culture Collection (Manassas, VA, USA). All strains were grown in tryptic soy broth (TSB), on tryptic soy agar [1.2% (w/v) agar] or on MacConkey's agar plates (containing the appropriate antibiotic as applicable) and stored for the long-term in 10% (w/v) glycerol at −80°C.

Procedure to screen for *E. coli* O157

Fecal samples (∼100 g) were collected immediately after defecation from a large (∼50 000 head) commercial feedlot on a single August day by walking in a 'Z' pattern through the cattle while the collector observed which cattle were defecating. Samples ($n = 6$) were collected from each of 10 pens ($n = 60$ total samples), with a pen average of 150 head. Samples were immediately placed in a sterile sealed bag and kept on ice for <48 h prior to *E. coli* O157 analysis. The presence of *E. coli* O157 in feedlot fecal samples was determined by our standard enrichment methodology using the REVEAL^{TM} O157 detection kit (Neogen Corporation, Lansing, MI, USA). Putative *E. coli* O157 colonies were picked from CHROMagar O157 plates and serotypes were determined by standard ELISA methods (Keen and Elder 2002). This method does not further differentiate O157:(X) and as such we use the term *E. coli* O157 to refer to isolates detected using this kit in this study.

Procedure for phage screening and isolation

Fecal sample phage analysis was begun within 96 h of collection as described below (Fig. 1). In all cases of phage screening and enrichment, ∼2 ml of 'upper phase' or chloroformed enrichment culture was stored at 4°C for future use.

Initial phage screen

A weighed sample of fecal material (∼1 g) was homogenized in 9 ml of sterile phosphate buffered saline [PBS (g l^{-1}); NaCl 7.65, Na_2HPO_4 7.24, KH_2PO_4 2.10, pH 7.4] containing 100 μl of $CHCl_3$. Once the solids had settled, 5 μL of this homogenate was spotted onto lawns of *E. coli* O157:H7 NCTC 12900 and *E. coli* B. The presence and number of plaques was noted after 16 h incubation at 37°C.

Enrichment method 1

Approximately 1 g of fecal sample was homogenized in 9 ml PBS containing 100 μl of $CHCl_3$ and left for 1 h at 25°C. An aliquot (300 μl) of the upper phase (PBS) was then added to 1.2 ml of a log-phase culture of 12900 and incubated overnight at 37°C with agitation. The following day, 100 μl of $CHCl_3$ was added to the culture before it was centrifuged in an Eppendorf tube at 3000 ***g*** for 15 min. The supernatant was collected and spot tested on 12900 as above.

Enrichment method 2

Samples that failed to yield O157:H7-infecting phages using enrichment method 1 were subjected to enrichment method 2. Fecal material (∼10 g) was added to 30–40 ml PBS, homogenized and allowed to sit at 25°C for 1 h before the addition of 100 μl $CHCl_3$. An aliquot (500 μl) of the upper phase was used to inoculate a 1.5 ml TSB-12900 log-phase culture, which was incubated overnight at 37°C with agitation. The following day, this enrichment culture was treated with $CHCl_3$ and centrifuged in

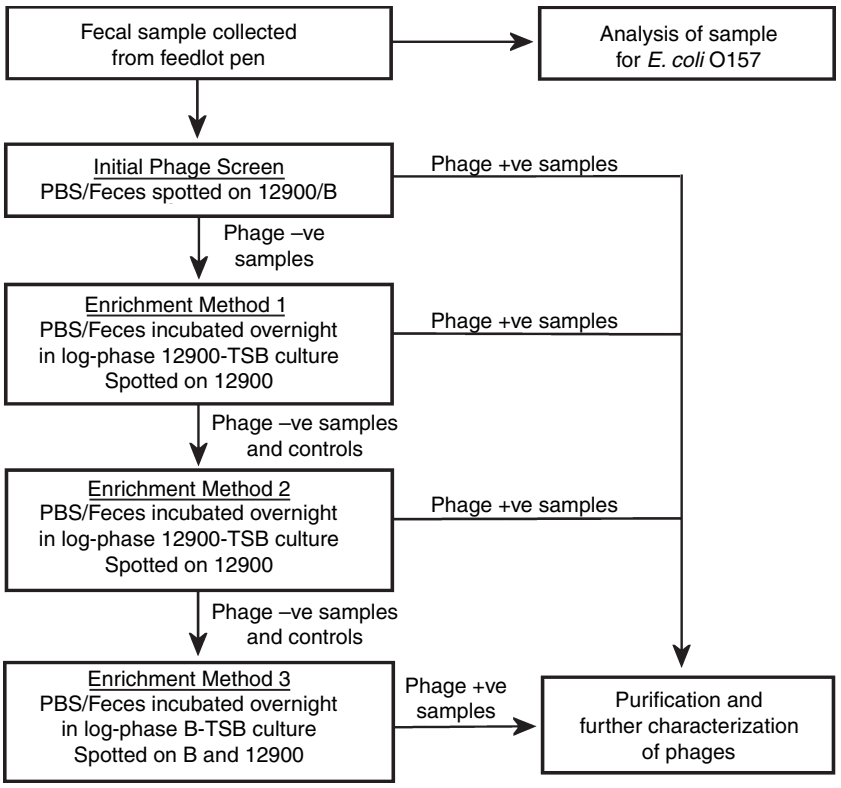

Figure 1 Fecal sample analysis: Samples were quantitatively (screen) and qualitatively (enrichment) analysed for the presence of *E. coli* O157 and O157:H7-infecting phages.

an Eppendorf tube at 3000 ***g*** for 15 min. The supernatant was collected and spot tested on 12900.

Enrichment method 3

All samples that tested negative for O157:H7-infecting phages after both enrichment methods 1 and 2 were put through a final enrichment protocol (enrichment method 3). This procedure was identical to enrichment method 2 except *E. coli* B was used as the host bacterium. Enrichment method 3 samples were spot tested on both B and 12900.

Results

Escherichia coli O157 was detected in ~27% (16/60) of the fecal samples and from 70% (7/10) of the feedlot pens sampled. Using our initial phage screen methodology (detection level >2000 PFU g^{-1} feces), phages capable of infecting the extensively studied laboratory strain *E. coli* B were detected in ~18% (11/60) of these fecal samples; in contrast, no O157:H7-infecting phages were initially detected (Table 1). In previous work we have noted that O157:H7-infecting phages are widely present, but often at such low concentrations they are not easily detected; each fecal sample was therefore subjected to enrichment. After enrichment (method 1) in the host *E. coli* O157:H7 NCTC 12900, O157:H7-infecting phages were detected in ~27% (16/60) of samples, with at least one phage-positive sample from each feedlot pen sampled (Table 1). Interestingly, both *E. coli* O157 and O157:H7-infecting phages were found in a small subset of these samples (61, 76, 106 and 109).

The 44 samples that failed to produce O157:H7-infecting phages after enrichment method 1 and two internal positive controls were subjected to enrichment method 2 in which ~10× the mass of feces was used, yielding an additional 17 samples containing O157:H7-infecting phages, five of which had tested negative by enrichment method one (62, 66, 69, 95 and 101).

A final enrichment (method 3) was carried out the 27 samples that had failed to yield O157:H7-infecting phages by both enrichment methods 1 and 2, this time using *E. coli* B as the host. Samples 69 and 120, which had previously been shown to contain O157:H7-infecting phages (enrichment method 2), were included here to examine the effect of host bacterium on phage enrichment. Samples enriched in this final procedure (method 3) were spot tested on both 12900 and B; ~21% (6/29) and ~93% (27/29) of these samples were found to contain phages active against 12900 and B, respectively. Interestingly, both sample 60 and 120 that had yielded O157:H7-infecting phages when enriched on 12900, did not yield

Table 1 Prevalence of *E. coli* O157 and O157:H7-infecting phage in fecal samples collected from a commercial feedlot with a population of ~50 000 head on the day of collection

Fecal sample	Feedlot pen	*E. coli* O157	Initial phage screen		Enrichment method 1 (12900)	Enrichment method 2 (12900)	Enrichment method 3 (B)	
			12900*	B*	12900*	12900*	12900*	B*
61	A	+	−	−	+	n/t	n/t	n/t
62		+	−	−	−	+	n/t	n/t
63		+	−	−	−	−	−	+
64		−	−	−	+	n/t	n/t	n/t
65		−	−	−	−	+	n/t	n/t
66		+	−	−	−	+	n/t	n/t
67	B	−	−	−	+	n/t	n/t	n/t
68		−	−	+	−	−	+	+
69		+	−	−	−	+	−	+‡
70		−	−	−	−	−	−	+
71		+	−	−	−	−	−	+
72		−	−	+	−	+	n/t	n/t
73	C	−	−	−	+	n/t	n/t	n/t
74		+	−	+	−	−	+	+
75		+	−	−	−	−	+	+
76		+	−	−	+	+†	n/t	n/t
77		+	−	−	−	−	+	+
78		−	−	+	−	−	−	+
79	D	−	−	−	−	+	n/t	n/t
80		−	−	−	−	−	−	+
81		−	−	−	−	+	n/t	n/t
82		−	−	−	−	+	n/t	n/t
83		−	−	+	−	−	−	+
84		−	−	+	+	+†	n/t	n/t
85	E	−	−	−	+	n/t	n/t	n/t
86		−	−	−	−	−	−	+
87		−	−	+	−	+	n/t	n/t
88		−	−	+	−	+	n/t	n/t
89		−	−	−	−	−	+	+
90		−	−	−	−	−	−	+
91	F	−	−	−	−	+	n/t	n/t
92		−	−	−	−	−	−	+
93		−	−	−	−	+	n/t	n/t
94		−	−	−	+	n/t	n/t	n/t
95		+	−	−	−	+	n/t	n/t
96		−	−	−	+	n/t	n/t	n/t
97	G	−	−	−	+	n/t	n/t	n/t
98		−	−	−	−	+	n/t	n/t
99		−	−	−	−	−	−	+
100		−	−	−	−	−	−	−
101		+	−	−	−	+	n/t	n/t
102		−	−	+	+	n/t	n/t	n/t
103	H	−	−	−	−	−	−	−
104		−	−	−	−	+	n/t	n/t
105		+	−	−	−	−	−	+
106		+	−	−	+	n/t	n/t	n/t
107		−	−	−	−	−	−	+
108		−	−	−	−	−	−	+
109	I	+	−	−	+	n/t	n/t	n/t
110		−	−	−	−	−	−	+
111		−	−	−	−	−	−	+
112		−	−	−	−	−	−	+
113		−	−	−	−	−	−	+

Table 1 (*Continued*)

Fecal sample	Feedlot pen	*E. coli* O157	Initial phage screen		Enrichment method 1 (12900)	Enrichment method 2 (12900)	Enrichment method 3 (B)	
			12900*	B*	12900*	12900*	12900*	B*
114	+	–	–	–	–	–	+	
115	J	–	–	+	+	n/t	n/t	n/t
116		–	–	–	+	n/t	n/t	n/t
117		–	–	–	–	–	–	+
118		–	–	+	+	n/t	n/t	n/t
119		–	–	–	–	–	+	+
120		–	–	–	–	+	–	+‡
Summary	7/10	16/60	0/60	11/60	16/60	17(19†)/60	6/60	27/60

*Bacterial strain on which sample was spotted.

†Samples positive in enrichment method 1 were re-examined in enrichment method 2 as positive controls.

‡Samples positive in enrichment method 2 were re-examined in enrichment method 3.

+, clear plaque; –, no plaque; n/t, not retested indicating + in a previous screen/enrichment.

these same phages when enriched on B. Of these 27 (previously phage negative) enrichment method 3 phage-containing samples, only six infected both O157:H7 and B (samples 68, 74, 75, 77, 89 and 119).

When considering the initial phage screen and all three enrichments, phages capable of infecting *E. coli* 12900 and/or B were detected in ~97% (58/60) of the samples examined in this study, of which 65% (39/60) contained O157:H7-infecting phages. Phages infecting O157:H7 were found in every feedlot pen and often in the vast majority of the samples for that pen. *E. coli* O157:H7-infecting phages were frequently found (~73% of the time) in those samples that tested positive for *E. coli* O157 (11/15 samples). In the three feedlot pens that tested negative for *E. coli* O157 (D, E, J), O157:H7-infecting phages were found in the majority of samples (13/18). Only 2/60 fecal samples examined failed to yield phages of either type.

Discussion

The practical application of phages to treat and control pathogens was originally demonstrated by d'Herelle ~80 years ago (d'Herelle 1919). After being largely ignored by Western Society since, the therapeutic use of these potent antibacterial agents in both human and animal disease has again become a focal point of applied research (Summers 2001; Sulakvelidze and Barrow 2005; Sulakvelidze and Kutter 2005). In support of our work to develop a phage-derived preharvest intervention treatment to control *E. coli* O157:H7, we systematically screened cattle fecal samples from a single (~50 000 head) commercial feedlot in the Southern Plains region of the United States for both *E. coli* O157 and O157:H7-infecting phages.

Escherichia coli O157 was detected in the majority of the pens screened (7/10) with a total of ~27% of the individual fecal samples being positive. It has been observed that fecal shedding of *E. coli* O157 varies widely according to season with levels typically low in winter and rising starkly during the summer months. Here, samples were collected during the month of August and as such a 25% incidence rate is consistent with levels found in previous studies (Elder *et al.* 2000; LeJeune *et al.* 2004; Low *et al.* 2005).

In the last 20 years, researchers have found that phage are much more numerous in many ecosystems than was traditionally thought and play a more central role in bacterial ecology (Fuhrman 1999; Ashelford *et al.* 2000; Danovaro *et al.* 2001; Breitbart *et al.* 2003; Brabban *et al.* 2005). The presence of phages in the guts of mammals (including ruminants) has been widely described (Dhillon *et al.* 1976; Breitbart *et al.* 2003; Callaway *et al.* 2006; Raya *et al.* 2006). Further, it has been suggested that phage in cattle help maintain microbial diversity and balance, allowing the ecology of the gut, particularly the rumen, to adapt to change (Klieve and Swain 1993; Swain *et al.* 1996). Although *E. coli* is a minor constituent of the ruminant microbiota, it is ubiquitous, and as one would expect, phages that infect O157:H7 and/or B were found in the majority of samples collected (~97%), although generally only after enrichment. This data, coupled with our previous phage screenings, illustrates the general prevalence of phage capable of infecting O157:H7 as well as the higher incidence of phages that can infect the standard lab strain B (Raya *et al.* 2004, 2006; Callaway *et al.* 2006). Our previous studies have demonstrated that phages isolated using *E. coli* B or K12 as host generally have much broader host ranges than do those isolated on pathogens, including O157:H7, although the efficiency of

plating of the former on wild strains is often much lower (Chibani-Chennoufi *et al.* 2004; unpublished data). Phage isolated against O157:H7 have a wide range of strain selectivity. Some infect the majority of tested O157:H7 strains but only a few diverse other *E. coli* (Bach *et al.* 2003; Raya *et al.* 2006; Sheng *et al.* 2006). For example, phage CEV1 infects 17/19 of tested O157:H7 strains and 9/72 of the ECOR strains, from 4/5 phylogenetic groups (Raya *et al.* 2006). In contrast, phage AR1 (initially reported to be highly specific for O157:H7) has been shown to infect 18/18 O157:H7 strains and 38/72 strains of the ECOR collection (Goodridge *et al.* 2003). Multiple different receptors may be used for binding to the various hosts in these cases, as has been shown for T4. Unlike O157:H7 the outer membrane of B only contains a novel core lipopolysaccharide (LPS) unshielded by an O-antigen; T4 binds efficiently to this LPS on *E. coli* B, but its equally efficient primary receptor on K12 strains is the outer membrane protein OmpC, which is not present in B, and it binds only weakly to the K12-specific LPS. The O157 version of OmpC is not recognized by T4 but is a primary receptor for AR1 (Prehm *et al.* 1976; Henning and Hashemolhosseini 1994; Goodridge *et al.* 2003).

Previously, we have described two phages, naturally resident in the guts of sheep, which seemed to confer a resistance to *E. coli* O157:H7 colonization. Phage CEV1 was isolated from a flock of 20 sheep from one farm in Texas; within 3 days, experimentally introduced *E. coli* O157:H7 ($\sim 10^9$ CFU per sheep) were no longer detected in the feces of these sheep. A second flock of sheep were subsequently found to contain a different O157:H7-infecting phage (CEV2), detected after enrichment in 20/39 members of that flock (Raya *et al.* 2004, 2006). Here we present confirmatory evidence that simple homogenization followed by spot testing of fecal samples does not begin to offer the level of detection that can be achieved using enrichments. A number of studies have examined the use of phages against O157:H7 *in vivo* either in model systems such as mice, or in livestock. In these experiments, researchers have consistently prescreened test animals for the presence of O157:H7, but rarely for the presence of naturally resident phages (Bach *et al.* 2003; Raya *et al.* 2006; Sheng *et al.* 2006). The results we present here imply that before carrying out *in vivo* experiments related to the eradication of pathogens (such as O157:H7) using any approach, it is crucial that researchers first screen by enrichment for phages; otherwise, endogenous phages may multiply after inoculation of the pathogenic bacteria and lead to false-positive results.

Cattle and other livestock are transiently colonized by *E. coli* O157:H7, shedding highly variable but detectable quantities for 30–60 days. This colonization/shedding occurs in clusters and it is thought that O157:H7 is introduced and passed among members of a herd or pen through contaminated feed, water, direct animal or fecal contact, and the farm/pen environment (Conedera *et al.* 2001; Van Donkersgoed *et al.* 2001; Bach *et al.* 2002; LeJeune *et al.* 2004). In a recent feedlot cattle study, it was noted that upon arrival only 1.4% of cattle shed O157:H7 but within 28 days this number had risen to 6.9%; no O157:H7 was detected in the pens before the cattle arrived (Khaitsa *et al.* 2006). Such research has improved understanding of the colonization/transfer process but our grasp of what is at the root of the transient nature of colonization is lacking. We would like to suggest that phage may, in part, explain this phenomenon. Previously, we have suggested that resident phages can prevent *E. coli* O157:H7 from colonizing the guts of sheep (Raya *et al.* 2006); here we suggest that they may play a central role in the transient nature of *E. coli* O157:H7 in cattle. Phages, by nature are predators of their host bacteria and as such, classical predator-prey relationship rules hold true. In the feedlot samples analysed here, high levels of O157:H7-infecting phages within a feedlot negatively correlate with detectable levels of *E. coli* O157; in the three feedlots that tested negative for *E. coli* O157:H7, the majority of the fecal samples contained O157:H7-infecting phages. We also note that in some samples *E. coli* O157:H7 and O157:H7-infecting phages were found to coexist. This data illustrate the ubiquitous nature of phages in the environment, demonstrated by the fact that no feedlot was phage-free, although levels may be extremely low and detectable only after enrichment. We have previously found that phages present at low concentrations in the ruminant gut (and fecal material) can rapidly reproduce once sufficient O157:H7 has been introduced either artificially or naturally (unpublished data). This phenomenon is a parallel to the co-existence of phages and their hosts at the very low levels in both marine and soil environments; phage numbers escalate after any expansion of their host bacterial population (Guixa-Boixereu *et al.* 1999; Ashelford *et al.* 2000). Our suggestion is also supported by the work of Smith *et al.*; in their studies using phage against enteropathogenic *E. coli* (EPEC) in young calves, the application of exogenous phage led to a 10^5 fold reduction in EPEC cell numbers typically within 48 h. In addition, the phage established themselves throughout the gut at concentrations as high as 10^8 PFU g^{-1} in the colon, showing the ability of phage to rapidly reproduce in the bovine gut in the presence of a suitable host (Smith and Huggins 1983). Perhaps more importantly in the context of our study was their observation that when new calves were introduced into a room in which calves had played host to high levels of EPEC-infecting bacteriophages, they became immune to EPEC infection within 3 h of entering the room, clearly

demonstrating the transmissible protection envisaged by d'Herelle; these bacteriophages were detected in the facility for up to a year (Smith *et al.* 1987).

If bacteriophages are to be effective therapeutic agents, both careful characterization of the phages of interest prior to their *in vivo* use and examination of the ecological effects of introducing an organism into a system as complex as the mammalian gastrointestinal tract are needed. The data we present here show that O157:H7-infecting phages may be more prevalent in the animal gut than was previously demonstrated. Further, we suggest that screening for phages by enrichment is needed before *in vivo* pathogen eradication studies using phage or any other regime are begun to avoid erroneous results. Finally, our data show that O157:H7-infecting phages and *E. coli* O157, can and sometimes do coexist in these systems, at low levels.

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