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Use of Heme and Hemoglobin by *Escherichia coli* O157 and Other Shiga-like-Toxin-Producing *E. coli* Serogroups

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The virulence properties of *Escherichia coli* O157 isolates were compared with those of Shiga-like-toxin-producing *E. coli* of non-O157 serogroups. The growth of all *E. coli* O157 isolates was stimulated by both heme and hemoglobin, and all produced enterohemolysin. The incidence of these properties was significantly lower in the non-O157 isolates. This may contribute to the greater virulence and higher incidence of human infection caused by *E. coli* O157.

Isolates of Shiga-like-toxin-producing *Escherichia coli* (SLTEC) have emerged as an important cause of human diarrheal illness (5). Most infections are caused by a single serogroup of *E. coli*, serogroup O157, first implicated as a human pathogen in 1982 following two outbreaks of hemorrhagic colitis in the United States (16). Isolates of this serogroup are recognized as an important cause of bloody diarrhea and hemorraghic colitis and are associated with the hemolytic-uremic syndrome (5, 8, 9, 16).

At least 40 different serogroups of *E. coli* can produce Shiga-like toxins (SLT) (5, 27). However, the incidence of human infection with *E. coli* O157 is greater than the incidence of infection with non-O157 serogroups (5, 17), and outbreaks are rare in the latter group (5). This is in marked contrast to the relative incidence of these isolates in food products. Non-O157 SLTEC has been isolated from both cattle and meat products with high frequency, whereas *E. coli* O157 is rarely isolated from food or cattle (5, 20, 24). This implies that humans are exposed much more often to non-O157 SLTEC than to *E. coli* O157. The low incidence of non-O157 infections in humans suggests that toxin production alone is insufficient to cause disease and that *E. coli* O157 isolates possess other factors that enhance their virulence (1).

Most *E. coli* O157 isolates produce enterohemolysin and possess the *eaeA* gene (1, 27) associated with adherence properties and their ability to produce attaching and effacing lesions (7, 10). The presence of these factors in non-O157 SLTEC isolated from both cattle and humans is variable, and the incidence is lower than that in serogroup O157 (1, 3, 27). However, some non-O157 isolates have both properties, suggesting that these factors alone cannot explain the greater virulence of *E. coli* O157. It is likely that virulence in *E. coli* O157 is a multifactorial phenomenon.

All bacteria have a nutritional need for iron and in iron-limited environments produce siderophores which remove iron from host iron-containing compounds such as transferrin and lactoferrin (6). Many enteric pathogens, e.g., *Vibrio cholerae* (21), *Campylobacter jejuni* (15), *Shigella flexneri* (13), and enteropathogenic *E. coli* (12), have been shown to utilize heme and/or hemoglobin as iron sources. We have shown that utilization of hemoglobin by enteropathogenic *E. coli* causes a marked enhancement of growth rate (12). Because bloody diarrhea is a common characteristic of SLTEC infection, we investigated whether *E. coli* O157 isolates can utilize heme or hemoglobin and if this ability was more common in this serogroup than in non-O157 SLTEC.

Twenty *E. coli* O157 isolates were examined; 19 of these were isolated from patients with bloody diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome. All isolates were distinct epidemiologically and consisted of several different phage and toxin types, although all belonged to flagellar type H7. The remaining isolate was *E. coli* O157 isolate 933, a kind gift of David Acheson. Sixteen human non-O157 SLTEC isolates were tested, including five O26, one O111, two O103, one O100, two O128, one O153, one O163, and three untypeable isolates. These were isolated from cases of bloody diarrhea and hemolytic-uremic syndrome. Seventeen cattle SLTEC isolates were tested; two were not serogrouped, and the remainder were untypeable with a range of 40 O group sera.

The ability of isolates to produce SLTs and enterohemolysin was confirmed by previously described methods (2, 11). Organisms were examined for the ability to use heme and hemoglobin by comparing growth in M9 medium supplemented with 2% Casamino Acids and made iron limited by the addition of ovotransferrin with growth in the same medium with either heme or hemoglobin added as an iron source. The method has been described previously in detail (12). Fisher's exact test was used to compare the incidences of the virulence properties in the groups.

All of the *E. coli* O157 isolates produced enterohemolysin and were able to utilize both heme and hemoglobin. The incidence of these properties in the non-O157 groups was variable and occurred at a significantly lower level than it did with the O157 isolates (Table 1). When a combination of factors were analyzed, namely, enterohemolysin production and heme or hemoglobin utilization, the occurrence of the combined virulence factors was also significantly higher in the O157 group (20 of 20 isolates) than in the cattle (2 of 17) and human (4 of 16) non-O157 groups ($P = 0.000000014$ and 0.00000014, respectively).

Shiga-like toxin II (SLT-II) was produced by all the O157 isolates; two also produced SLT-I. Both SLT-I and -II were produced by four of the human non-O157 isolates, five produced SLT-II only, and seven produced SLT-I only. Among the cattle isolates, only one produced both toxins, seven produced SLT-II only, and nine produced SLT-I only.

The similarity of properties among the O157 isolates is not surprising considering the clonal origin of this serogroup (26). Since SLT-producing *E. coli* O157 commonly produces bloody

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TABLE 1. Occurrence of virulence properties in three serogroups of SLTEC

Serogroup	Total no. of isolates	No. of isolates producing hemolysin	Enterohemolysin production		Stimulation of growth by heme		Stimulation of growth by hemoglobin	
			No. of isolates (%)	P^a	No. of isolates (%)	P	No. of isolates (%)	P
O157	20	0	20 (100)		20 (100)		20 (100)	
Human non-O157	16	1	11 (69)	0.011	5 (31)	0.0000072	3 (19)	0.00000024
Cattle non-O157	17	0	11 (65)	0.001	2 (12)	0.00000014	1 (6)	0.0000000013

[a] P values are for comparison of human or cattle non-O157 serogroup values with O157 serogroup values, determined by Fisher's exact test.

diarrhea, the ability of isolates of this serogroup to use heme or hemoglobin as an iron source may enhance virulence by stimulating rapid multiplication, assisting in bowel colonization. A tentative model for the role of heme or hemoglobin utilization is described.

Following ingestion of the O157 isolate, organisms adhere to and colonize the bowel mucosa; this may be mediated in part by the *eaeA* gene (4, 23). SLTs which bind to receptors on the bowel mucosa are elaborated and translocated into the cell interior, causing cessation of protein synthesis and cell death (14). Toxin delivery to the mucosa may be assisted by intimate adherence, a characteristic of *E. coli* O157 and some non-O157 serogroups (10). Cell death leads to mucosal damage, causing release of blood into the bowel lumen; at this stage, blood in the feces may not be apparent. Lysis of erythrocytes by enterohemolysin liberates heme and hemoglobin. Utilization of these compounds results in rapid multiplication of the organism. As multiplication takes place, further toxin production occurs, causing greater damage, releasing increasing amounts of blood, and resulting in growth stimulation.

The reduced incidence of infection caused by non-O157 isolates may be due to a number of factors such as their inability to colonize the bowel through lack of adhesins such as *eaeA*, which is present in only a proportion of non-O157 serogroups. Adherent isolates may be unable to lyse erythrocytes to release heme and hemoglobin or may be unable to use these compounds when they are released. We have shown that these properties occur significantly less frequently in non-O157 serogroups. Such isolates may therefore be unable to colonize or multiply sufficiently in the bowel to secrete the levels of toxin necessary to produce diarrhea.

Since heme and hemoglobin utilization was found among a small proportion of the non-O157 serogroups, it cannot solely explain the increased virulence of *E. coli* O157 serogroups, and these properties are likely to act in conjunction with other virulence factors. Some serogroup O26 and O111 isolates share similar properties with O157 in that they are *eaeA* and enterohemolysin positive (18, 27) and their growth is stimulated by hemoglobin and/or heme, and yet these serogroups are not as common as *E. coli* O157 in cases of human infection. One explanation of the reduced virulence may be related to the type of toxin produced. All of the O157 isolates elaborated SLT-II, and in two cases SLT-I was also produced; isolates that produce SLT-I alone are rare. However, all of the O26 serogroup isolates produced SLT-I only, a finding confirmed by others (18). Of the two forms of toxin, SLT-II is more toxic than SLT-I in a mouse model (22, 25). Furthermore, in vitro, we have found that for many SLTEC isolates the yield of SLT-II is greater than that of SLT-I (unpublished data). Therefore, SLT-II production may be a greater virulence attribute than production of SLT-I, accounting in part for the increased virulence of *E. coli* O157. Diarrheal cases and rare outbreaks attributed to isolates of non-O157 serogroups may occur when large numbers of organisms which may counteract the reduced virulence of these isolates are ingested.

We have noted that the incidence of heme and hemoglobin utilization in the non-O157 SLTEC isolates is considerably lower than among enteropathogenic *E. coli* isolates and normal flora human *E. coli* isolates tested previously (12). It is possible that this ability is beneficial for colonization of the human but not the cattle intestinal tract, and hence, there is a low incidence of these properties in cattle SLTEC. *E. coli* O157 may represent a clone of organisms which is virulent for humans because of the production of SLT-II, adhesive properties, and the abilities to release iron-containing compounds from erythrocytes and to use these compounds.

These in vitro findings must be confirmed in relevant in vivo models, such as that recently described by Sjogren et al. (19). Furthermore, the use of molecular techniques to generate mutants with inactivated hemolysin or heme and hemoglobin uptake or utilization systems will be useful in determining the role of these potential virulence factors in disease caused by SLTEC.

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