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Evaluation of a QIAamp DNA stool purification kit for Shiga-toxigenic *Escherichia coli* detection in bovine fecal swabs by PCR

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SUMMARY

A commercial kit intended for Taq polymerase inhibitor removal was tested to detect Shiga-toxigenic *Escherichia coli* (STEC) by polymersase chain reaction (PCR) directly from cattle fecal samples. Forty-five samples were analysed for the presence of *stx* genes. Results were compared to those obtained by two other methods: amplification of DNA purified by a non-commercial procedure (heat lysis protocol), and amplification of DNA from samples cultured in solid media, commonly used in our lab. Identical numbers of positive samples (33/45, 73 %) were obtained with the QIAamp DNA stool purification kit and the culturing procedure, suggesting an adequate removal of inhibitors that interfere in PCR amplification from the feces. Besides, the number of positive samples detected using DNA purified by the non-commercial protocol was lower, 25/39 (64%) than that achieved by using the kit. In conclusion, the use of the QIAamp DNA stool purification kit provided a rapid *stx* gene detection by PCR in bovine fecal samples.

Key words: PCR, STEC, shiga-toxin, stool, bovine

RESUMEN

Evaluación del *kit QIAamp DNA stool purification* para la detección de *Escherichia coli* productor de toxina Shiga en hisopados de materia fecal bovina por PCR . Un *kit* comercial diseñado para la eliminación de inhibidores de la polimerasa Taq fue ensayado para la detección de STEC por PCR en muestras fecales de bovinos. Cuarenta y cinco muestras fueron evaluadas por la presencia de genes *stx*. Los resultados fueron comparados con aquéllos obtenidos por otros dos métodos: amplificación de ADN purificado por un procedimiento no comercial (protocolo de lisis por calor), y amplificación de ADN de muestras cultivadas en medio sólido, comúnmente usado en nuestro laboratorio. El mismo número de muestras positivas (33/45, 73 %), fueron obtenidas con el *QIAamp DNA stool purification kit* y el procedimiento de cultivo, sugiriendo una eliminación adecuada de inhibidores que interfieren con la amplificación en materia fecal. Por otro lado, el número de muestras positivas detectadas usando ADN purificado por el protocolo no comercial fue menor, 25/39 (64%). En conclusión, el uso del *kit QIAamp DNA stool purification* permitió una detección rápida de genes *stx* por PCR en muestras fecales bovinas.

Palabras clave: PCR, STEC, toxina shiga, heces, bovino

INTRODUCTION

Rapid and sensitive microorganism detection in domestic animals such as cattle is often required to prevent infection of non-infected animals, reduce cross-contamination of derived products, or to diminish the risk of human infection.

Detection of microorganisms in fecal samples by PCR is considered difficult due to the presence of Taq polymerase inhibitors. Several protocols have been used on human samples to purify DNA for PCR, including direct DNA extraction from feces, DNA extraction with previous enrichment in selective media, and the use of commercial kits that involve spin columns (5, 6, 10). Bovine samples are not exempt from this problem; time consuming protocols, such as bacterial isolation by selective plating and detecting target sequences in samples by PCR (5), are used to avoid inhibitors interference. However, the suitability of spin columns has not been evaluated for animal feces.

A group of serotypes of shiga toxin-producing *E. coli* (STEC), with *E. coli* O157 as the prototype strain, is an important food-borne pathogen, whose presence in cattle feces has been demonstrated. In addition,

serogroups other than O157, some of which are present in cattle, are also frequently associated with human illness. Additional quality controls, by means of systematic testing before animal slaughtering, will probably be necessary to reduce the risk of meat contamination. Thus, PCR could be an advantageous technique to apply in testing programs. In this study a commercial kit for the elimination of Taq polymerase inhibitors in stool samples was evaluated to observe if it may be useful for the detection of STEC in bovine fecal samples.

MATERIALS AND METHODS

Processing of samples

Forty-five rectal swabs were randomly collected from live calves prior to slaughter, from July 1999 to December 2000, in different slaughterhouses in Buenos Aires province. Cotton-tipped swabs were used. Swabs were transported in Cary Blair medium and resuspended in 5 ml of saline solution. Samples were conserved at -20°C until processing.

STEC detection

QIAamp DNA stool purification kit protocol: Samples were processed for isolation of DNA as described by the manufacturer (QIAamp DNA stool mini kit, Qiagen, Germany). Briefly, ASL buffer (provided by the kit) was added to 200 µl of rectal swab suspension and the sample was homogenized by vortexing. Heat lysis at 70°C and centrifugation at 13000 rpm were performed to pellet stool particles prior inhibitor adsorption onto a solid matrix/inhibiteEX tablets. After absorption of inhibitors and DNA-degrading substances, the inhibitEX reagent was pelleted by centrifugation and supernatant containing DNA was treated with 15 µl of proteinase K (20 mg/ml). Following DNA precipitation by two volumes of ethanol, DNA was purified on QIAamp spin columns and eluted with 200 µl of Elution buffer (provided by the kit).

Heat lysis protocol: Twenty microliters of proteinase K (10mg/ml) and 50 µl of 10% SDS were added to 200 µl of fecal swab suspension. Samples were mixed and incubated 1h at 65°C. Lysis was performed by placing in boiling water for 10 min. DNA was precipitated by the addition of 2 volumes of 100% ethanol and centrifugation for 5 min at 13,000 rpm. Finally, DNA was resuspended in 50 µl of distilled water.

Direct culture on TSA protocol: Rectal swabs were streaked onto TSA (Triptic Soy Agar) plates and cultivated overnight at 37°C. Bacteria were suspended in 5 ml of water, and a 1/5 dilution was lysed at 100°C and centrifuged 5 min at 13,000 rpm to eliminate cellular debris. Five microliters of the supernatant were used for PCR amplification. A fraction of the bacterial growth was conserved at -20°C.

Sensitivity

A negative confirmed STEC sample (1ml), was contaminated with different number of bacteria ranging from 10^6 to 10^0 CFU of a $stx1^+/stx2^+$ STEC strain and processed using Heat lysis and QIAamp DNA stool purification Kit protocols. The samples with different STEC bacteria concentrations were plated on TSA and processed as described for the Direct culture on TSA procedure.

DNA concentration and purity

Purified DNA from the Heat lysis and QIAamp DNA stool purification protocols were measured in a GeneQuant *pro* (Amersham Pharmacia biotech) spectrophotometer at 260nm, and the purity of the DNA in each sample estimated by the ratio A260/A280.

PCR

Five microliters of the purified DNA were used for amplification of the *stx1, stx2*, *eae* and *rfb* genes. Primers used to detect *stx1* (GAAGAGTCCGTGGGATTAC and AGCGATGCAGCTAT TAATAA) and *stx2* (CTTCGGTATCCTATTCCCGG and GGATGCA TCTCTGGTCATTG) were described by Pollard *et al* (9) and by Blanco *et al* (1), respectively. For *eae* gene amplification, primers that amplify a sequence common to the STEC and enteropathogenic *Escherichia coli* (EPEC) strains were used (3). To amplify the *rfb* gene coding for O157 LPS, primers described by Paton and Paton (8), were used. Stx genes were amplified in a single reaction at an annealing temperature of 55ºC and 2.5 mM $MgCl_2$. Other conditions used were as described by the authors. PCR products were analysed by electrophoresis on 2% agarose gels followed by ethydium bromide staining.

Negative PCR samples were re-tested by PCR using different volumes of template (1 µl, 7.5 ml, and 5 µl, of 1/50 and 1/100 dilutions) and by the addition of bovine serum albumin (BSA) to the PCR mixture to at final concentration of 0.1 mg/ml, as suggested by the kit instructions. In order to detect the presence of inhibitors in these samples, 1 pg of purified DNA from a known positive strain was added.

Isolation of STEC strains

Individual colonies from the fraction conserved at -20°C and streaked onto MacConkey agar, were selected and analysed by PCR to find the *stx*1 or *stx*2 positive isolates. At least 20 colonies per sample were analysed as already described (4).

Statistical analysis

Results were evaluated using the *t* test.

RESULTS

The average nucleic acid yield was 0.9±0.4 µg using the QIAamp DNA stool purification kit, and 4.8±4.3 µg using the Heat lysis protocol. However, the A260/A280 value obtained by QIAamp DNA stool purification kit, was higher than those obtained using the Heat lysis protocol (1.40±0.03 vs 1.26±0.02, $p<0.001$).

Samples showing amplicons of one or both *stx* genes amplified were considered positive. Identical numbers of positive samples (33/45) were detected by the Direct culture on TSA and QIAamp DNA stool purification kit protocols, while, twenty-five samples (25/39) were positive according to the Heat lysis protocol (Table 1). Using the Heat lysis protocol, only five positive samples were detected with no dilution of the template, while 18 positive samples were detected after diluting DNA 1/50, and two more after diluting DNA 1/100 (Table 1). Not all procedures gave positive PCR results for the same sample. By adding the results from all the protocols assayed, a total of 40/45 *stx*-positive samples were detected. Nine samples gave positive results by only one procedure (3 by QIAamp DNA stool purification kit, and 6 by the Direct culture on TSA protocol). A representative gel showing amplification products is shown in Figure 1.

Seven samples were positive according the Direct culture on TSA protocol, but negative by QIAamp DNA stool purification kit. These samples were assayed with different volumes of template, but no positive result was obtained. Addition of BSA to the PCR mixture did not allowed amplification in these samples. Inhibition controls performed by spiking these samples with pure *stx* positive DNA indicated that inhibitors were not present.

The average time for diagnosis was 24 h, for the QIAamp DNA stool purification kit protocol, similar to that for the Heat lysis protocol. However, using this latter method, it was frequently necessary to re-test more diluted samples which lengthen diagnosis time.

Eleven samples that resulted positive by more than one method were found to differ in their toxin genotype (Table 2). Seven samples had single *stx* genotype results by both Heat lysis and solid media protocols, but were *stx1/stx2* when assayed by the QIAamp DNA stool purification kit (samples 13, 14, 19, 23, 27, 32 and 34). On the other hand, three samples were positive for both *stx* loci only by the Direct culture on TSA method (samples 6, 12 and 26) and of a single genotype according to the two other protocols. Sample 9 was classified as *stx2* by Heat lysis and the QIAamp DNA stool purification kit, but identified as *stx1* by the Direct culture on TSA protocol. The genotypes obtained for the remaining samples were identical for all protocols.

The highest sensitivity corresponded to the Direct culture on TSA procedure, which was capable of detecting a single bacterium. In the case of Heat lysis protocol, the sensitivity was 10^4 bacteria detected, while 10^3 bacteria were detected by using the commercial kit.

STEC isolates were identified in 85% of the positive samples. In three animals (27, 34 and 41, Table 2) isolates with different genotypes, *stx1/stx2* and *stx1 or stx2* were isolated, while the *stx1* gene was identified by all the protocols in these samples, the *stx2* gene was detected only by using the QIAamp DNA stool purification kit (except sample 41). A *stx2* strain was isolated in samples identified as *stx1/stx2* by more than one protocol (4, 8, 20, 21), which indicates that this genotype was much more abundant than the *stx1* or *stx1/stx2* strains.

Other STEC-related genes were also evaluated by PCR using two protocols. The *rfb* O157 gene was tested in all samples processed by QIAamp DNA stool purification kit and the Direct culture on TSA protocols. Six positive samples were detected by QIAamp DNA stool purification protocol, and 5 by the Direct culture on TSA protocol. The *eae* gene was evaluated in these *rfb* O157 positives samples. All, except one, was found to be *eae* positive.

DISCUSSION

According to the results presented in this study, QIAamp DNA stool purification kit allows a rapid PCR identification of STEC in bovine feces. This kit involves the elimination of inhibitors by heat lysis and their binding to a solid matrix which is precipitated by centrifugation. In spite of the fact that this procedure was initially designed for human feces, the present results demonstrate its efficacy in animal feces. The same number of samples were identified as positive by the QIAamp DNA stool purification kit and solid sample suspension procedures. Time to diagnosis is an important parameter, especially in human outbreaks and in the international commerce of animal products. QIAamp DNA stool purification kit proved to be faster than the solid media culture protocol, although the later procedure showed the highest analytical sensitivity.

The fact that 7 samples were positive by the Direct culture on TSA protocol and negative by QIAamp DNA stool purification kit or Heat lysis protocol, could be explained by the lower total amount of microorganisms, and by the increase of STEC number after culturing. In turn, other 7 samples were negative by the Direct culture on TSA protocol but positive by the QIAamp DNA stool purification kit. Although we do not know the reason for this, it could be explained on the basis of the low ability of specific isolates to survive or spread in the conditions of the Direct culture on TSA protocol causing a decrease of their proportion in the sample.

The differences observed in the toxin genotypes identified by the solid sample suspension protocol and PCR performed on purified DNA, could be due to the existence of animals colonized by more than one STEC strain. The isolation of more than one strain in three animals supports this issue. In addition, another sample was identified as *stx1*$^+$/*stx2*$^+$ by all protocols, though only two *stx2*$^+$ strain could be isolated. Thus, the QIAamp DNA stool purification kit protocol allows an efficient amplification of both loci in the same sample when compared with the other protocols assayed. Consequently, DNA obtained by this procedure is suitable for multiloci detection by PCR, since a greater detection of *stx1/stx2* samples was obtained compared with the Direct culture on TSA. Due to a variable abundance of STEC in each animal, a more exhaustive analysis of colonies could be needed to detect more than one STEC isolate in each *stx1/stx2* sample. Additionally, the genetic instability of toxin genes encoded by phages (11), could contribute to the different genetic composition.

Other virulence-related genes as *rfb* O157 were detected in similar proportion when samples were processed by QIAamp DNA stool purification kit or the Direct culture on TSA protocol. A recent study (1) describes a multi-gene detection single PCR putting together the rapid detection of several virulence genes and serogrouping of tested strains. This type of approach, combined with an optimal and rapid purification of DNA from clinical samples, is a useful tool to identify potential dangerous strains present in the sample.

A lower number of positive samples was identified when PCR was performed using Heat Lysis protocol without culture. This kind of protocol was already used by other authors (1, 2, 7). However several samples were identified as positive only after diluting DNA. The dilution of samples reduces inhibitory interferences that affect amplification, but can diminish target sequences below detection limit levels.

On conclusion, the use of spin columns with Taq-polymerase-inhibitor removal reagent would facilitate a rapid detection of STEC by PCR without previous enrichment, making available same-day results.

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Figure 1. Representative agarose gel showing *stx1* and *stx2* amplification products. Lanes. M, molecular weight marker (from top to bottom, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp); 1 to 4, series of four samples processed by each protocol.

Table 1. Comparison of PCR results obtained by the different protocols.

Conditions of template and performance during fecal swab analysis	Solid media suspension (n =45)	Heat lysis (n =39)	QIAamp DNA stool purification kit (n =45)
Undiluted	33	5	33
1/50	-	18	ND
1/100	-	2	ND
Total *stx* positive Samples detected	33 [1]	25 [1]	33 [1]
%+	73	64	73
Diagnosis time	48 h.	24 h.[2]	24 h.

[1] Percentages were significantly different according to the Student's t-test ($p<0.001$). [2]without considering the need of further dilution of negative samples. Nd: not done

Table 2. Results of the protocols assayed to detect *stx* genes by PCR in bovine feces

Sample	Direct culture on TSA	QIAamp DNA stool purification Kit	Heat lysis			*Stx* genotype of the strain isolated
		Undiluted	Undiluted	1/50	1/100	
1	Stx2	Stx2	Stx2	Nd	Nd	Stx2
2	-	Stx2	-	Stx2	Nd	-
3	-	Stx2	Stx2	Nd	Nd	-
4	Stx1/stx2	Stx1/stx2	-	Stx1/stx2	Nd	Stx2
5	Stx2	Stx2	-	Stx2	Nd	Stx2
6	Stx1/stx2	Stx2	-	-	stx1	Stx1/stx2
7	-	Stx2	-	Stx2	Nd	-
8	Stx1/stx2	Stx1/stx2	-	Stx1/stx2	Nd	Stx2
9	Stx1	Stx2	-	Stx2	Nd	Stx2
10	Stx1/stx2	Stx1/stx2	-	Stx1/stx2	Nd	Stx1/stx2
11	Stx1/stx2	Stx1/stx2	-	Stx1/stx2	Nd	Stx1/stx2
12	Stx1/stx2	Stx2	-	Stx2	Nd	Stx2
13	Stx1	Stx1/stx2	Stx1	Nd	Nd	Stx2
14	Stx1	Stx1/stx2	Stx1	Nd	Nd	Stx1
15	Stx2	Stx2	-	Stx2	Nd	Stx2
16	-	Stx2	-	-	-	-
17	Stx2	Stx2	-		stx2	Stx2
18	-	-	-	-	-	-
19	Stx1	Stx1/stx2	-	-	-	Stx2
20	Stx1/stx2	Stx1/stx2	-	Stx2	Nd	Stx2
21	Stx1/stx2	Stx1/stx2	Nd			Stx2
22	Stx2	Stx2	-	Stx2	Nd	Stx2
23	Stx1	Stx1/stx2	-	Stx1	Nd	Stx2

24	Stx2	Stx2	-	Stx2	Nd	Stx2
25	Stx2	Stx2	-	Stx2	Nd	Stx2
26	Stx1/stx2	Stx2	Nd			Stx2
27	Stx1	Stx1/stx2	-	Stx1	Nd	Stx1 stx1/stx2
28	Stx1	-	-	Stx1	Nd	-
29	-	Stx2	-	Stx2	Nd	Stx2
30	Stx1	-	-	-	-	-
31	-	Stx2	-	-	-	-
32	Stx1	Stx1/stx2	-	-	-	Stx1/stx2
33	Stx2	-	-	-	-	Stx2
34	Stx1	Stx1/stx2	Nd			Stx1 Stx1/stx2
35	-	Stx2	-	-	-	-
36	-	-	-	-	-	-
37	-	-	-	-	-	-
38	-	-	-	-	-	-
39	Stx2	-	-	-	-	Stx2
40	Stx1	-	Nd			-
41	Stx1/stx2	-	Nd			Stx2 Stx1/stx2
42	Stx2	Stx2	Nd			Stx2
43	Stx2	Stx2	Stx2	-	-	Stx2
44	Stx2	-	-	-	-	Stx2
45	-	-	-	-	-	-

Nd: not done. -: negative