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Evaluation of Enzyme-Linked Immunosorbent Assays and a PCR Test for Detection of Shiga Toxins for Shiga Toxin-Producing 
*Escherichia coli* in Cattle Herds

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Antigen capture enzyme-linked immunosorbent assays (ELISAs) for the detection of Stx1 and/or Stx2 in cattle feces were validated in comparison to the Vero cell cytotoxicity neutralization test (as a “gold standard”) applied in the course of a monitoring program for Shiga toxin-producing *Escherichia coli* in German cattle herds as a prescreening test and compared to MK1/MK2 PCR as an alternative prescreening test.

Several enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of Shiga toxin-producing *Escherichia coli* (STEC), some of which use the P1 glycoprotein from hydatid cyst fluid, or Gb3, for Shiga toxin capture (1, 2, 16). Recently, monoclonal antibodies (MAbs) to verocytotoxins from hydatid cyst fluid, or Gb3, for Shiga toxin capture (VCNA) which served as the “gold standard.” The assays were validated for the detection of Stx1 and/or Stx2 in cattle feces. These ELISAs were used in a monitoring program as a prescreening test and compared to MK1/MK2 PCR (13).

A total of 100 *E. coli* field strains isolated from cattle were tested in the STEC-ELISAs, the Vero cell cytotoxicity assay (VCA), and the Vero cell cytotoxicity neutralization assay (VCNA) which served as the “gold standard.” The assays were performed as described by Konowalchuk et al. (14), with some modifications (8, 17). The cytopathic effect (CPE) and the neutralization of the CPE were measured by determining cell densities in a Coulter Z2 counter (Coulter Electronics Inc., Miami, Fla.). The specificity of the VCA for the toxic production of *E. coli* field isolates was determined by neutralization tests of the CPE on Vero cells (ATCC CRL1587) by MAbs 13C4 (ATCC CRL1794) and 11E10 (ATCC CRL1907).

The ELISAs utilized hydatid cyst fluid of *Echinococcus granulosus* for detecting the Shiga toxins, as described previously (18). A total of 200 μl of supernatant of the *E. coli* field strains or preenriched fecal samples of cattle were directly investigated in this assay. Each sample was tested in quadruplicate. Positive controls (*E. coli* C600-J1 for Stx1 and *E. coli* C600-W34 for Stx2) and a negative control (*E. coli* C600) were tested sixfold on each plate (blanks in each corner). The MAbs 13C4 and 11E10 were used for the specific detection of Stx1 and Stx2. Bound MAbs were detected with goat anti-mouse peroxidase conjugate and 3,3’5,5’tetramethylbenzidine (TMB; KPL, Gaithersburg, Md.) as the substrate. The reactions were stopped by adding 0.5 M sulfuric acid. Optical densities (ODs) were read at 450 nm with a reference filter at 620 nm. The mean ODs of positive and negative controls and of the samples were calculated from the blank-corrected OD readings (OD value − blank value), and an index was determined for each sample according to the following equation:

\[
\text{index} = \frac{\text{mean of OD}_{\text{sample}} - \text{mean of OD}_{\text{negative control}}}{\text{mean of OD}_{\text{positive control}} - \text{mean of OD}_{\text{negative control}}}
\]

The study showed that Stx1- and Stx2-specific ELISAs can be utilized for the detection of these Shiga toxins. All 34 *E. coli* strains which produced Stx1 (as shown by the VCNA results) were also specifically recognized in the respective ELISAs. The low cutoff value (index, 0.02) calculated by a two-graph receiver operating characteristic (ROC) analysis (CMDT, 1.0) divided the population very well into Stx1-positive and Stx1-negative *E. coli* strains. The estimated sensitivity and specificity values were 100% (*p* = 100% and *p* = 100%, where *p* is the lower confidence limit and *p* is the upper confidence limit) relative to the gold standard (Fig. 1). The area under the curve (AUC) of the ROC resulted in a value of 1.0, indicating that the usefulness of the Stx1 ELISA seems equal to that of the VCNA for detection of Stx1 in terms of test performance, but it is much easier to handle under routine conditions.

The *E. coli* strains producing Stx2 were also very well detected by the Stx2 ELISA. An index value of ≤0.040 was obtained from 63 *E. coli* isolates. These 63 isolates tested negative for Stx2 in the VCNA. A total of 37 isolates exceeded the cutoff in the Stx2 ELISA, but four of them yielded negative results in the VCNA for Stx2 (Fig. 2). Interestingly, these four strains were also positive for Stx1 by VCNA and the presence of the stxl and stx2 genes was demonstrated by PCR (using primer pair LP30 and LP31 and primer pair LP43 and LP44) (reference 5 and data not shown). A high level of Stx1 expression may have suppressed the expression of Stx2. The ELISAs detected both toxins, but the indices for Stx2 just exceeded the cutoff value, while the index values for Stx1 were high. Yet the sensitivities of the ELISAs can be adjusted in such a way that
they are higher than the sensitivity of the VCNA when the *E. coli* strains have the potential for producing both toxins Stx1 and Stx2. With an AUC of 0.993 ($p_l = 0.989$, $p_u = 0.998$), the Stx2 ELISA also seems to be a good test for the detection of Stx2. For the further analysis of field samples for Stx2 by ELISA, the cutoff value was set to an index value of 0.04.

Running a combination of the VCA and the VCNA in the laboratory requires a lot of experience. Conducting both assays is expensive, and standardization is difficult. The test protocol is time-consuming. In the reading of the CPE by microscopic examination, a certain degree of arbitrariness cannot be avoided. Thus, the use of a cell counter facilitated some progress towards increased repeatability with respect to the counting of live versus dead cells. In contrast, ELISAs are quick and simple tests. Since the ELISAs yielded similar and sometimes perhaps even better results than the gold standards, the ELISAs can be used instead of the VCA-VCNA. Mitomycin C has been used to enhance Stx production by *E. coli* and to increase the sensitivity of an ELISA aiming at the detection of Stx (12). Biohazard aspects related to the use of mitomycin C, a carcinogenic substance, need to be considered.

A total of 1,030 fecal samples from cattle obtained over a period of 3 years (9) were prescreened in parallel by PCR using the primers MK1/MK2 (13) and the Stx1 and Stx2 ELISAs. On each visit, fecal samples (rectal swabs) were taken from each cattle and transferred into sterile tubes. The samples were shaken in the sampling tubes with 2 ml of phosphate buffer and then 50 μl of each sample was transferred into 3 ml of modified Trypticase Soya broth with mitomycin C (100 ng/ml) as a preenrichment for the ELISA. In parallel, 50 μl was transferred into Luria-Bertani broth as a preenrichment for the MK1/MK2-PCR without mitomycin C, a known PCR inhibitor. ELISA results were determined on the basis of appropriate cutoff values calculated for Stx1 and Stx2 by ROC analysis. Hence, agreement in the results for both test systems (PCR and ELISA) was found for 751 samples (72.9%), yielding a $\kappa$ of 0.452 ($p_l = 0.401$, $p_u = 0.503$) for the comparison between PCR and ELISA (Table 1). $\kappa$ measures the ratio of the actual agreement between observers who interpret data (7, 15). The $\kappa$ value calculated for the comparison between MK1/MK2-PCR and Stx1 and Stx2 ELISAs indicates a moderate or fair level of agreement.

The PCR detected an *stx* gene in 236 samples that were negative for the respective Shiga toxin in the ELISA. Therefore, the sensitivity of PCR seemed to be substantially higher than the sensitivity of the ELISA. The lower sensitivity of the

FIG. 1. Comparison of the Stx1 ELISA with the VCNA. The index value obtained in the Stx1 ELISA was plotted against the percentage of surviving cells in the VCNA for each individual sample tested.
ELISA can be explained by the absence of the Stx gene product or a suppression of the expression of Stx in these strains, although the stx gene is present. Alternatively, after overnight growth these samples could have contained very few STEC cells, which may have been sufficient for PCR detection of toxin genes but not for the detection of the toxin itself by ELISA. It also seems possible that some isolates produced Stx2 variants that MAb 11E10 failed to recognize. It is believed, however, that the MK1/MK2-PCR recognizes all genes of the known stx subtypes. Some isolates reacted positively in the Stx ELISA but were not detected by PCR. In these samples, the PCR was not as sensitive as the ELISA or the ELISA result was a false positive. The possibility that cross-reacting epitopes in nontoxic proteins related to the Stx may exist in feces, which could explain false-positive ELISA reactions, was described previously (3). On the other hand, fecal samples sometimes cause problems in PCR as they may contain inhibitors (4, 6, 11). Heme is regarded as the most inhibitory substance in blood and has also been detected in fecal samples. Therefore, we believe that PCR-negative–ELISA-positive reactions are more likely to reflect false-negative PCR than false-positive ELISA results.

In conclusion, the agreement of the ELISAs results with those of the MK1/MK2-PCR was only moderate. In our monitoring program, the PCR detected more than 25% more stx-positive samples. Therefore, this PCR should be favored as the prescreening test. An improvement of sensitivity by the detection of positive samples could be increased if the PCR were combined with the Stx ELISAs. However, a combination of both tests may increase the costs of the prescreening procedure.

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