J Vet Diagn Invest 15:94–99 (2003)

Comparison of four techniques for the detection of *Clostridium perfringens* type D epsilon toxin in intestinal contents and other body fluids of sheep and goats

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Abstract. Polyclonal capture enzyme-linked immunosorbent assay (PC-ELISA), monoclonal capture ELISA (MC-ELISA), mouse neutralization test (MNT), and counterimmunoelectrophoresis (CIEP), were compared for their ability to detect epsilon toxin in intestinal contents and body fluids of sheep and goats. When used to evaluate intestinal contents of sheep artificially spiked with epsilon prototoxin, PC-ELISA detected 0.075 mouse lethal dose $(MLD)_{50}$/ml, whereas the MNT, MC-ELISA, and CIEP detected 6, 25, and 50 MLD_{50}/ml, respectively. Amounts of epsilon toxin detected by PC-ELISA, MC-ELISA, MNT, and CIEP in sheep pericardial fluid artificially spiked with epsilon prototoxin were 0.075, 0.75, 6, and 200 MLD_{50}/ml, respectively. For assaying epsilon toxin in aqueous humor, PC-ELISA and MC-ELISA detected 0.075 MLD_{50}/ml, whereas CIEP detected 200 MLD_{50}/ml (MNT was not evaluated). When 51 samples of intestinal contents of sheep and goats (32 positive and 19 negative to MNT) were analyzed by the other 3 techniques, the relative sensitivity of PC-ELISA, MC-ELISA, and CIEP was 93.75, 84.37, and 37.50%, respectively. The specificity of PC-ELISA, MC-ELISA, and CIEP was 31.57, 57.89, and 84.21%, respectively. The absolute sensitivity of PC-ELISA, MC-ELISA, CIEP, and MNT was 90.90, 69.69, 15.15, and 54.54%. The absolute specificity of the 4 techniques was 100%. These results show that there is a marked inconsistency among techniques routinely used to detect *Clostridium perfringens* epsilon toxin. Until more consistent results are achieved, the diagnosis of enterotoxemia should not only be based solely on epsilon toxin detection, but also on clinical and pathological data.

Epsilon toxin is one of the 4 major toxins of *Clostridium perfringens* together with alpha, beta, and iota.[10] This toxin is produced by *C. perfringens* types B and D, and it is responsible for enterotoxemia in sheep,[10] goats,[13] and probably cattle.[15]

All types of *C. perfringens* may be normal inhabitants of the intestine of various animal species.[10] Thus, the mere presence of this microorganism in intestinal contents from affected animals is not of diagnostic significance. When large amounts of undigested carbohydrates, usually due to sudden changes in the diet (other factors may also be involved), reach the duodenum, *C. perfringens* proliferates and produces large amounts of toxins that act locally and/or are absorbed into the systemic circulation.[2]

Traditionally, the definitive diagnosis of enterotoxemia is based on the detection of preformed *C. perfringens* toxin(s) in intestinal contents,[6,12] and according to some authors,[5] in other body fluids such as pericardial fluid. Small numbers of *C. perfringens* present in the intestine of healthy animals may produce minute amounts of epsilon toxin.[1] Therefore, the demonstration of this toxin in intestinal contents may not be diagnostic for enterotoxemia.

Histopathology is a valuable aid for the diagnosis of enterotoxemia in sheep because *C. perfringens* type D characteristic histopathological changes (i.e., perivascular proteinaceous edema and occasionally focal symmetrical encephalomalacia) are frequently present in the brain.[3] In other species, however, these histopathological changes are not consistently found, and the definitive diagnosis of enterotoxemia is commonly based only on the detection of significant levels of epsilon toxin in intestinal contents.

The techniques most commonly used to detect epsilon toxin in intestinal contents and other body fluids are the mouse neutralization test (MNT),[12] several enzyme-linked immunosorbent assays (ELISAs),[4,8,9,16] and counterimmunoelectrophoresis (CIEP).[6] There is little information available in the literature about the comparative ability of these methods to detect epsilon toxin in intestinal contents and other body fluids. An evaluation and comparison of 4 techniques used to detect *C. perfringens* type D epsilon toxin in intestinal contents and other body fluids of sheep and goats is presented here.

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Table 1. Comparison of the ability of 4 techniques to detect *Clostridium perfringens* type D epsilon toxin in sheep intestinal contents, pericardial fluid, and aqueous humour before and after being artificially spiked with different concentrations of epsilon prototoxin. Each sample originated from a pool of 10 clinically normal sheep. Samples 11 (spiked with 0.0075 MLD_{50}/ml* of epsilon prototoxin) and 12 (nonspiked) of the 3 specimens were negative for all tests (except for MNT that was not performed on aqueous humor).

Sample	Subsample	Epsilon toxin (MLD_{50}/ml)	PC-ELISA	MC-ELISA	CIEP	MNT
Intestinal contents	1	200	+	+	+	+
	2	100	+	+	+	+
	3	50	+	+	+	+
	4	25	+	+	−	+
	5	12.5	+	−	−	+
	6	6	+	−	−	+
	7	3	+	−	−	−
	8	1.5	+	−	−	−
	9	0.75	+	−	−	−
	10	0.075	+	−	−	−
Pericardial fluid	1	200	+	+	+	+
	2	100	+	+	−	+
	3	50	+	+	−	+
	4	25	+	+	−	+
	5	12.5	+	+	−	+
	6	6	+	+	−	+
	7	3	+	+	−	−
	8	1.5	+	+	−	−
	9	0.75	+	+	−	−
	10	0.075	+	−	−	−
Aqueous humor	1	200	+	+	+	nd
	2	100	+	+	−	nd
	3	50	+	+	−	nd
	4	25	+	+	−	nd
	5	12.5	+	+	−	nd
	6	6	+	+	−	nd
	7	3	+	+	−	nd
	8	1.5	+	+	−	nd
	9	0.75	+	+	−	nd
	10	0.075	+	+	−	nd

* MLD_{50}/ml = mouse lethal dose fifty of epsilon toxin per milliliter of sample; PC-ELISA = polyclonal capture ELISA; MC-ELISA = monoclonal capture ELISA; CIEP = counterimmunoelectrophoresis; MNT = mouse neutralization test; nd = not done.

Materials and methods

Samples collected from normal animals and spiked with epsilon toxin. Samples of small intestine contents were collected from 10 clinically normal, adult Merino sheep at slaughter and were pooled before aliquoting them into 12 subsamples. Eleven of these subsamples were spiked with epsilon prototoxin to obtain final concentrations of the prototoxin ranging from 0.0075 to 200 mouse lethal dose fifty per milliliter (MLD_{50}/ml). The remaining subsample was used as a nonspiked control (Table 1). Samples of pericardial fluid and aqueous humor were also collected from the same animals and treated as described for intestinal contents (Table 1).

Intestinal contents from animals with a presumptive diagnosis of enterotoxemia. Seventeen samples of intestinal contents from 15 sheep (small intestine) and 1 goat (ileum and caecum content), with a presumptive diagnosis of enterotoxemia were used (Table 2). These samples had been sent to different diagnostic laboratories for confirmation of the diagnosis. The presumptive diagnosis of enterotoxemia had been based upon history, clinical signs, and brain histopathology.

Intestinal contents from animals with experimental enterotoxemia. Seventeen samples of intestinal contents (both colon and small intestine) from 9 goats with experimental enterotoxemia were used (Table 3). These samples have been described previously.[14]

Twelve samples of small intestine contents from sheep that did not die of enterotoxemia or of causes related to intestinal disease.

Each sample was divided into 4 aliquots and frozen until required for processing using each of the diagnostic tests at the corresponding laboratory.

Diagnostic techniques

Most of the samples were processed by PC-ELISA, MC-ELISA, CIEP, and MNT (Tables 1–3).

The PC-ELISA[a] based on previously described methods[16] was performed at the Animal Research Institute in Brisbane, Australia, where this technique is routinely used as a stan-

Table 2. Comparison of the ability of 4 techniques to detect *Clostridium perfringens* type D epsilon toxin in samples of intestinal contents from 15 sheep and 1 goat with a presumptive diagnosis of enterotoxemia.*

Sample	PC-ELISA	MC-ELISA	CIEP	MNT
1	+	−	−	+
2	+	+	−	+
3	+	+	−	−
4	+	+	+	−
5	+	+	−	+
6	+	+	+	+
7	+	+	+	+
8	+	+	−	+
9	+	+	−	+
10	+	+	−	+
11	+	+	+	+
12	+	+	+	nd
13	−	−	+	+
14	−	−	+	+
15	−	−	+	−
16†	+	+	+	+
17‡	+	+	−	+

* PC-ELISA = polyclonal capture ELISA; MC-ELISA = monoclonal capture ELISA; CIEP = counterimmunoelectrophoresis; MNT = mouse neutralization test; nd = not done.

† Goat case, ileum content.

‡ Goat case, caecum content.

Table 3. Comparison of the ability of 4 techniques to detect *Clostridium perfringens* type D epsilon toxin in 17 samples of intestinal contents (colon and small intestine) from 9 goats with experimental enterotoxemia.*

Animal	Sample	PC-ELISA	MC-ELISA	CIEP	MNT
1	colon content	+	+	+	+
2	colon content	+	+	−	+
2	s.i. content	+	+	−	+
3	colon content	−	−	+	−
3	s.i. content	−	−	+	nd
4	colon content	+	+	−	nd
4	s.i. content	−	−	+	nd
5	colon content	+	+	−	−
5	s.i. content	+	+	−	+
6	colon content	+	+	+	+
6	s.i. content	+	+	−	+
7	colon content	+	+	−	−
7	s.i. content	+	+	+	nd
8	colon content	+	+	−	nd
8	s.i. content	−	−	+	nd
9	colon content	+	+	−	−
9	s.i. content	+	+	−	+

* PC-ELISA = polyclonal capture ELISA; MC-ELISA = monoclonal capture ELISA; CIEP = counterimmunoelectrophoresis; MNT = mouse neutralization test; s.i. = small intestine; nd = not done.

dard test for diagnosis of enterotoxemia. Affinity-purified ovine antiepsilon toxin antibodies were prepared as previously described.[16] Briefly, highly purified epsilon prototoxin was converted to toxoid with formalin and used to hyperimmunize a sheep. Antibodies from the sheep serum were purified on an affinity chromatography column made by coupling purified epsilon toxin to AH Sepharose[b] with diethyl-3 (3-dimethyl amino propyl) carbodiimide, using the method described previously.[17] Affinity-purified epsilon toxin antibodies were conjugated to horseradish peroxidase by the periodate method.[11] For the PC-ELISA, samples diluted in buffer containing fetal calf serum were added to ELISA plates coated with affinity-purified ovine antiepsilon toxin antibodies and incubated overnight at 4 C. The plates were washed, affinity-purified ovine antiepsilon toxin antibody conjugated to horseradish peroxidase was added, and the plates were incubated at 37 C for 90 minutes. After a final washing, the plates were incubated at 30 C for 15 minutes, stopped with 1 M H_3PO_4 and read in an ELISA reader[c] at 450 nm. Optical densities of 1.0 or higher indicated positive results.[16]

The MC-ELISA was performed using a commercial ELISA kit[d] according to the manufacturer's instructions. Briefly, the samples were added to the wells of the ELISA plates to which a *C. perfringens* epsilon toxin monoclonal antibody was bound and the plates were incubated for 60 minutes at room temperature. A washing step was performed and horseradish peroxidase conjugated with antiepsilon toxin antibody was added to the plates before another hour of incubation at room temperature. After an additional wash step, a substrate/chromogen solution was added to each well, and the plates were incubated for 15 minutes at room temperature. The results were read in an ELISA reader[b] with a 450-nm filter. Positive or negative readings were obtained according to the instructions of the manufacturer.

Counterimmunoelectrophoresis, as previously described,[6] was performed at the Elizabeth MacArthur Agricultural Institute, Camden, Australia, where this technique is routinely used as a standard test for diagnosis of enterotoxemia. The test fluids (undiluted and diluted 1:2 and 1:4) were added to wells made in an agarose plate, close to the cathode of an electrophoresis unit. Ten microliters of horse anti-*C. perfringens* type D antiserum[c] were placed in the series of wells closest to the anode. After applying a constant current of 10 mA and 250 v for 60 minutes, the plates were examined for precipitin bands between the wells. The plates were further examined after standing overnight at room temperature. Purified epsilon toxin[e] was used as a positive control. The formation of bands in the form of fine lines with either the neat sample or any of the dilutions was considered as a positive result.

The MNT was performed as a slight modification of the technique previously described by Sterne and Batty.[12] Briefly, the samples were centrifuged at 14,000 × *g* for 40 minutes at 4 C, filtered through a 0.22-μm filter and the filtrate was trypsinized with 0.1% trypsin[f] at 37 C for 40 minutes. The trypsinized filtrates were mixed in equal parts with *C. perfringens* antitoxin types A–D.[g] After 30 minutes of incubation at room temperature, 0.5 ml of each sample was injected intraperitoneally into each of the two 20–25 g Quackenbush mice. The results were read 48 hours after inoculation, with each mouse categorized as either dead or alive.

Relative sensitivity and specificity were calculated for each technique. Relative sensitivity of a given technique was considered the percentage of samples positive to the MNT

Table 4. Ability of PC-ELISA, MC-ELISA, and CIEP to detect epsilon toxin in samples of known MNT status, and ability of these three techniques plus MNT to detect epsilon toxin in samples spiked or non-spiked with epsilon prototoxin.*

		MNT status			Epsilon toxin		
		+	−	Total	+†	−‡	Total
PC-ELISA	+	30	13	43	30	0	30
	−	2	6	8	3	15	18
	Total	32	19	51	33	15	48
MC-ELISA	+	27	8	35	23	0	23
	−	5	11	16	10	15	25
	Total	32	19	51	33	15	48
CIEP	+	12	3	15	5	0	5
	−	20	16	36	28	15	43
	Total	32	19	51	33	15	48
MNT	+	na	na	na	12	0	12
	−	na	na	na	10	14	24
	Total	na	na	na	22	14	36

* PC-ELISA = polyclonal capture ELISA; MC-ELISA = monoclonal capture ELISA; CIEP = counterimmunoelectrophoresis; MNT = mouse neutralization test.

† Samples spiked with epsilon toxin.

‡ Samples from healthy animals nonspiked with epsilon toxin and samples from animals dying from causes other than intestinal disease.

that were also positive for the given technique. Relative specificity of a given technique was the percentage of samples negative to the MNT that were also negative for the given technique.

Absolute sensitivity was calculated as the percentage of samples spiked with epsilon toxin (intestinal content, pericardial fluid, and aqueous humor labeled 1–11 in Table 1) that gave a positive result to each technique. Absolute specificity was calculated as the percentage of samples from healthy animals nonspiked with epsilon toxin (intestinal content, pericardial fluid, and aqueous humor labeled 12 in Table 1 and the 12 samples from animals dying from causes other than intestinal disease) that gave negative results to each technique.

Results

The results of the 4 techniques to detect epsilon toxin in body fluids spiked with epsilon prototoxin are shown in Table 1. The PC-ELISA and the CIEP showed the highest and the lowest sensitivity, respectively, for the 3 body fluids. The PC-ELISA detected as little as 0.075 MLD_{50}/ml of epsilon toxin in intestinal contents, pericardial fluid, and aqueous humor, whereas the CIEP detected only as little as 50 MLD_{50}/ml of epsilon toxin in intestinal content, and 200 MLD_{50}/ml in pericardial fluid and aqueous humour.

The results of testing intestinal contents of 15 sheep and a goat with a presumptive diagnosis of enterotoxemia for epsilon toxin are shown in Table 2. With one exception, both ELISAs detected the same samples as positive or negative, whereas the CIEP and the MNT produced variable results.

Table 5. Relative sensitivity and specificity of PC-ELISA, MC-ELISA, and CIEP, and absolute sensitivity and specificity of these 3 techniques plus MNT.*

	Relative		Absolute	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
PC-ELISA	93.75	31.57	90.90	100
MC-ELISA	84.37	57.89	69.69	100
CIEP	37.5	84.21	15.15	100
MNT	na	na	54.54	100

* PC-ELISA = polyclonal capture ELISA; MC-ELISA = monoclonal capture ELISA; CIEP = counterimmunoelectrophoresis; MNT = mouse neutralization test; na = not available.

The results obtained from testing the intestinal contents of 9 goats with experimental enterotoxemia for epsilon toxin using the 4 techniques are shown in Table 3. Identical results were found with both ELISAs, although the results were highly variable with CIEP and MNT.

The 12 intestinal content samples from sheep that died of causes other than enterotoxemia or intestinal disease were negative for epsilon toxin when evaluated by the 4 diagnostic techniques.

The comparison among the results of 3 diagnostic techniques with MNT and of 4 techniques to detect epsilon toxin in samples that may or may not contain epsilon toxin is shown in Table 4.

Relative sensitivity and specificity of PC-ELISA, MC-ELISA, and CIEP and absolute sensitivity and specificity of these 3 techniques with MNT are shown in Table 5.

Discussion

Traditionally, a diagnosis of enterotoxemia is based upon detection of epsilon toxin in intestinal contents and/or other body fluids, such as peritoneal or pericardial fluid.[5,6,12] Different techniques are routinely used to detect epsilon toxin in body fluids.[4,6,8,9,12,16]

In the present study the 4 techniques tested showed marked differences in their ability to detect epsilon toxin in intestinal content and other body fluids. The PC-ELISA was the most sensitive technique regardless of fluid type. The MC-ELISA was the second most sensitive technique when used to test pericardial fluid and aqueous humor but was less sensitive than the MNT for the detection of epsilon toxin in intestinal contents. The CIEP was the least sensitive of the technique for body fluids but worked best for intestinal contents. A few samples from sheep with a presumptive diagnosis of enterotoxemia (Table 2) and from goats with experimental enterotoxemia (Table 3) were

found to be negative to PC-ELISA or MC-ELISA or both, and yet they were CIEP- and/or MNT-positive. Because the antisera used in the CIEP and the MNT consisted of polyclonal antibodies against *C. perfringens* type D that are not specific for epsilon toxin, it is possible that these tests detected, in addition to epsilon toxin, other substances present in the intestinal contents, which the more specific ELISAs did not. The reason for the differences in sensitivity of the tests for the detection of epsilon toxin is yet to be determined, although differences in the reagents used in the different techniques (i.e., monoclonal vs. polyclonal antobodies) may be responsible for at least part of these differences.

All the types of *C. perfringens* can be normal inhabitants of the small intestine of ruminants; therefore, it has been suggested that some epsilon toxin (up to 250 LD_{50}/ml) may be found in the intestinal contents of healthy sheep.[1] It is possible then that these small amounts of epsilon toxin in the intestines of healthy animals can produce false positives. However, in the present study, none of the techniques gave positive results on any of the 12 samples from animals dying from causes other than enterotoxemia. Although the presence of *C. perfringens* type D was not investigated in this relatively small set of samples, the results suggest that no epsilon toxin is detected when the amount present in the intestine is below the limit that produces disease. This indicates that the specificity of the 4 techniques evaluated is very high.

The PC-ELISA described here uses a polyclonal, although affinity-purified antibody for capture. It is therefore not possible to be absolutely certain that positive reactions were due to detection of epsilon toxin alone. However, the results from the 12 samples from animals dying from causes other than enterotoxemia and nonspiked with epsilon toxin suggest that the PC-ELISA is highly specific and that no positive results were obtained when epsilon toxin was absent in the samples.

The specificity and sensitivity of the various tests were calculated relative to MNT (the most widely used technique) results. These measures of test accuracy need to be interpreted with care. For instance, when the PC-ELISA detected 43 positive samples, only 30 of these were positive for the MNT. On the basis of the results presented in Table 1, it is likely that the sensitivity of MNT is moderate at best. Using artificially spiked intestinal contents, the efficiency of MNT to detect epsilon toxin was also only moderate (6 MLD_{50}/ml) in comparison with PC-ELISA (0.075 MLD_{50}/ml). Nonetheless, these results do not necessarily mean that PC-ELISA is a better diagnostic technique because it could detect concentrations of toxin that are insufficient to produce disease. The amount of epsilon toxin that can produce disease in sheep has not been established; however, as little as 0.3 ng of activated epsilon toxin is sufficient to kill a mouse.[18] The limit of toxin detection of the PC-ELISA used in this study has been reported to be 32 ng/ml.[18] Others[7] compared the efficiency of CIEP with that of MNT in the detection of epsilon toxin in intestinal contents of goats and they found that the limit of detection of CIEP was 50 MLD_{50}/ml, the same value found in the present study.

It has been reported that epsilon toxin is fairly stable in intestinal contents, especially if these samples are kept in containers and not in the intestine itself. The stability of the toxin is highest when the samples are preserved at −20 C and it decreases progressively when the samples are stored at 4 C or at room temperature, respectively. To avoid any loss of toxin activity, each sample was aliquoted in 4 subsamples (each to be tested by the 4 techniques evaluated here), and the subsamples were maintained at −20 C until processed. It is possible, however, that there was some loss in toxin activity during transport of the samples to the other laboratories.

The results of this study show that there is a marked inconsistency among the various techniques routinely used to detect *C. perfringens* epsilon toxin. Until more information is available about the reliability of these techniques, the diagnosis of enterotoxemia should not only be based solely on epsilon toxin detection, but also on clinical and pathological data. A larger sample should be evaluated to further validate these techniques, and the threshold for epsilon toxin toxicity in intestinal contents and other body fluids should be determined.

Acknowledgements

This work was funded by the University of Queensland, Australia and by Fondo Nacional de Ciencia y Tecnología (Project 01-03591), Argentina. F. A. Uzal was supported by the National Institute of Agricultural Technology and the National Council of Scientific and Technical Research, from Argentina. The MC-ELISA kit was kindly provided by Dr. A. Ginter, BioX, Brussels, Belgium and the PC-ELISA reagents were kindly provided by Dr. W. Weddell, Wallaceville Animal Research Centre, New Zealand. We thank Dr. S. More for the critical reading of this manuscript.

Sources and manufacturers

a. Wallaceville Animal Research Centre, Wallaceville, New Zealand.
b. Pharmacia, Uppsala, Sweden.
c. Titertek Multiskan, Flow Laboratories, Kirkham, UK.
d. BioX, Brussels, Belgium.
e. Wellcome Diagnostics, Dartford, UK.
f. Difco, Kansas City, MO.
g. Pragma, West Wickham, UK.

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