The Veterinary Journal 197 (2013) 694–698

Contents lists available at SciVerse ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Is the prevalence of *Clostridium difficile* in animals underestimated? ☆

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ARTICLE INFO

Article history:
Accepted 7 March 2013

Keywords:
Clostridium difficile
Bacteriological culture
Enrichment
Prevalence
Recovery rate

ABSTRACT

Reported prevalence rates of *Clostridium difficile* infection in animals differ considerably depending on the nature of the study and the population surveyed. The methods used to recover this organism from faecal samples may account for some of the prevalence variation. The objective of this study was to assess the performance of two different methods of detecting *C. difficile* in animal faeces in comparison with a conventional isolation procedure ('ethanol shock' of faecal samples followed by culture on a single plate of solid selective medium). Samples were obtained from two populations of pigs where the expected prevalence rate of *C. difficile* infection was anticipated to differ, namely, 'high prevalence' (<7-day old piglets) and 'low prevalence' (2–3-month old pigs).

The first alternative detection method required culturing faecal samples on 10 (instead of one) plate of solid selective medium after ethanol shock, while the second method included an intermediate enrichment step in selective broth prior to ethanol shock and subsequent plating. Both alternative methods considerably increased bacterial recovery in tested samples from both surveyed populations and highlighted the existence of a considerable proportion ($\geqslant$22%) of false negatives. The results confirm previous suggestions that the procedure used to isolate *C. difficile* can have a significant impact on prevalence data for this pathogen.

Introduction

The relatively recent emergence of *Clostridium difficile* as a human and animal pathogen and the suggestion that pets and livestock might act as possible sources of hypervirulent zoonotic strains have prompted considerable research into the epidemiology of this opportunistic anaerobe in animal populations (Debast et al., 2009; Norman et al., 2011; Janezic et al., 2012; Koene et al., 2012). Reported prevalence rates for *C. difficile* in animals vary considerably depending on the country in which the study was performed, the species and population surveyed, and even on the sampling procedure (Weese, 2010; Keessen et al., 2011a). Furthermore, it has been suggested that the methods used to detect *C. difficile* in faeces may account for some of the variation in prevalence (Weese, 2010; Dubberke et al., 2011; Keessen et al., 2011a). Although there is currently no 'gold standard' or ISO procedure for detecting *C. difficile* (Keessen et al., 2011a,b), culturing faeces is widely used both in human and animal medicine as this method is relatively inexpensive and facilitates the recovery of individual isolates that can then be genotyped and further characterised.

Isolation procedures for *C. difficile* are far from standardised, and variations to the most commonly used method of culture (on a single plate of solid selective medium after an 'ethanol shock' step) have been proposed. Most such variations include an enrichment step in selective broth before and/or after ethanol shock (Arroyo et al., 2005; Rodriguez-Palacios et al., 2006, 2011; Norman et al., 2009, 2011; Thakur et al., 2010; Weese et al., 2010b; Hopman et al., 2011; Costa et al., 2012; Schoster et al., 2012; Susick et al., 2012). The aim of this study was to compare the performance of different culture procedures used to recover *C. difficile* from porcine faeces. The conventional 'single-plate' procedure was compared against (1) simultaneous culture of each faecal sample on 10 plates of solid selective medium after ethanol shock, and (2) plate culture following two ethanol shock stages separated by a phase of enrichment in selective broth. The relative merits of these methods in the context of epidemiological studies are discussed.

Materials and methods

Samples and study design

Faecal samples were collected using dry cotton-tipped swabs (Nirco) from two pig populations consisting of 1–7-day old and 2–3-month-old animals, respectively. All animals were clinically normal at time of sampling. As previous studies demon-

☆ Preliminary results were presented as an Abstract at the 4th International *Clostridium difficile* Symposium, Bled, Slovenia, September 2012.

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1090-0233/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tvjl.2013.03.053

strated that the prevalence of *C. difficile* in <15-day old piglets is typically high (Álvarez-Pérez et al., 2009b; Avbersek et al., 2009; Thakur et al., 2010; Weese et al., 2010b; Hopman et al., 2011; Keessen et al., 2011b), the younger population surveyed was considered a 'high prevalence' (HP) population, while the older pigs were designated a 'low prevalence' (LP) population.

Isolation of Clostridium difficile from faecal samples

We compared the performance of conventional culture with two alternative methods in the following two experiments (Fig. 1).

Experiment 1

A total of 36 rectal swabs (18 from each of the HP and LP pig populations) were cultured on solid medium following ethanol shock (Álvarez-Pérez et al., 2009b). Briefly, the tip of each rectal swab was submerged for 30 s in a 1.5 mL microtube containing 1 mL of 70% ethanol (v/v; Panreac). The tubes were then sealed and incubated at room temperature for 20 min. After incubation, the microtubes were vortexed and a 75 μL aliquot from each was plated onto solid medium containing cycloserine and cefoxitin as selective agents (CLO agar; bioMérieux). In this experiment the alternative method involved taking ten 75 μL aliquots (i.e. the previous, and nine additional, aliquots) from each microtube after ethanol shock and plating these on CLO agar. In both conventional and alternative procedures, the inoculated plates were incubated under anaerobic conditions for 48 h at 37 °C.

Experiment 2

Twenty swab samples from each of the HP and LP populations were cultured in parallel following the conventional procedure described above. The alternative method for this experiment was based on that described by Arroyo et al. (2005) with minor modifications. The tip of each rectal swab was introduced into a 10 mL glass tube containing 5 mL of liquid selective broth for *C. difficile* (40 g/L proteose peptone, 5 g/L disodium hydrogen phosphate, 1 g/L potassium dihydrogen phosphate, 0.1 g/L magnesium sulfate, 2 g/L sodium chloride, 6 g/L fructose, 1 g/L sodium taurocholate, 250 mg/L D-cycloserine, and 8 mg/L cefoxitin; pH 7.4; TecLaim). After 8 days of incubation at 37 °C under anaerobic conditions, 2 mL of the liquid culture was mixed with 2 mL of absolute ethanol (Panreac) and incubated for 1 h under agitation (200 rpm) at room temperature.

Finally, tubes were centrifuged at 1520 *g* for 10 min, the supernatants were discarded, and the precipitates collected using sterile cotton-tipped swabs and plated onto the solid medium (CLO agar) described previously. Inoculated plates were incubated under anaerobic conditions for 48 h at 37 °C.

In both experiments, *C. difficile* was identified based on colony morphology, typical odour, and a positive reaction in a rapid specific immunoassay, which simultaneously detects the production of glutamate dehydrogenase and toxins A and B (C. Diff Quik Chek Complete, Techlab).

Statistical analysis

Fisher's exact tests were used for comparison of proportions, and *P*-values $\leqslant 0.05$ were considered significant. Sensitivity, specificity, the positive predictive value (PPV), and the negative predictive value (NPV) were calculated for the conventional in comparison with the two alternative isolation methods (i.e. '10-plate' culturing and enrichment prior to plate culturing). The results of the two alternative methods were used as references to define true and false positive and negative samples in the conventional procedure.

Results

The results of experiment 1 are shown in Tables 1 and 2. If the 10 plates cultured/sample are taken into account (alternative method 1), *C. difficile* was recovered from a total of 16 samples, 11 of which were from the HP and 5 from the LP population. This difference between populations was close to but not statistically significant ($P = 0.092$). However, if only the first of the 10 cultured plates were kept for each sample (the usual procedure in several studies), the number of positive cultures would have been eight (i.e. 7 and 1, from the HP and LP populations, respectively), and the difference between the populations would be significant ($P = 0.041$). The difference in the recovery of *C. difficile* between

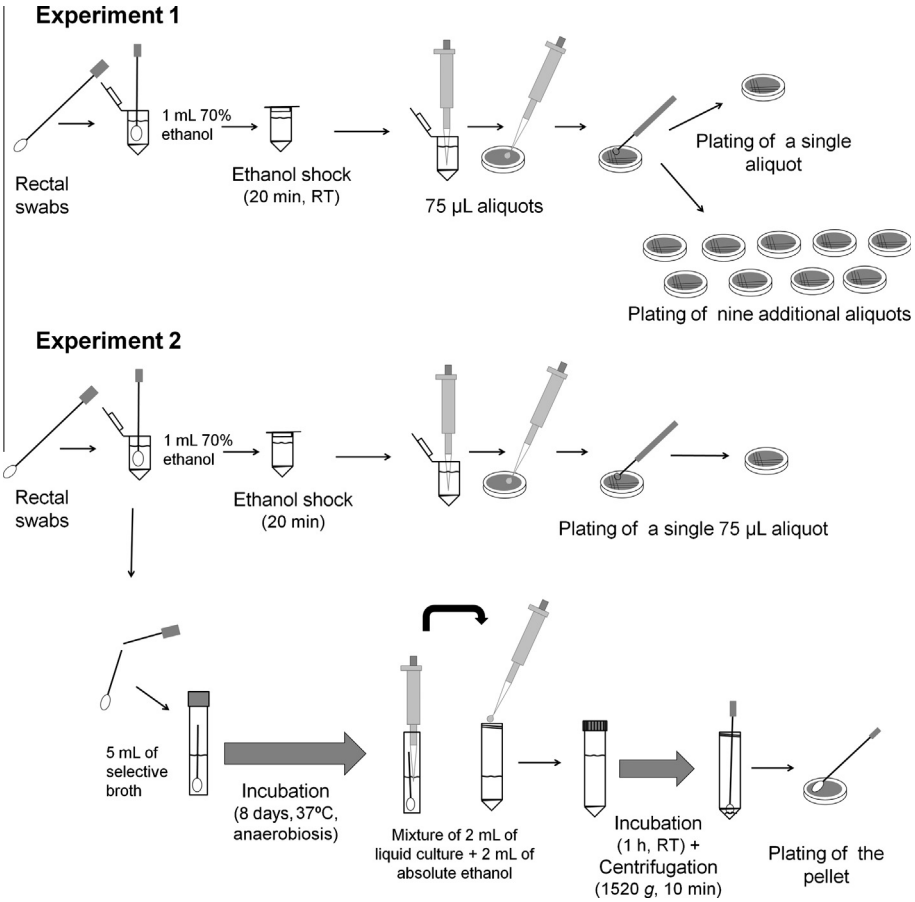

Fig. 1. Schematic representation of experiments 1 and 2. A detailed description of the procedures is given in the materials and methods. RT, room temperature.