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Comparison of Vero Cell Assay and PCR as Indicators of the Presence of Verocytotoxigenic *Escherichia coli* in Bovine and Human Fecal Samples

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Comparisons were made between Vero cell assay (VCA) and PCR as indicators for the detection of verocytotoxigenic *Escherichia coli* (VTEC; also known as Shiga-like toxin-producing *E. coli*) and as predictors of VTEC isolation from bovine and human fecal samples. Fecal samples were collected as part of a survey on the prevalence of VTEC on dairy farms in southern Ontario (J. B. Wilson et al., J. Infect. Dis., 174:1021–1027, 1996). A total of 2,655 samples were examined by VCA and PCR, 2,153 originating from cattle and 502 originating from humans. Overall, 36.2% of the samples were positive in the VCA and 38.7% were positive by PCR. Of the VCA-positive samples screened, 41.6% yielded a VTEC isolate. For both human and bovine samples, a significant positive association between PCR result and VCA titer ($P = 0.0001$) was found. In addition, there was a significant positive association between the PCR result and VTEC isolation from VCA-positive samples for cattle (odds ratio = 9.1, $P < 0.0001$). For bovine samples positive in the VCA, VCA titer was significantly associated with the probability of obtaining a VTEC isolate. Agreement between VCA and PCR was good for both bovine and human samples (kappa = 0.69 and 0.64, respectively). The sensitivity and specificity of the PCR with respect to the VCA for bovine samples were 82.0 and 86.5%, respectively, and those for human samples were 59.3 and 98.1%, respectively. Although correlation between VCA and PCR results was not absolute, when used in conjunction, these tests complemented one another as predictors of VTEC isolation.

Verocytotoxin-producing *Escherichia coli* (VTEC; also known as Shiga-like toxin producing *E. coli*) is a known cause of serious illness in humans and a major public health concern (4, 17). Rapid and reliable laboratory tests are needed to facilitate early diagnosis, expedite outbreak investigations, and establish the incidence of VTEC carriage in humans and animals.

The Vero cell assay (VCA) (5) is well recognized as a highly sensitive means of detecting verocytotoxins (10). There are several major drawbacks to the VCA, however, including lack of specificity (16, 19, 23), the need for maintenance of Vero cells, and a 48-h turnaround time for results. The PCR targeting VT gene sequences is one alternative test that has gained prominence (1, 3, 9, 11, 13, 18–21). In comparison to the VCA, PCR is a more specific, more rapid procedure that can produce results within one working day. However, the usefulness of PCR, relative to the VCA, in predicting VTEC isolation needs to be formally evaluated.

The primary objective of this study was to compare VCA and PCR as indicators for the detection of VTEC and as predictors of VTEC isolation in bovine and human fecal samples. In addition, certain aspects of the protocol used to screen samples for VTEC isolates were evaluated. Samples used in this study were obtained as part of a study on the prevalence of VTEC in Ontario dairy farm families (26).

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MATERIALS AND METHODS

Samples. Stool specimens originating from 502 humans and 2,153 cattle were obtained on 80 dairy farms in 12 southern Ontario counties, as part of a larger study reported elsewhere (26). Testing of fecal samples for the presence of VT, VT genes, and individual VTEC isolates was undertaken in a multistage manner. Fecal samples were placed in 9 ml of MacConkey broth (Difco Laboratories, Detroit, Mich.) and incubated overnight at 37°C. Following incubation, 500 μl of MacConkey broth culture was added to 5 ml of brain heart infusion broth (BHIB; Difco). BHIB cultures were incubated overnight at 37°C. The remaining MacConkey broth culture was stored at 4°C.

Vero cell assay. BHIB culture filtrates from all 2,655 samples were screened for verocytotoxicity by using a VCA described by Clarke and coworkers (5), with the following modifications. Two hundred microliters of Eagle's minimal essential medium (Gibco, Burlington, Ontario, Canada), containing 5% inactivated fetal bovine serum (Gibco), was added to each well of a flat-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark). One milliliter of BHIB culture was filtered through a 0.2-μm-pore-size Acrodisc 4192 syringe filter (Gelman Sciences, Ann Arbor, Mich.). A 50-μl sample of filtered BHIB was added to the top well, and then 1:5 dilutions were made in each of three subsequent wells of the plate. A 100-μl aliquot of a suspension of Vero cells (containing approximately 4×10^5 cells per ml) was then added to each well. The plates were incubated at 37°C in an atmosphere of 5% CO_2 for 48 h. Microtiter plates were examined with an inverted microscope at 24 and 48 h to estimate the degree of verocytotoxicity. Wells having 50% or greater cytotoxicity, compared to a standard control well, were considered positive. Wells exhibiting less than 50% cytotoxicity in the 1:5 dilution were classified as weak. The VCA titer endpoint was recorded at 48 h of incubation as negative, weak, 1:5, 1:25, 1:125, or 1:625.

PCR. The PCR procedure was conducted for all 2,655 cultured fecal samples. Extracts from BHIB cultures were prepared as described previously (21), with slight modification. Briefly, 1 ml of the overnight BHIB culture was placed in a microcentrifuge tube and centrifuged at 12,000 × *g* for 1 min, and the supernatant fluid was decanted. The pellet was resuspended in 1 ml of FA buffer (Difco) and centrifuged again. The supernatant fluid was again decanted, and the pellet was resuspended in 500 μl of sterile distilled water. The samples were then placed in a boiling water bath for 10 min and immediately placed on ice. The samples were centrifuged at 12,000 × *g* for 1 min, and the supernatant fluid was used in the PCR procedure.

Generic VT synthetic oligonucleotide primers (21), designed to target con-

TABLE 1. PCR result and VTEC isolation rate according to VCA titer of BHIB culture of bovine fecal samples

VCA titer of BHIB filtrate	No. (%) of samples				
	Total	PCR positive	VTEC isolate positive		
			Total	PCR positive	PCR negative
Negative	1,114	150 (13.5)	NT[a]	NT	NT
Weak	105	49 (46.7)	NT	NT	NT
1:5	180	120 (66.7)	14 (7.8)	12	2
1:25	249	210 (84.3)	52 (20.9)	48	4
1:125	189	172 (91.0)	87 (46.0)	84	3
1:625	316	301 (95.3)	238 (75.3)	232	6
Total	2,153	1,002	391	376	15

[a] NT, not tested.

TABLE 2. PCR result and VTEC isolation rate according to VCA titer of BHIB culture filtrate of human fecal samples

VCA titer of BHIB filtrate	No. (%) of samples				
	Total	PCR positive	VTEC isolate positive		
			Total	PCR positive	PCR negative
Negative	475	9 (1.9)	0	0	0
Weak	6	1 (16.7)	1 (16.7)	0	1
1:5	12	9 (75.0)	4 (33.3)	3	1
1:25	3	2 (66.7)	0	0	0
1:125	4	2 (50.0)	2 (100.0)	2	0
1:625	2	2 (100.0)	2 (100.0)	2	0
Total	502	25	9	7	2

served sequences in VT1, VT2, and VT2e (edema disease toxin), were used in the PCR procedure to screen BHIB culture extracts. The procedure was performed with the PCR Core Reagents kit (Perkin-Elmer Cetus, Norwalk, Conn.) and the protocol described previously (21). The PCR mixture contained 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 200 mM (each) dATP, dCTP, dGTP, and dTTP; 0.1 μM each primer; 0.31 U of AmpliTaq; and 1.5 mM $MgCl_2$; 5 μl of prepared sample extract was added to the mixture for a final reaction volume of 25 μl. Submarine gel electrophoresis was used to detect the amplified product from the PCR.

VTEC isolation. To isolate VTEC from bovine samples, MacConkey broth cultures corresponding to initial BHIB culture filtrates that had a VCA titer of 1:5 or greater in the initial BHIB culture filtrate were streaked onto MacConkey agar plates (Difco). These plates were then incubated at 37°C overnight. Five representative individual colonies were selected from each plate and inoculated individually into microcentrifuge tubes containing 1 ml of BHIB. In addition, a mixture of colonies, termed a "sweep," from the initial streak area was inoculated into 1 ml of BHIB. BHIB tubes were incubated overnight at 37°C and centrifuged at 12,000 × *g* for 1 min, and 100-μl volumes of the resulting culture supernatant fluids were screened for VT activity by the VCA as described above. Samples which had VCA-positive isolates among the initial five colonies selected did not undergo any further screening.

Bovine samples not yielding VTEC isolates from individual colonies but having a VCA titer of 1:25 or greater in the BHIB sweep culture supernatant fluid were subjected to further testing. Successive groups of five additional colonies were selected from the MacConkey plate and assessed for cytotoxicity in the VCA as described above. This was repeated for each sample until either a positive isolate was identified or a maximum of 20 colonies were tested.

For the isolation of VTEC from human samples, MacConkey broth cultures corresponding to initial BHIB culture filtrates that had a weak or higher titer in the VCA, or were positive by PCR, were streaked onto MacConkey agar plates. Twenty colonies and three sweeps were assessed for cytotoxicity by the VCA as described above. If no VTEC isolate was obtained, an additional 20 colonies were selected from the MacConkey agar plate and screened for cytotoxicity by VCA.

Presumptive VTEC isolates were confirmed as VTEC by the generic VT PCR procedure as described above for BHIB cultures.

Statistical analyses. The data were entered into computer files (dBase IV; Ashton Tate, Torrance, Calif.) and analyzed with SAS version 6.04 for personal computers (SAS Institute Inc., Cary, N.C.) and Lotus 123 version 2.3 (Lotus Development Corp., Cambridge, Mass.). Data for bovine and human samples were analyzed separately. Logistic regression analysis was used to evaluate the relationship between the VCA titer of the initial BHIB cultures and the probability of obtaining a VTEC isolate, as well as the relationship between VCA titer and PCR result obtained from the initial BHIB cultures.

The kappa statistic (7) was used to evaluate the agreement beyond chance between the VCA and PCR results from initial BHIB cultures. The sensitivity and specificity of the PCR relative to VCA were also calculated. These calculations were performed after dividing VCA titers into two groups: those greater than or equal to 1:5 and those less than 1:5.

Fisher's exact test (6) was used to determine the association between PCR result and VTEC isolation from bovine initial BHIB cultures that had a VCA titer of 1:5 or higher and from human initial BHIB cultures which either were positive by PCR or had a weak or higher VCA titer.

Finally, combination theory (8) was used to calculate the proportion of bovine samples that would have yielded a VTEC isolate if fewer than five colonies had been tested per sample.

RESULTS

VCA and PCR tests were conducted for all 2,655 samples, 2,153 originating from cattle and 502 originating from humans (Tables 1 and 2). A total of 934 bovine samples (43.4%) and 27 human samples (5.4%) were screened for VTEC isolates based on the criteria described above. Of those screened, 41.9% (391 of 934) of bovine samples and 33.3% (9 of 27) of human samples yielded a VTEC isolate (41.6% overall). A total of 1,002 (46.5%) bovine samples and 25 (5.0%) human samples were classified as PCR positive. PCR correctly identified 96.2% (376 of 391) of bovine samples and 77.8% (7 of 9) of human samples which yielded a VTEC isolate.

The proportion of PCR-positive samples and the proportion of VTEC isolates increased significantly with increasing VCA titer (Tables 1 and 2). Logistic regression analysis indicated that there was a significant positive association between VCA titer and PCR result for both species ($P = 0.0001$). Similarly, for samples positive in the VCA, there was a positive association between VCA titer and the probability of obtaining a VTEC isolate for cattle ($P = 0.0001$) and humans ($P = 0.0838$). The proportion of samples yielding a VTEC isolate increased as the VCA titers of the BHIB sweep supernatants increased (Tables 3 and 4). This proportion was consistently equal to or higher than the proportion of samples yielding isolates found at the corresponding VCA titers of the initial BHIB culture filtrates (Tables 1 and 2).

There was moderate agreement between the VCA and PCR results on the initial BHIB culture filtrates for cattle and humans (kappa = 0.69 and 0.64, respectively). In bovine samples, the sensitivity and specificity of the PCR with respect to the

TABLE 3. VTEC isolation with respect to VCA sweep titer from bovine fecal samples

VCA sweep titer	No. (%) of samples	
	Total	VTEC isolate positive
Not done	2	NA[a]
Negative	301	12 (4.0)
Weak	59	11 (18.6)
1:5	101	27 (26.7)
1:25	164	87 (53.0)
1:125	138	106 (76.8)
1:625	169	148 (87.6)
Total	934	391

[a] NA, not applicable.

TABLE 4. VTEC isolation with respect to VCA sweep titer from human fecal samples

VCA sweep titer	No. (%) of samples	
	Total	VTEC isolate positive
Negative	28	2 (7.1)
Weak	2	0 (0)
1:5	4	3 (75.0)
1:25	2	2 (100.0)
1:125	1	1 (100.0)
1:625	1	1 (100.0)
Total	38	9

VCA on BHIB culture supernatant fluids were 82.0 and 86.5%, respectively. The corresponding values of relative sensitivity and specificity for human specimens were 59.3 and 98.1%. There was a positive association between PCR result and isolation of VTEC for VCA-positive bovine BHIB culture filtrates (odds ratio = 9.10, $P < 0.001$). A trend towards increased isolation of VTEC from PCR-positive human samples was also observed but was not statistically significant (odds ratio = 3.5, $P = 0.32$).

There were 340 bovine fecal samples from which VTEC isolates were obtained by testing the initial five colonies. The proportion of VTEC colonies among the five tested colonies from these samples was one of five in 116 samples, two of five in 75 samples, three of five in 55 samples, four of five in 47 samples and five of five in 47 samples. The expected number of bovine fecal samples that would have yielded a VTEC isolate if fewer than five colonies been tested is shown in Table 5.

DISCUSSION

The VCA has been a standard procedure for the detection of VTs since the initial discovery of this toxin family by Konowalchuk and colleagues (14). However, the constraints inherent in tissue culture, as well as prolonged turnaround time for results and lack of specificity, have limited the routine use of the VCA procedure for detection of VTEC in diagnostic laboratory settings. The advent of VTEC-specific PCR techniques has provided the opportunity to develop new procedures for the detection of VTEC which will address many of the shortcomings of the VCA. Screening of fecal samples for the presence of VTEC by VCA and PCR incorporates two inherently different strategies; the VCA detects toxin production, while PCR detects toxin gene sequences.

In this study, we formally evaluated the usefulness of the PCR procedure, relative to the VCA, both for the detection of VTEC and as a predictor of VTEC isolation in a diagnostic setting. The proportions of all samples classified as positive by VCA and PCR were comparable, 36.2% (961 of 2,655) and 38.7% (1,027 of 2,655), respectively. Based on the logistic regression analysis, a significant association was observed between PCR and VCA results on initial BHIB cultures. Although the two tests were correlated, this correlation was not absolute, since the two tests were not always classifying the same samples as positive, as reflected in the kappa statistic and the sensitivity and specificity of the PCR with respect to the VCA.

TABLE 5. Theoretical result of testing fewer than five colonies from VTEC-positive bovine fecal samples

Proposed no. of colonies to test	No. expected to yield at least one VTEC isolate[a]
1	171 (50.3)
2	242 (71.2)
3	286 (84.1)
4	317 (93.2)

[a] Based on 340 bovine samples for which a VTEC isolate was obtained among the first five colonies tested. Numbers in parentheses were obtained by dividing the corresponding values in the second column by 340 and represent the percentages of samples which would have been expected to yield at least one VTEC isolate if fewer than five colonies had been tested.

Bovine samples were screened for VTEC based on the VCA titer, regardless of the PCR result. Therefore, direct comparison between PCR and VCA in predicting isolation of VTEC could not be made. However, based on the odds ratio for bovine samples, those with a positive PCR result which were also VCA positive were nine times more likely to yield an isolate than were samples with a negative PCR result. A similar trend was found with human samples; however, the results were not statistically significant. This may have been the result of the smaller numbers of human samples tested.

The proportion of samples yielding a VTEC isolate increased as the VCA titers of both the initial and sweep BHIB cultures increased. This relationship was particularly evident for the sweep titer. The logistic regression further reflected this significant association between increasing VCA titer and isolation rate. Thus, the opportunity to use a specific VCA titer as a cutoff point to screen samples for VTEC isolates exists. Furthermore, the sweep BHIB culture was a better predictor of VTEC isolation. Thus, in order to maximize efficiency, the sweep BHIB titer alone could be used to determine which samples should be selected for screening. Of the VCA-positive bovine samples screened for VTEC, 58.1% did not yield an isolate. This concurs with other studies, which have shown that the VCA is highly sensitive for detection of VTs (16, 19, 23) but, in the absence of neutralization of VTs, lacks specificity. Serum neutralization was not utilized in this study; therefore, results for some VCA-positive samples may have been due to non-VT cytotoxicity. However, failure to obtain isolates from VCA-positive samples may also be attributable to low VTEC numbers, which would decrease the probability of finding an isolate, since maximums of only 20 and 40 colonies were tested for bovine and human samples, respectively. Clearly the chance of obtaining a VTEC isolate would have been increased if more colonies had been tested in the VCA; however, the workload precluded screening of additional colonies from VCA-positive samples.

In contrast with the VCA, the use of PCR resulted in a moderate number of false negatives but low numbers of false positives. Certain samples that were PCR positive did not yield an isolate. PCR-positive but VCA-negative results may have been due to the presence of nonviable, or viable but nonculturable, organisms (12), or possibly low levels of VT production (16) or lack of toxin synthesis despite the presence of intact VT gene sequences. Results for samples which were both VCA and PCR positive but from which no isolate was obtained may have been due to low numbers of VTEC with respect to background flora, again reflecting the limitations imposed by testing 20 to 40 colonies. Multiple copies of VT genes coupled with high levels of toxin synthesis may account for the inability to obtain an isolate in this type of sample.

A number of factors may have led to false-negative PCRs in samples yielding VTEC isolates. PCR was performed on extracts of mixed cultures from BHIB, which may have contained minute quantities of culture components and high concentrations of DNA that may interfere with the PCR (22). In addi-

tion, it is possible that inhibitors present in fecal samples (3, 25) may have been carried over through the various subculture steps and caused false negatives. The limit of detection of this PCR procedure using pure cultures is 10^4 VTEC organisms per ml (21); thus, low numbers of VTEC may have been responsible for some false-negative reactions. The background flora present in the sample may have further reduced the limit of detection by PCR (2).

Traditional DNA extraction techniques may be used to enhance VTEC detection by PCR (9); however, the complexity of these procedures precludes their routine use for diagnostic samples. A number of commercial products, developed to improve the recovery of DNA from a sample, are currently under evaluation in our laboratories. Many of these products employ techniques that are rapid and simple and may easily be introduced into a diagnostic laboratory protocol. Their use, in combination with immunocapture techniques and methods to increase the sensitivity of detection of the PCR product, may improve the limit of detection of PCR to the point where it may replace the VCA for screening of samples for VTEC.

The variability in the concentration of VTEC in bovine fecal samples was reflected by the number of isolates obtained from each animal when five colonies were screened. For most samples where an isolate was obtained, only one or two colonies were confirmed as VTEC. In developing the protocol for this study our aim was to maximize the number of isolates obtained while effectively utilizing laboratory resources. This balance appears to have been achieved, since in an investigation of the value of selecting five colonies, combination theory analysis showed that selecting fewer than four colonies would have greatly reduced the overall number of isolates found in this study. More-stringent criteria were used to screen human samples as a precautionary measure.

Difficulties arise in isolating VTEC without the advantage of visible markers, since it is impossible to distinguish VTEC from *E. coli* background flora and since VTEC may be present in very low numbers. Methods that may increase isolation rate over blind selection of colonies have been described (15, 24); however, the complexity of these tests often precludes their use in large surveys.

In summary, both VCA titer and PCR result were found to be of value in identifying samples most likely to yield a VTEC isolate. When used in conjunction, the different detection strategies of these two methods complemented one another. The formal statistical tests used in this study were of value in comparing both the efficacy of VCA and PCR and the efficacy of the screening protocol utilized.

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