

Evaluation of three enzyme immunoassays and toxigenic culture for diagnosis of *Clostridium difficile*-associated enteritis in piglets

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Summary

The aim of this study was to compare test performances of three commercial enzyme immunoassays (EIAs) for A and B toxin detection and that of a simple toxigenic culture protocol to the cytotoxicity assay (CTA) as the gold standard for diagnosis of *Clostridium difficile*-associated enteritis in piglets. A total of 73 piglets submitted to the Veterinary School of Universidade Federal de Minas Gerais were included in this study. Intestinal content was collected from 62

diarrheic and 11 non-diarrheic piglets, 1 to 7 days old. Vero cells were used in the CTA protocol to detect A and B toxins. Fecal samples were inoculated on cycloserine-cefoxitin fructose agar for isolation of *C. difficile*. The EIAs were performed according to the manufacturers' instructions. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated for each EIA and for toxigenic culture against CTA. The CTA was positive for 22 of the 73 samples (30.1%). Sensitivities of all EIAs

and toxigenic culture for the piglet samples were low (41% to 64%), whereas specificities were 80% to 98%. These results suggest that the EIAs and toxigenic culture protocol tested are not suitable for diagnosis of *C. difficile* infection in individual piglets.

Keywords: swine, *Clostridium difficile* toxins A and B, neonatal diarrhea, colitis

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Resumen - Evaluación de tres inmunoensayos de enzimas y de cultivo toxigénico para el diagnóstico del *Clostridium difficile* asociado con la enteritis en lechones

El propósito de este estudio fue comparar el desempeño de tres pruebas comerciales de inmunoensayo de enzimas (EIAs por sus siglas en inglés) y el del protocolo del cultivo toxigénico simple con el del ensayo de citotoxicidad (CTA por sus siglas en inglés) como el estándar de oro para el diagnóstico del *Clostridium difficile* asociado con la enteritis en lechones. Un total de 73 lechones enviados a la Escuela Veterinaria de la Universidad Federal de Minas Gerais se incluyeron en este estudio. Se recolectó el contenido intestinal de 62 lechones diarreicos y 11 no diarreicos de 1 a 7 días de edad. Se utilizaron células Vero en el protocolo CTA para detectar toxinas A y B. Se inocularon muestras fecales en agar fructuosa-cicloserina-cefoxitina para el aislamiento de *C. difficile*. Las EIAs se desarrollaron de

acuerdo a las instrucciones del fabricante. Se calcularon la sensibilidad, especificidad, valor predictivo positivo, y valor predictivo negativo para cada EIA y para el cultivo toxigénico contra el CTA. El CTA resultó positivo para 22 de las 73 muestras (30.1%). Las sensibilidades de todas las EIAs y el cultivo toxigénico para las muestras de los lechones fueron bajas (41% a 64%), mientras que las especificidades fueron de 80% a 98%. Estos resultados sugieren que las EIAs y el protocolo de cultivo toxigénico probados no son adecuados para el diagnóstico de la infección por *C. difficile* en lechones individuales.

Résumé - Évaluation de trois épreuves immuno-enzymatiques et d'une méthode de culture toxigénique pour le diagnostic d'entérite associée à *Clostridium difficile* chez les porcelets

L'objectif de la présente étude était de comparer les performances de trois épreuves

immuno-enzymatiques commerciales (EIA) et d'un protocole de culture toxigénique à l'épreuve de cytotoxicité (CTA) considérée comme l'épreuve étalon pour le diagnostic de l'entérite associée à *Clostridium difficile* chez les porcelets. Au total, 73 porcelets soumis à la Faculté vétérinaire de l'Universidade Federal de Minas Gerais ont été inclus dans l'étude. Le contenu intestinal a été prélevé de porcelets diarrhéiques (62) et non-diarrhéiques (11), âgés de 1 à 7 jours. Des cellules Vero ont été utilisées dans l'épreuve CTA afin de détecter les toxines A et B. Des échantillons de fèces ont étéensemencés sur gélose cycloserine-cefoxitin-fructose pour l'isolement de *C. difficile*. Les EIA ont été effectuées selon les instructions des fabricants. La sensibilité, la spécificité, la valeur prédictive positive, et la valeur prédictive négative ont été calculées pour chaque EIA et pour la culture toxigénique versus le test CTA. Ce dernier était positif pour 22 des 73 échantillons (30,1%). Les sensibilidades de toutes les EIA et de la culture toxigénique pour les échantillons de porcelets étaient faibles (41% à 64%), alors que les spécificités étaient de 80% à 98%. Ces résultats suggèrent que les EIA et le protocole de culture toxigénique testés ne sont pas appropriés pour le diagnostic de l'infection à *C. difficile* chez des porcelets pris individuellement.

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Clostridium difficile is a spore-forming, anaerobic, gram-positive bacillus that has been recognized as responsible for 95% of all pseudomembranous colitis cases and most cases of antibiotic-associated diarrhea in humans.¹ In veterinary medicine, this organism is considered the most important uncontrolled cause of neonatal diarrhea in pigs in some countries, including the United States and Brazil.^{2,3} In addition, recent studies also showed that the strains isolated from humans suffering from *C. difficile* infection (CDI) have a high genetic relatedness to strains of animal origin,⁴ suggesting that CDI is a zoonosis.

For most authors, the “gold standard” for diagnosis of CDI is the cytotoxicity assay (CTA), but this test is both labor intensive and time consuming. Therefore, commercial enzyme immunoassays (EIAs) remain the most common method used for diagnosis of CDI in humans and animals.^{5,6} Recently, toxigenic culture has also been described as a sensitive method for human samples,⁷ but little is known with regard to its application for samples from domestic animals.

Despite the importance of *C. difficile* as a swine enteropathogen and even as a potential zoonotic agent, no established guidelines are available for diagnosing CDI, and performance is unknown for most commercially available detection methods.⁸ In light of this fact, the aim of the present study was to compare test performances of three different EIAs and toxigenic culture to the CTA as the gold standard.

Materials and methods

Ethics approval for this study was granted by the Animal Experiments Committee of the Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

The piglets included in this study were submitted to the Veterinary School of Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, for routine diagnosis of piglet neonatal diarrhea. Live animals were euthanized and necropsied, and intestinal content was collected in sterile containers and stored at -20°C until tested up to 7 days later. A total of 73 samples from 32 farms were included in the study, with 62 samples from diarrheic piglets and 11 from non-diarrheic piglets.

The CTA for *C. difficile* A and B toxins was performed with Vero cells (ATTC CCL 81) as described previously.⁹ Briefly, fecal samples were diluted 1:4 in phosphate-buffered

saline (pH 7.0) and centrifuged at 3000g for 5 minutes at 4°C. The resulting supernatant was filtered through a 0.22- μ m pore size filter and diluted twofold until a dilution of 1:1024 was reached. Serial dilutions and parallel samples with *Clostridium sordellii* antitoxin (National Institute for Biological Standards and Control, Hertfordshire, England) were added onto the Vero cell monolayers. The cells were examined after 24 hours of incubation at 37°C in a 5% CO₂ incubator. A specimen was considered positive if at least 90% of the cells were rounded and the effect was neutralized by antitoxin at the same dilution in a parallel sample.

For toxigenic culture, the fecal samples were subjected to alcohol shock,¹⁰ and 50- μ L aliquots were inoculated onto cycloserine-cefoxitin fructose agar (CCFA) plates (Hi-media, Mumbai, India) supplemented with 7% horse blood and 0.1% sodium taurocholate (Sigma-Aldrich Co, St Louis, Missouri). After incubation in an anaerobic chamber at 37°C for 72 hours, colonies with morphology suggestive of *C. difficile* and a positive Gram stain were subjected to a previously described multiplex polymerase chain reaction (PCR) for a housekeeping gene (*tpi*), toxins A (*tcdA*) and B (*tcdB*), and binary toxin genes (*cdtB*).¹¹ In addition, all toxigenic isolates in the PCR were tested by CTA for in vitro toxin production as previously described.⁵ *Clostridium difficile* ATCC 9689 was used as a control for the PCR and toxigenic culture.

Three commercial enzyme immunoassays (EIAs) for A and B toxin detection were tested: *Clostridium difficile* Tox A/B II (Techlab Inc, Blacksburg, Virginia), Remel ProSpecT *Clostridium difficile* Toxin A/B Microplate Assay (Oxoid, Hampshire, United Kingdom), and Ridascreen *Clostridium difficile* toxins A/B (R-Biopharm, Darmstadt, Germany). All EIAs were performed according to the manufacturers' recommendations. The sensitivity, specificity, positive predictive value, negative predictive value, and 95% confidence intervals were calculated for each EIA and for toxigenic culture against CTA (Stata 12; StataCorp LP, College Station, Texas).

Results

The CTA was positive in 22 of the 73 samples (30.1%). Six of the positive samples were from non-diarrheic piglets. Sensitivities of the EIAs evaluated were \leq 63.6%, and specificities were 80.3% to 98% (Table 1).

Clostridium difficile was isolated from 13 of the 73 samples (18.3%), with 10 strains toxigenic by PCR. All toxigenic isolates were positive for the *tcdA* and *tcdB* genes, and one was also positive for the binary toxin gene (*cdtB*). One toxigenic strain isolated from a diarrheic piglet was negative for A and B toxins by CTA. All PCR-toxigenic strains were able to produce toxin in vitro. Sensitivity and specificity of toxigenic culture are provided in Table 1.

Discussion

All EIAs tested had sensitivities < 65% when used for piglet fecal samples. This undesirable EIA performance with regard to piglet fecal samples is not surprising and was previously reported for other EIAs.^{8,12} Some authors attributed the low specificity of EIAs for swine fecal samples to inhibitors in animal feces; however, to date, no evidence confirms this possibility.^{8,13} In contrast, similar to a previous report,⁸ false-positive results varied widely among EIAs, suggesting that the incorrect results were due to the test and not to an interfering substance in the samples. It is also interesting to note that an older version of the Techlab EIA was previously tested on porcine fecal samples, with a sensitivity of 91% and a specificity of 86% reported.¹⁴ In the present study, the new version of the Techlab test had a much lower sensitivity (59.1%), but the specificity was 98.0%.

In contrast to a previous report,⁸ the sensitivity of toxigenic culture in this study was low (40.9%). Unfortunately, there is no standard method for toxigenic culture of *C. difficile*, making it difficult to compare reported results. A great variety of media have been reported, in addition to differences in isolation protocol, such as the use of alcohol shock and variations in incubation time. Accordingly, this study reports a simple isolation method that would be more applicable for diagnosis than previously reported protocols.^{5,8,15} In this protocol, the samples were subjected to alcohol shock, plated on CCFA supplemented with 0.1% sodium taurocholate, and incubated for 72 hours. It is well known that some *C. difficile* strains fail to grow on CCFA because of susceptibility to one or both antibiotics used in the medium.¹⁶ In addition, the use of CCFA, even with supplemental taurocholate, may result in variable sensitivity for recovery of *C. difficile* spores, compared with other isolation protocols, such as use of pre-enrichment broth.¹⁷ All these factors might

Table 1: Comparison of three commercial enzyme immunoassays and toxigenic culture to the cytotoxicity assay (CTA) as the gold standard for diagnosis of *Clostridium difficile*-associated diarrhea in piglets*

Method	Piglets samples (n = 73)			
	% (95% CI)			
	Sensitivity	Specificity	PPV	NPV
Toxigenic culture	41.0 (23.3-61.3)	98.0 (89.7-99.7)	90.9 (59.6-98.2)	79.7 (67.8-87.5)
Ridascreen <i>Clostridium difficile</i> toxins A/B†	54.5 (34.7-73.1)	88.2 (76.7-94.4)	76.7 (43.8-83.7)	81.8 (69.7-89.8)
<i>Clostridium difficile</i> Tox A/B II Microplate Assay‡	59.1 (38.7-76.7)	98.0 (89.7-99.6)	92.9 (68.5-98.7)	84.7 (73.5-91.8)
Remel ProSpecT <i>Clostridium difficile</i> Toxin A/B§	63.6 (42.9-80.2)	80.3 (67.5-88.9)	58.3 (38.8-75.5)	83.6 (70.9-91.5)

* 73 piglets 1 to 7 days old were submitted to the Veterinary School of Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, for routine diagnosis of piglet neonatal diarrhea. The CTA for *C. difficile* was positive in 22 of the piglets and negative in 51.

† R-Biopharm, Darmstadt, Germany.

‡ Techlab Inc, Blacksburg, Virginia.

§ Oxoid, Hampshire, United Kingdom.

PPV = positive predictive value; NPV = negative predictive value.

have contributed to the low sensitivity of the toxigenic culture protocol tested and suggest that this protocol is not acceptable for diagnosis of CDI in piglets, in contrast to the previous reports for human samples.^{6,7}

All toxigenic isolates in the present study were positive for the *tcdA* and *tcdB* genes, whereas one strain was also positive for the binary toxin gene (*cdtB*). It is interesting to note that all the piglet *C. difficile* isolates that were considered to be toxigenic by PCR were also able to produce toxins A and B in vitro. This result suggests a good correlation between toxin gene detection by PCR and in vitro toxin production in porcine *C. difficile* strains. Therefore, the toxin production test after isolation may not be necessary when A and B toxin genes have been detected. The removal of this step would save time and reduce the cost of diagnosis. Further studies with a larger number of strains are needed to confirm this hypothesis.

In the present study, one toxigenic strain isolated from a diarrheic piglet was negative for A and B toxins by CTA. Several hypotheses should be considered. First, the toxins might not have been detected by CTA, which does not exhibit 100% sensitivity.⁵ Second, the piglet might have been an asymptomatic carrier, and other enteropathogens might

have been responsible for the diarrhea.

Another possibility is that A and B toxins that had been present were degraded by fecal proteases. It should be emphasized that the time between sample collection and processing in the present study was short (only 7 days). In a previous study, A and B toxins remained detectable in piglet fecal samples for at least a month at -20°C.⁸ These data suggest that failure to detect A and B toxins using the EIAs in the present study was not caused by storage conditions. Some authors contend that protease activity in animal fecal specimens may cause rapid toxin degradation such that toxin may not be detectable by EIAs or CTA; to date, to the authors' knowledge, there is no study confirming this hypothesis.¹³

The sensitivity and specificity of all the EIAs tested were unacceptable for testing individual piglet samples. Similar results have been reported in humans, and some studies suggest that at least a two-step algorithm is needed to reliably diagnose CDI;⁷ however, there is no consensus thus far on which tests should be used in each step. One possible approach is use of an EIA with a high sensitivity as the primary test, followed by CTA as the confirmatory test for positive samples. Another option is use of a high-specificity method associated with a large number of

samples from each swine farm. In the present study, one of the EIAs had a specificity of 98% (95% CI, 89.7%-99.6%) for piglet samples, allowing a great degree of confidence in the positive results, with a PPV of 92.9% (95% CI, 68.5%-98.7%) and an NPV of 84.7% (95% CI, 73.5%-91.8%). Therefore, we suggest this EIA (Techlab) might be useful for screening for CDI in a herd when multiple samples are collected.

Implications

- Under the conditions of this study, sensitivity and specificity of the three EIAs tested are unacceptable for diagnosis of *C. difficile* in individual piglet fecal samples.
- Use of a high-specificity EIA associated with a large number of samples from each swine farm could be used to screen for *C. difficile* infection in a herd when multiple samples are collected.

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Conflict of interest

None reported.

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