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Pre-harvest Detection and Control of Verotoxin (Shigatoxin)-producing *Escherichia coli*

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Introduction

Verotoxin-producing *Escherichia coli* (VTEC) are a diverse group of *E. coli* belonging to over 200 serotypes, and defined by production of one or more Verotoxins (VTs), also known as Shigatoxins (Stxs). VTEC pathogenic for humans cause diarrhea, bloody diarrhea and the hemolytic uremic syndrome. Most serious human infections are caused by enterohemorrhagic *E. coli* (EHEC), a virulent sub-group of VTEC which includes the predominant serotype, *E. coli* O157:H7, and others such as O26:H11 and O111:H- [33].

Human VTEC infections are linked frequently to foods, water, animals and environmental sources contaminated with the manure of infected but healthy animals [45,46,68]. Domestic and wild ruminants are the major animal reservoirs of VTEC [7]. Cattle are particularly important in areas of intensive cattle production; they excrete 30-50 kg of manure per day, which may contain up to 10^7 *E. coli* O157:H7 per gram [24,41,52]. Since *E. coli* O157:H7 is prevalent in many cattle herds and feedlots, particularly in the summer [23,29,65], the load of this pathogen entering the environment is substantial. It is even greater if all VTEC are included [65,69].

Recently introduced interventions to control *E. coli* O157:H7 and other VTEC in beef slaughter and processing have had little impact on the incidence of human disease. In part, this reflects the importance of sources other than beef meats, such as animal contact, and contaminated water, produce and environments [46,47,68]. Consequently, there is growing emphasis on the pre-harvest epidemiology and control of VTEC in cattle and other animal reservoirs.

Detection of VTEC in animal reservoirs and environments

As knowledge of the epidemiology of VTEC in animals has increased, so too has awareness of the importance of the sampling plan, sample type, sample size and test methods for reliable detection of VTEC in animals [16,49]. Test methods have evolved along two main approaches; one focused on *E. coli* O157:H7, and the other on all VTEC. Cultural, immunological and molecular methods continue to be developed for both approaches. Most test methods are qualitative, and since animal-related samples frequently have fewer VTEC than found in human clinical samples, some form of enrichment culture is needed. Newer methods, such as real-time PCR, offer hope for direct detection and quantitation.

Sampling: Detection of individual animals shedding *E. coli* O157:H7 and other VTEC typically has relied on testing of fecal samples, where samples >1 g provide more reliable results [58]. Recent evidence for preferential colonization of cattle at the terminal colon and rectum [5,50], has led to a recto-anal mucosal swab sampling method that potentially can distinguish between colonized animals and transient carriers [57]. Sampling for prevalence by pens, lots or herds typically is a proportional sample of fecal samples from the rectum or from fecal pats, based on expected prevalence. Other samples, from water troughs [42], hides, the oral cavity [3,36], or from a novel rope sampling device [61], appear to be reliable alternatives that may simplify detection of *E. coli* O157:H7 and other VTEC at the pen, lot or herd level. Longitudinal sampling every two to four weeks is recommended to detect the peak prevalences when fecal samples are the sample of choice [6,16,29,43].

Detection of *E. coli* O157:H7: Methods for detection of *E. coli* O157:H7 rely mainly on presence of the O157 antigen and other partially selective properties, such as inability to ferment sorbitol within 24 h, lack of β-glucuronidase activity and resistance to tellurite, cefixime and cefsulodin [16,18,35]. Consequently, the most commonly used plating medium for isolation of *E. coli* O157:H7 is sorbitol-MacConkey agar containing cefixime and tellurite (CT-SMAC), although several commercially available chromogenic agars may have greater selectivity for *E. coli* O157:H7 [44]. Colonies typical of *E. coli* O157:H7 on these media can be tested for the O157 antigen by simple agglutination tests. Putative *E. coli* O157:H7 isolates can then be characterized by biotyping, standard O and H serotyping and/or tested for VTs or for selected genetic markers by PCR or colony blot hybridization [35,53]. Notably, methods relying on the sorbitol-negative and β-glucuronidase-negative properties of *E. coli* O157:H7 will not allow detection of the sorbitol-positive, β-glucuronidase-positive *E. coli* O157:H- strains causing human disease in parts of Europe[35].

Perhaps the greatest advance in reliable isolation of *E. coli* O157:H7 has been immunomagnetic separation (IMS), utilizing magnetic beads coated with antibodies to *E. coli* O157:H7 to capture the bacteria in enrichment cultures. Being 100-fold more sensitive than other isolation methods [15], IMS is now used widely, can be automated, and adapted for enumerating *E. coli* O157:H7 in bovine feces [24,31]. Newer automated IMS systems designed for food testing may also be useful for testing samples from animals and other sources.

Numerous immunoassays are available as screening tests for *E. coli* O157:H7 [18]. Many have good test sensitivity when applied to enrichment cultures, but most detect the O157 antigen, and give false-positive results when non-VTEC of this serogroup, or antigenically-related organisms, are present. Greater specificity can be achieved with immunoassays incorporating monoclonal antibodies [67].

Molecular methods, particularly PCR, have proven useful as screening tests for *E. coli* O157:H7, and for confirmatory identification of putative isolates. Target sequences include those of the rfb_{O157} locus, intimin γ and the $fliC_{H7}$ flagella gene [20,26,27]. More recently, several real-time PCR methods for detection and/or characterization of *E. coli* O157:H7 have been developed [8,30,56,59]. In addition to reducing test time, real-time PCR offers the possibility of direct quantification of the organism

Detection of all VTEC. Detection of all VTEC requires testing for VTs or VT genes. Mixed cultures can be screened for VTs by the Vero cell cytotoxicity assay or VT immunoassays, and for VT genes by PCR [70]. Although more rapid and more suitable for high through-put than the cytotoxicity assay, immunoassays and PCR generally require overnight enrichment culture to obtain sufficient numbers of VTEC to meet the limits of detection of the respective tests. This may be reduced for real-time PCR.

While screening tests can provide evidence of VTEC in mixed cultures, isolating VTEC from these cultures is laborious. VTEC other than *E. coli* O157:H7 have few features other than VT production or VT genes to differentiate them from other *E. coli*. Consequently, isolation relies on testing numerous individual colonies for VT production or VT genes by the above tests, or by colony hybridization [25,35,53] or colony immunoblotting [2]. VT-positive isolates can then be identified by biotyping, serotyping and molecular typing. Due to limitations in the feasibility of testing high numbers of colonies, the rates of isolation of VTEC from VTEC-positive mixed cultures of samples other than human clinical specimens has often been low [32,55]. However, isolation rates greater than 90% have been reported [2]. Recently, IMS and multiplex PCR assays have been applied to some non-O157 VTEC, including the more common EHEC serogroups O111 and O26 [31,51,60].

Serological Diagnosis: Infection of cattle with *E. coli* O157:H7 induces strong antibody responses to the O157 antigen [34,40]. Similar responses to the LPS antigens of other VTEC, particularly those that are pathogenic in cattle (O111, O26, O103) also occur (unpublished). However, since most cattle experience infection with these and other VTEC in the first year of life, serological diagnosis has limited application. A competitive assay employing a monoclonal antibody to the O157 antigen of *E. coli* O157:H7 appears to provide greater specificity than indirect assays in detecting recent, first infections with *E. coli* O157:H7 [40]. Little is known of bovine serological responses to other relevant antigens, although calves and adult cattle have antibodies to VT1, but few have antibodies to VT2 [9]. Most adult cattle also have serum antibodies to intimin, Tir, and EspA of *E. coli* O157:H7 (unpublished).

Control of VTEC in animals

Pre-harvest control measures to reduce the public health risks arising from VTEC in animal reservoirs must address not only entry of VTEC into the food chain at slaughter, but also hide and environmental contamination. The nature and timing of interventions ideally will be designed to limit the spread of infection within herds or lots. Calves may be infected by *E. coli* O157:H7 soon after birth and most experience infection in their first year [28,29,41,65]. In both beef and dairy cattle, highest rates of infection occur soon after weaning and in young adult animals, with duration of infection in most animals being four weeks or less [6], although some cattle may remain infected for longer periods [6]. In feedlots, infection rates are highest soon after induction and decrease during the fattening period. However, the heavy bacterial load in the feedlot environment and survival of VTEC in manure [38] and water troughs [42] contribute to re-infections, and the prolonged pen-level infections seen in feedlots [37,43,48]. Effective interventions applied at the time of weaning, feedlot induction and other times of co-mingling are likely to reduce the spread of infection and fecal, hide, water tough and environmental contamination.

Numerous potential interventions to control VTEC have been evaluated, primarily in cattle or sheep infected with *E. coli* O157:H7. These include competitive exclusion, probiotics, prebiotics, synbiotics, antibiotics, other antimicrobials, colicins, vaccination, bacteriophage therapy, sodium chlorate, diet, water treatment, and other farm management practices [3,63], several of which appear to have promise.

<u>Probiotics:</u> Treatment of 8-10 weeks-old calves with a mixture of three selected probiotic *E. coli* three days after oral inoculation with EHEC O157:H7, O26:H11 or O111:NM substantially reduced or eliminated fecal shedding of EHEC O157:H7 and O111:NM beyond 8 and 6 days after treatment, respectively. Shedding of EHEC O26:H11 was unaffected [64]. In addition, a selected probiotic *Lactobacillus acidophilus* strain (NPC 747) fed to feedlot cattle reduced the number of *E. coli* O157:H7-shedding animals by about 40-50%, compared to untreated cattle, and the prevalence of this organism in pens and on hides at slaughter [1,10].

<u>Antibiotics</u>: Although the use of antibiotics in agriculture is currently under scrutiny, treatment of cattle with neomycin sulphate for two days dramatically reduced the prevalence of *E. coli* O157:H7 and numbers of generic *E. coli* in feces, although generic *E. coli* counts returned to near normal levels five to seven days after treatment [22]. In commercial feedlots, neomycin sulphate reduced the numbers of cattle shedding *E. coli* O157:H7 from 46% to 0%, as well as hide prevalence, and was almost as effective when administered in the water supply [1].

<u>Bacteriophage therapy</u>: Evidence that bacteriophage (phage) therapy was effective in controlling *E. coli* diarrhea in calves, piglets and lambs [62] has prompted investigations of phages to control *E. coli* O157:H7 in cattle. Several phages lytic for *E. coli* O157:H7 *in vitro* have proven less effective *in vivo* or in microaerophilic conditions [4,14,39]. However, a panel of six *E. coli* O157:H7-specific phages administered orally to calves experimentally infected with *E. coli* O157:H7 resulted in elimination of the organism from feces in six to eight days. Most infected but untreated calves shed *E. coli* O157:H7 for 10 to >14 days [66]. Phage therapy may therefore be effective in cattle, but will require considerable research to address regulatory requirements.

<u>Sodium chlorate</u>: Sodium chlorate (100 mM) given to cattle in drinking water over a 24 h period substantially reduced populations of *E. coli* O157:H7 in the gastrointestinal tract, without any apparent effect on the total anaerobic population or rumen fermentation [12,13]. This bacteriocidal effect results from reduction of chlorate to chlorite in bacteria with a respiratory nitrate reductase, and thus effects many *E. coli* and some *Salmonella* spp. Consequently, total *E. coli* population in feces and on hides are also affected.

<u>Vaccines</u>: The bovine immune response to the O157 antigen, or the murine immune response to the identical O-polysaccharide antigen of *Salmonella* Landau do not confer strong protective immunity [17,34]. However, some success has been obtained with vaccines containing adherence factors of *E. coli* O157:H7. Vaccination with secreted type III proteins (EspA, EspB, Tir and others) of *E. coli* O157:H7 significantly reduced the numbers of cattle shedding, and the levels and duration of shedding by experimentally challenged cattle, and the animal prevalence of *E. coli* O157:H7 in a feedlot setting [54]. In addition, a vaccine containing *E. coli* O157:H7 intimin

reduced colonization and intestinal lesions in challenged suckling piglets [19], indicating intimin is also a candidate vaccine antigen.

Other interventions: Despite considerable research, other potential interventions have had little or only moderate success, including those involving dietary manipulations [3,11,21,63], water trough hygiene [42], and farm management practices [3,63].

Conclusions

Recent improvements in detection methods for *E. coli* O157:H7, have facilitated investigation of the pre-harvest epidemiology and control of this important pathogen. Methods for nonO157 VTEC are less well-developed. Better, more economical methods would enable the wider scale of testing that is needed for on-farm studies, including the evaluation of intervention strategies. Several of the more promising intervention strategies investigated for pre-harvest control of VTEC are pathogen-specific. Interventions that would also control other pathogens would be highly desirable, but this is challenging until we know more about the adaptation of these human pathogens in their animal reservoirs. It seems, however, that no single intervention will effectively control bacterial pathogens in this dynamic environment.

Abstract

Les méthodes actuelles d’isolement et de détection ont contribué à l'étude des *E. coli* O157:H7 et d'autres *E. coli* producteurs de vérotoxine (ECPV) provenant des animaux et de l’environnement. Néanmoins, des méthodes améliorées et plus économiques sont nécessaires, tout particulièrement pour la quantification des agents pathogènes. Les interventions d’avenir dans le domaine du contrôle des ECPVà la ferme sont dans la plupart des cas spécifiques à chaque agent pathogène, et incluent certains probiotiques, antibiotiques, bactériophages, chlorure de sodium par voie orale et les vaccins.

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