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William Laegreid
University of Nebraska-Lincoln, wlaegrei@uwyo.edu

Mark Hoffman
National Animal Disease Center, USDA Agricultural Research Service, Ames, Iowa

James Keen
U.S. Meat Animal Research Center, USDA Agricultural Research Service, Clay Center, Nebraska, jkeen3@unl.edu

Robert Elder
U.S. Meat Animal Research Center, USDA Agricultural Research Service, Clay Center, Nebraska

Jimmy Kwang
U.S. Meat Animal Research Center, USDA Agricultural Research Service, Clay Center, Nebraska

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Development of a Blocking Enzyme-Linked Immunosorbent Assay for Detection of Serum Antibodies to O157 Antigen of *Escherichia coli*

WILLIAM LAEGREID,[1]* MARK HOFFMAN,[2] JAMES KEEN,[1] ROBERT ELDER,[1] AND JIMMY KWANG[1]

U.S. Meat Animal Research Center, USDA Agricultural Research Service, Clay Center, Nebraska,[1] *and National Animal Disease Center, USDA Agricultural Research Service, Ames, Iowa*[2]

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The O157 antigen of *Escherichia coli* shares structural elements with lipopolysaccharide (LPS) antigens of other bacterial species, notably *Brucella abortus* and *Yersinia enterocolitica* O9, a fact that confounds the interpretation of assays for anti-O157 antibodies. To address this problem, a blocking enzyme-linked immunosorbent assay (bELISA) was designed with *E. coli* O157:H7 LPS as the antigen and a monoclonal antibody specific for *E. coli* O157, designated 13B3, as the competing antibody. The bELISA had equivalent sensitivity to, and significantly higher specificity than, the indirect ELISA (iELISA), detecting anti-O157 antibodies in sera from cattle experimentally inoculated with O157:H7. Only 13% of sera from naive heifers vaccinated for or experimentally infected with *B. abortus* had increased anti-O157 bELISA titers, while 61% of anti-O157 iELISA titers were increased. The bELISA is a sensitive and specific method for the detection of serum antibodies resulting from exposure to *E. coli* O157.

Escherichia coli O157:H7 is a recently emergent pathogen which is capable of causing severe disease and death in humans (1). Serious outbreaks as well as sporadic cases of *E. coli* O157:H7 infection resulting in hemorrhagic colitis, thrombocytopenia, and hemolytic uremic syndrome have been reported with increasing frequency since 1982 (5, 26). The gastrointestinal tracts of clinically healthy cattle are major reservoirs of *E. coli* O157:H7. Outbreaks have been associated with ground beef as well as with various other foods, such as apple cider, which may have been contaminated with bovine feces (1, 33). Epidemiologic studies indicate that a significant proportion of cattle herds contain individuals which shed *E. coli* O157:H7 in their feces, suggesting a serious potential risk of meat and environmental contamination (16). However, since fecal shedding of *E. coli* O157:H7 by cattle is intermittent, the actual prevalence of infection and the associated risks are likely to be higher than estimates based on fecal cultures (3, 10, 14).

Given the intermittent nature of fecal shedding in cattle and the limited duration of shedding in humans, it is logical that other diagnostic techniques, such as serology, might be useful adjuncts to bacteriologic culture methods for detecting infection with *E. coli* O157:H7. Both cattle and humans seroconvert to O157 antigen following oral infection with *E. coli* O157:H7 (2, 19). Several studies have demonstrated the utility of anti-O157-antibody detection in cases of hemorrhagic colitis and hemolytic uremic syndrome where bacteriologic culture failed to detect O157:H7 in feces (4, 7, 8). In addition, an association between exposure to cattle and the prevalence of anti-O157 serum antibodies in healthy human populations has been documented (25). These studies used highly purified O157 lipopolysaccharide (LPS) antigen in indirect hemagglutination or indirect enzyme-linked immunosorbent assays (iELISA) to quantify anti-O157 serum antibodies, and these techniques appear to be reasonably sensitive and specific when applied to populations at risk. However, serologic cross-reactivity between *E. coli* O157 LPS and LPSs of several other gram-negative bacteria has been reported; this cross-reactivity brings about false-positive results which complicate the interpretation of clinical and epidemiologic studies utilizing these indirect tests. Bacterial species known to cross-react with *E. coli* O157 LPS include *Escherichia hermannii*, *Citrobacter freundii*, *Salmonella* $O30_1$ (group N), *Vibrio cholerae* O1, *Yersinia enterocolitica* O9, and *Brucella abortus*, as well as other *E. coli* O serotypes (6, 21–23). This cross-reactivity may affect both the specificity of the results obtained from indirect tests and the sensitivity, as indicated by the relatively high negative cutoff titers reported for O157 iELISA (2, 19). The purpose of this study was to determine the sensitivity and specificity of detection of anti-O157 antibodies by utilizing highly O157-specific monoclonal antibody (MAb) in a blocking ELISA (bELISA) format.

MATERIALS AND METHODS

Bacterial strains. *E. coli* O157:H7 (ATCC 43895) was utilized for preparation of LPS antigen and for immunization of cattle. Calves were infected with *E. coli* O157:H7 86-24, *E. coli* O157:H7 87-23 (a Shiga toxin type 2 negative variant isolated from the same outbreak), or wild-type *B. abortus* (9, 17, 30). *B. abortus* 19 vaccine was obtained from a commercial source (Professional Biological Co., Denver, Colo.).

MAb. The production and characterization of MAb to O157 LPS has been previously described (32). One immunoglobulin G3 (IgG3) MAb, designated MARC 13B3, specific for an epitope of the O157 antigen not shared by *B. abortus* or *Y. enterocolitica*, was selected in preliminary studies for development of the bELISA.

LPS purification. Crude O157 LPS was prepared from a late-log-phase culture in brain heart infusion media as previously described (20). Briefly, the suspensions were washed twice with double-distilled H_2O (ddH_2O) and then resuspended in 2 ml of 0.25 M EDTA–0.5 M Tris-HCl (pH 7.2) and incubated at room temperature for 30 min. After the volume had been adjusted to 10 ml with ddH_2O, the suspension was centrifuged (9,000 × *g*, 10 min) and the pellet was discarded. Crude LPS was precipitated from the supernatant by the addition of 2.5 vol of acetone, the resulting suspension was centrifuged at 10,000 × *g* for 30 min, and the pellet was dried under vacuum and then resuspended in ddH_2O. Highly purified LPS was prepared from this suspension by ultracentrifugation (105,000 × *g*, 16 h) that was repeated until no UV (340 to 220 nm) absorption peaks were detected (11). LPS prepared in this manner contained no detectable protein and showed a typical distinct laddering pattern upon gel electrophoresis (data not shown).

iELISA. iELISA was performed essentially as described by Reymond et al. (25). Round-bottom 96-well plates (Immulon 2; Dynex, Chantilly, Va.) were coated with 1 μg of purified LPS per ml in phosphate-buffered saline (PBS) (pH 7.2), first for 1 h at 37°C and then overnight at 4°C in a humidified chamber. Plates were washed five times with PBS, blocked with 1% gelatin for 1 h at 37°C, and washed again five times with PBS. Sample sera were initially diluted 1:80 and

* Corresponding author. Mailing address: USDA, ARS, U.S. Meat Animal Research Center, P.O. Box 166, State Spur 18D, Clay Center, NE 68933. Phone: (402) 762-4177. Fax: (402) 762-4375. E-mail: laegreid@aux.marc.usda.gov.

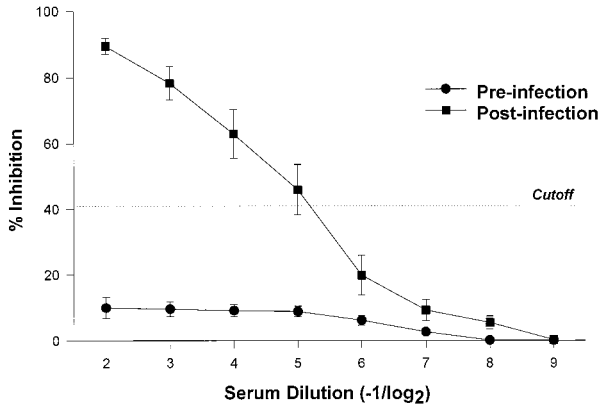

FIG. 1. Inhibition of MAb MARC 13B3 binding to *E. coli* O157:H7 LPS by immune and nonimmune cattle sera. Points are averages ± SEM, $n = 19$.

then diluted twofold in fluorescent trepomonal antibody hemagglutination buffer (BBL Microbiology Systems, Cockeysville, Md.) with 0.1% Tween 80 and 0.5% horse serum (PBS-T-HS). A total of 100 μl was added to wells and incubated for 1 h at 37°C, and the plates were washed five times with PBS-T-HS. Bound anti-O157 antibody was detected by the addition of 100 μl of a 1:1,280 dilution of peroxidase-labeled goat anti-bovine IgG heavy and light chain (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and incubation at 37°C for 1 h, followed by the addition of 100 μl of 0.3% 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (ABTS) (Kirkegaard & Perry Laboratories). After 15 min, the reaction was stopped by adding 50 μl of 1% sodium dodecyl sulfate. The difference between the specific optical density at 405 nm (OD_{405}) and that at 490 nm, as measured by an automated microplate reader (Bio-Tek Instruments Inc., Winooski, Vt.), was used for analysis. Cutoff values were determined for pre- and postinoculation sera from a subset of experimentally infected cattle by nonparametric receiver-operator characteristic (ROC) analysis of the OD at a 1:320 sample dilution and were comparable to those previously reported (15). Titer was defined as the reciprocal of the highest twofold dilution of test serum resulting in an OD greater than or equal to the cutoff value. All sera were tested in duplicate.

bELISA. Optimal concentrations of O157 LPS and MARC 13B3 MAb were determined by checkerboard titration to provide near-maximal binding of MARC 13B3. Plates were coated with highly purified LPS diluted in 0.5 mM carbonate buffer, pH 9.6, for 1.5 h at 37°C and then washed five times with PBS-T-HS. Sample sera were diluted twofold in PBS-T-HS, and 100 μl was added to each well and incubated for 45 min at 37°C; then plates were washed six times with PBS-T-HS. Ascites containing MARC 13B3 (100 μl of a 1:7,000 dilution) were added to each well and incubated for 15 min at 37°C; the plates were washed six times with PBS-T-HS, and bound MARC 13B3 was detected by the addition of 100 μl of peroxidase-labeled rabbit anti-mouse IgG diluted 1:1,500 (Kirkegaard & Perry Laboratories), incubation for 15 min, washing eight times with PBS-T-HS, and the addition of 100 μl of ABTS for 15 min. The reaction was stopped by adding 50 μl of 1% sodium dodecyl sulfate. Results were expressed as percent inhibition of MARC 13B3 binding relative to the inhibition caused by fetal calf serum (FCS) according to the following formula: $[OD_{405/490}\ (FCS) - OD_{405/490}\ (sample)]/OD_{405/490}\ (FCS) \times 100$ = percent inhibition. Cutoff values were determined with a subset of experimentally infected cattle sera by nonparametric ROC analysis (15). Titer was defined as the reciprocal of the highest twofold dilution of test serum resulting in percent inhibition greater than 40.9% of the inhibition caused by FCS alone. Animals were considered seropositive if they had bELISA or iELISA titers ($-1/\log_2$) greater than or equal to 2 or 6.3, respectively. All sera were tested in duplicate.

Animals. Three conventionally reared, 550-kg, mixed-breed beef steers were immunized by three subcutaneous injections of 10^8 CFU of heat-killed *E. coli* O157:H7, approximately 3 weeks apart. A group of 14 conventionally reared beef calves were infected by oral inoculation of 10^{10} CFU of *E. coli* O157:H7. These calves received three inoculations at 3-week intervals over a course of 7 weeks. Five additional calves served as controls for the first 6 weeks and were inoculated with *E. coli* O157:H7 during week seven. All cattle were negative by fecal culture for *E. coli* O157:H7 prior to initiation of experiments. Animals which had positive preinoculation titers by both iELISA and bELISA were excluded from ROC analysis (31). For evaluation of cross-reactivity in O157 ELISA tests, serum samples were obtained from a herd of 20 mixed-breed beef heifers in central Nebraska immediately prior to initial immunization with *B. abortus* 19 and again 42 days following immunization. Additional pre- and postinoculation samples were obtained from three calves which were experimentally infected with wild-type *B. abortus.*

Statistical analysis. Cutoff values and test sensitivity and specificity were determined by nonparametric ROC analysis by the method of Greiner, executed on an Excel 5.0 spreadsheet supplied by the author (15). Differences between titers and inhibition percentages were evaluated by Mann-Whitney U test with the Astute software module (University of Leeds, United Kingdom) and Excel 5.0 (Microsoft, Bellevue, Wash.). Proportions of animals responding serologically were compared by means of McNemar's exact test with Yates's correction (27). Sensitivity, specificity, likelihood ratios, and confidence intervals (CIs) were calculated by standard methods (12, 28). Reverse cumulative distribution plots were generated by the method of Reed et al. (24).

RESULTS

Sera from experimentally infected calves strongly inhibited binding of MARC 13B3 MAb to O157 LPS (Fig. 1). At a dilution of 1:4, postinfection sera inhibited MARC 13B3 binding 92.8% ± 3.27% (mean ± standard error of the mean [SEM]; range, 70 to 97%), while preinfection sera inhibited binding 8.7% ± 3.94% (range, 0.25 to 35%). Inhibition by postinfection sera was significantly greater than by corresponding dilutions (up to 1:256) of preinfection sera ($P \leq 0.05$). Postinfection sera at dilutions of 1:64 inhibited MARC 13B3 binding significantly more than 1:4 dilutions of preinfection sera ($P \leq 0.05$). Nonparametric ROC analysis of inhibition by 1:4 dilutions of pre- and postinfection sera resulted in a point estimate optimum cutoff value of 40.9% inhibition of MARC 13B3 binding (95% CI = 15 to 84%). ROC analysis of the absorbance values from iELISA tests performed with 1:320 dilutions of the same serum samples resulted in a point esti-

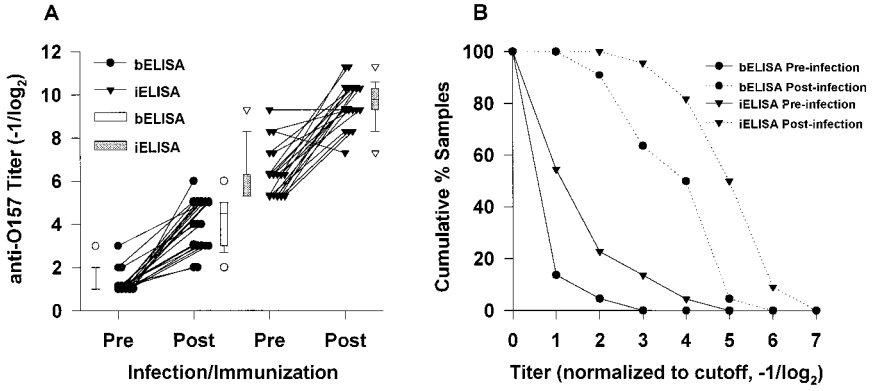

FIG. 2. Serum antibody responses to *E. coli* O157 antigen of calves infected per os ($n = 19$) or immunized ($n = 3$) with *E. coli* O157:H7. (A) Paired pre- and postinfection/immunization bELISA and iELISA titers. Summary box plots indicate the median titer (solid line), 25 to 75% range (box), and 5 to 95% range (error bars) for each set of titers. Open symbols indicate data points outside the 5 to 95% range. (B) Cumulative distribution of anti-O157 bELISA and iELISA titers of calves infected orally with *E. coli* O157:H7 ($n = 19$) or immunized with heat-killed *E. coli* O157:H7 ($n = 3$).

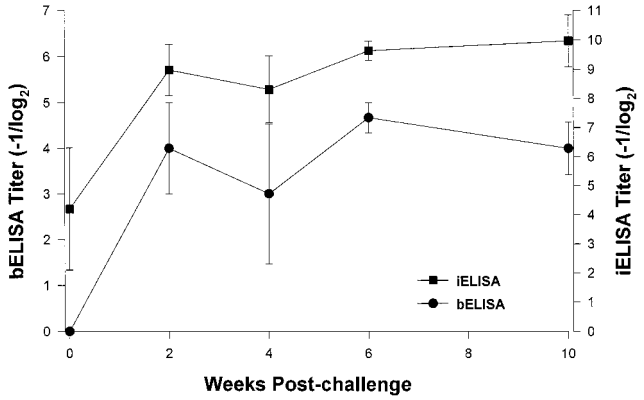

FIG. 3. Serologic responses of calves ($n = 3$) following oral inoculation with *E. coli* O157:H7 86-24, as indicated by bELISA and iELISA titers. Points are geometric means ± SEM.

mate optimum cutoff value of 1.21 A_{405} (95% CI = 0.44 to 1.69). These values were used to define titer endpoints.

Prior to oral infection or parenteral immunization with *E. coli* O157:H7, significantly fewer cattle (3 of 22) were seropositive by bELISA than were positive by iELISA (12 of 22; $P = 0.008$). Mean titers ($-1/\log_2$) ± SEM for positive cattle were 2.25 ± 0.25 and 7.59 ± 0.42 for bELISA and iELISA, respectively. All cattle were seropositive by both tests 10 to 12 weeks postinoculation, with average titers of 4.09 ± 0.25 (bELISA) and 9.66 ± 0.21 (iELISA). Average increase in titer (mean difference between pre- and postinoculation titers) was 2.91 ± 0.25 and 3.41 ± 0.37, respectively (Fig. 2A). The cumulative distributions of titers, normalized to cutoff, as measured by bELISA and iELISA were not different (Fig. 2B). Sensitivities of both the bELISA and the iELISA were 100% on this set of samples. Specificity of the bELISA was 86.4% (95% CI, 72 to 100%) while the specificity of the iELISA was 45.5% (95% CI, 24.6 to 66.3%). Likelihood ratios for bELISA and iELISA were 7.3 (95% CI, 5.4 to 9.2) and 1.83 (95% CI, 0.1 to 3.7), respectively, based on pre- and postinoculation titers. bELISA and iELISA evaluations of antibody responses to experimental oral infection of a subset of calves monitored over time were parallel (Fig. 3).

All 23 animals in a group of cattle were seropositive to O157 by iELISA, with a mean titer (± SEM) of 7.65 ± 0.20, prior to vaccination with *B. abortus* 19 or experimental infection with wild-type *B. abortus*. Following infection or vaccination, 20 of 23 animals had increases in iELISA titer; the mean increase was 1.96 ± 0.24 ($P = 0.043$), significantly increasing the group mean titer to 9.60 ± 0.25 ($P = 0.001$) (Fig. 4A). Only 12 of 23 cattle were anti-O157 seropositive by bELISA, with a mean titer of 3.25 ± 0.28. The proportion of animals seropositive for O157 by bELISA (12 of 23) did not increase following *B. abortus* infection or vaccination; though three animals did have increased titers, this did not significantly affect the group mean titer of 3.42 ± 0.19 (Fig. 4A). Cumulative distribution of bELISA titers did not change, while a clear shift to higher cumulative iELISA titers is evident following *B. abortus* infection or immunization (Fig. 4B). The induction of cross-reactive antibodies following *B. abortus* vaccination was confirmed by Western blots in which reactivity to O antigen of O157 LPS was present with post- but not prevaccination sera (data not shown).

DISCUSSION

Specificity is a critical element of serologic testing, especially in cases where supportive data, such as culture or epidemiologic associations, may be lacking. The bELISA described in this study is based on a MAb for the *E. coli* O157 antigen which lacks cross-reactivity with other *E. coli* serotypes and with antigens of other gram-negative bacteria which share determinants with O157, such as *Y. enterocolitica* O9 and *B. abortus* (32). Use of this MAb in the bELISA format provides a high level of specificity for serologic testing of anti-O157 antibody. This is best demonstrated by results obtained with sera from cattle experimentally infected with or vaccinated against *B. abortus*. Increased iELISA titers against O157, compared to prevaccination titers, were present in *B. abortus*-immune cattle, and antibodies cross-reactive with O antigen were detected by Western blot that had detected *E. coli* O157 LPS. These cross-reactive antibodies did not affect bELISA titers, confirming the specificity of this assay. Although sera from animals infected with other bacteria which cross-react with *E. coli* O157 were not tested, results similar to those obtained with *B. abortus* antisera would be expected based on the specificity of the MAb MARC 13B3. An exception to this would be animals infected with group N *Salmonella* spp. which have O antigens identical

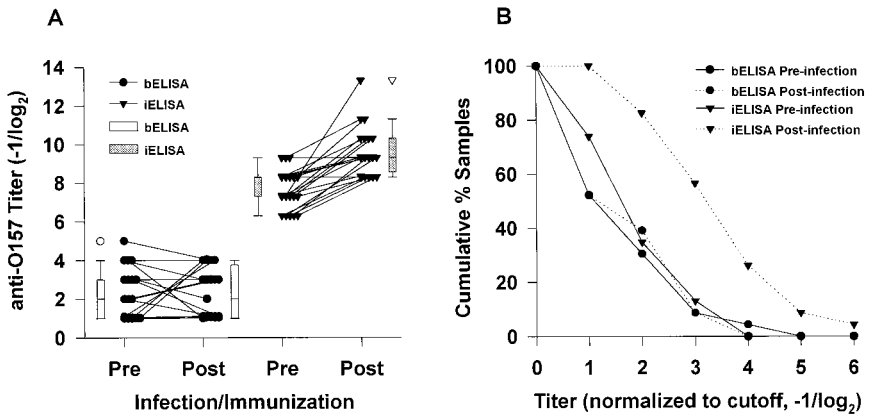

FIG. 4. Serum antibody reactivity to *E. coli* O157 antigen of calves vaccinated ($n = 20$) or infected ($n = 3$) with *B. abortus*. (A) Paired pre- and postvaccination/infection bELISA and iELISA titers. Summary box plots indicate the median titer (solid line), 25 to 75% range (box), and 5 to 95% range (error bars) for each set of titers. Open symbols indicate data points outside the 5 to 95% range. (B) Cumulative distribution of anti-O157 bELISA and iELISA titers of calves vaccinated with *B. abortus* 19 ($n = 20$) or infected with wild-type *B. abortus* ($n = 3$).

to those of *E. coli* O157 (22). Differentiation of these infections on the basis of serologic response to O antigens, regardless of test format, will be problematic. Fortunately, group N *Salmonella* are not commonly isolated from cattle or other livestock species in the United States (13).

The O157 bELISA format has several advantages, other than specificity, over iELISA. First, the lack of cross-reactivity allows for efficient, single-dilution screening of serum samples, greatly increasing sample throughput for serologic surveys. Second, sera from species other than cattle can be used with this format without modification, which facilitates its use with other livestock species, and with wildlife and humans (data not shown). Third, the format includes negative-control information in the calculations, resulting in internal normalization which simplifies comparisons between assays done on different plates or at different times. These attributes contribute to the overall usefulness of the O157 bELISA for diagnostic and epidemiologic applications.

Calves have been shown to seroconvert strongly and persistently to O157 antigen following experimental infection with *E. coli* O157:H7 (19). The effect of seroconversion to O157 on *B. abortus* serology was recognized by Stuart and Corbel, who described positive *B. abortus* agglutination tests with sera from rabbits immunized against *E. coli* O157 as well as with sera from cattle experimentally infected with *E. coli* O157 (29). The significance of this finding was recently challenged by Johnson et al., who found that cross-reactivity to *B. abortus* was rare in cattle naturally infected with *E. coli* O157:H7 (18). The discrepancies between these studies may be ascribed to differences in infectious dose, subtle differences in technique, or other factors. It is clear from the present study, however, that there are O157-cross-reactive antibodies induced by either vaccination or experimental infection of cattle with *B. abortus* and that these antibodies have the potential to significantly influence the results of O157 iELISA tests. It is possible that infection of cattle with other bacteria which cross-react with O157 could give similar false-positive results on O157 iELISA tests. The specificity of the O157 bELISA should allow for a greater degree of confidence in a positive test result.

In conclusion, a bELISA for anti-O157 antibodies has been developed which has a high degree of specificity and a sensitivity equivalent to that of the less specific iELISA and which is applicable to cattle and other species. This serologic test will be a useful tool for clinical diagnosis and epidemiologic studies of *E. coli* O157 infections of cattle and other livestock, with potential for use with other species, including humans. Given the limited duration and intermittent nature of shedding of *E. coli* O157:H7 in cattle, development of the anti-O157 antibody-specific bELISA test provides an important new tool for epidemiologic studies of *E. coli* O157 infections.

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