Anaerobes in animal disease

A comparative study on the use of selective media for the enumeration of *Clostridium perfringens* in poultry faeces

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**A B S T R A C T**

Isolation and fast detection of *Clostridium perfringens* is essential in veterinary medical diagnostics and veterinary research, as it allows to recommend suitable treatment options after antimicrobial resistance determination, and is essential to study pathogenesis. In this study four selective media were tested for the enumeration of, and selectivity towards *C. perfringens* in faecal samples from poultry. The routinely used Columbia agar with 5% sheep (CBA), Shahidi-Ferguson-perfringens agar (SFP), tryptose sulphite cycloserine agar (TSC), and a novel chromogenic medium, CHROMagar™ *C. perfringens* (CHCP), were tested. Overall, no difference in *C. perfringens* recovery could be observed between the selective media. The limit of quantification was 10^3 CFU/mL for all agars. CHCP showed the highest specificity, especially when low *C. perfringens* loads were present in the faeces, with TSC being the second most specific selective medium. Both CBA and SFP allowed considerable growth of other faecal microbiota and were not specific for *C. perfringens*. On CHCP, differentiation of *C. perfringens* from other faecal bacteria was straightforward due to the appearance of *C. perfringens* as orange colonies, with other bacteria being absent or appearing as blue/green colonies. On TSC, *C. perfringens* appeared as black colonies, but longer incubation periods were sometimes needed for the black colour to develop. Therefore, CHCP can be recommended when timely and easy identification and enumeration of *C. perfringens* from complex samples, such as faeces, is needed.

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1. Introduction

In diagnostic bacteriology laboratories, a new generation of molecular tools has become available that partly replace traditional methods [1,2]. Despite these advances, isolation of bacterial strains is still indispensable for multiple purposes. First, isolation of causative bacterial strains is often needed in routine diagnostics for antimicrobial susceptibility testing and thus recommending treatment options for medical doctors and veterinarians. Secondly, bacterial isolates are essential to establish links between bacteria and diseases, as isolates are needed to perform infection trials and fulfill Koch’s postulates, to identify genomic elements that are crucial for disease, and to develop control methods, including antigen selection for vaccine development [3–6].

In veterinary medical diagnostics, one of the bacteria that is often encountered in intestinal and faecal samples is *Clostridium perfringens*. This is a Gram positive, non-motile, spore-forming, anaerobic rod-shaped bacterium that is commonly found in water, soil and the intestinal tract of healthy animals [7]. However, it is also one of the most common pathogens, causing a variety of important human and animal diseases, ranging from histotoxic to enteric infections [8,9]. In poultry, *C. perfringens* causes necrotic enteritis (NE), which is one of the most important enteric diseases of broilers. NE can also occur in layers and replacement pullets [10]. In order to isolate this bacterium from the faeces or intestinal digesta, a medium is needed that suppresses outgrowth of concomitant microbiota but permits growth of *C. perfringens* as easily differentiated colonies. Several selective media are being used for isolating *C. perfringens* from complex samples, but none of them seems to be ideal, because of either partial inhibition of *C. perfringens* or permission of growth of too
many other bacterial species, hampering isolation and quantification of C. perfringens [12]. In order to allow growth of C. perfringens in a readily recognizable form and thus improve the ease of identification of its colonies, various supplements are used. Most common is the use of sheep blood, egg yolk or sulphite in the agar plates. When grown on agar containing sheep blood, the haemolytic activity of C. perfringens causes a typical narrow zone of complete haemolysis inside a larger zone of partial haemolysis [13]. However, growth of other haemolytic bacteria (amongst others Bacillus sp., Enterococcus sp., some E. coli strains and Clostridium bifermentans) can impede the correct identification of C. perfringens on blood agar plates [14–17]. Supplementation of agar plates with egg yolk results in opaque zones around the C. perfringens colonies, which is due to its lecithinase activity [13]. However, Clostridium sordelli and C. bifermentans produce enzymes that are closely related to C. perfringens alpha toxin (lecithinase), and can therefore lead to false positives [18]. In addition to sheep blood and egg yolk, also sulphite is commonly used to aid in the isolation of C. perfringens. Sulphite has the additional advantage that it is inhibitory to many bacteria, while C. perfringens is not inhibited by this substance but reduces the sulphite to hydrogen sulphide, which leads to precipitation of iron sulphide, resulting in black colonies [19–21].

Recently, a novel chromogenic agar has been developed for selective isolation of C. perfringens (CHROMagar™ C. perfringens, CHCP) but this agar has not yet been benchmarked in scientific literature. The aim of the current study was to compare this novel chromogenic agar to three commonly used agars (CBA, SFP and TSC), to determine the recovery rate of and specificity towards C. perfringens, as well as the ease of differentiation between C. perfringens and contaminating bacteria present in complex samples.

2. Material and methods

2.1. Media preparation

Columbia blood agar (CBA) was prepared using Columbia blood agar base (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood, 12 mg/L kanamycin sulphate and 30 000 IU/L polymyxin B sulphate. Shahadi-Ferguson Perfringens (SFP) agar and tryptose sulphite cycloserine (TSC) agar were prepared using Perfringens Agar Base (Sigma-Aldrich) supplemented with either 5% sterile egg yolk emulsion (Oxoid, Basingstoke, UK), 12 mg/L kanamycin sulphate and 30 000 IU/L polymyxin B sulphate for SFP or 400 mg/L d-cycloserine for TSC. Sodium metabisulphite and ferric ammonium citrate are present in both media. CHROMagar™ C. perfringens (CHCP) was prepared according to the manufacturer’s instructions. Briefly, CHCP base was supplemented with CHCP supplement 1 to a final concentration of 2000 mg/L, as well as CHCP supplement 2 to a final concentration of 120 mg/L. Sterile supplements were added after sterilization of the respective media, after which they were dispersed in 120 × 120 mm petri dishes, air dried at room temperature and stored at 4°C for maximum 30 days.

2.2. Bacterial strains and culture conditions

A collection of 44 bacterial strains was used to assess the growth and colony appearance on CHCP (Table S1). This included 28 C. perfringens strains isolated from 7 different animal species, four Clostridoides difficile strains, one Bacillus subtilis, one Bacillus amyloliquefaciens, one Enterococcus gallinarum, one Entrococcus faecalis, four chicken isolates of Escherichia coli, one Lactobacillus crispatus, one Lactobacillus vaginalis and one Lactobacillus salivarius strain. Bacteria were routinely grown on Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated sheep blood and anaerobically incubated, except the Bacillus, Lactobacillus and E. coli strains that were both aerobically and anaerobically incubated. To assess the selectivity of CHCP towards C. perfringens, all strains listed in Table S1 were plated on CHCP and incubated overnight at 37°C under anaerobic conditions.

From this collection, two pathogenic C. perfringens type G strains isolated from necrotic enteritis lesions (CP56 and EHE-NE18) and two commensal C. perfringens type A strains isolated from healthy broilers (CP10 and CP20) were grown overnight in brain heart infusion broth (BHI; Sigma-Aldrich, Steinheim, Germany) at 37°C under anaerobic conditions. From each overnight culture, bacterial suspensions were prepared in sterile phosphate-buffered saline (PBS) to obtain 107, 105, 103 or 101 CFU/mL for use in the C perfringens isolation experiments using spiked chicken faeces. The number of C. perfringens in the final suspensions was determined by plating 10-fold serial dilutions and colony counting.

2.3. Evaluation of the selective media for isolation and quantification of C. perfringens from chicken faeces

Four selective media (CBA, TSC, SFP and CHCP) were compared for their ability to isolate and quantify a known concentration of C. perfringens present in chicken faeces. Therefore, faecal samples were collected from healthy chickens housed at the Faculty of Veterinary Medicine, Ghent University, Belgium. To account for differences in microbial composition between individual birds, the experiment was performed on six different days, each time using a fresh faecal sample from a healthy chicken (one chicken per day, six different birds in total). Furthermore, to account for differences in the ability of C. perfringens strains to outgrow the residing microbiota, each faecal sample was spiked with four different C. perfringens strains (CP10, CP20, CP56 or EHE-NE18). More specifically, each faecal sample was collected in a sterile tube and subsequently divided into 200 mg aliquots (17 aliquots per sample), which were spiked with respectively 1800 μL of the C. perfringens strain dilutions expected to be 107, 105, 103 or 101 CFU/mL, as described above. As a negative control, faeces were spiked with sterile PBS only, to determine the level of C. perfringens present in the collected faeces. In two faecal samples, no C. perfringens could be detected in the unspiked aliquots, whereas in the other four faecal samples, some C. perfringens colonies could be observed (2–12 counted colonies in 6 × 20 μL droplets, not expected to have major influence on the results of the spiking experiments). After homogenization of the C. perfringens-spiked faecal aliquots by vortex mixing, C. perfringens was enumerated by plating 10-fold serial dilutions and colony counting. Therefore, 10-fold dilutions of the faeces were prepared by diluting 20 μL of each sample with 180 μL PBS. For each dilution, six droplets of 20 μL were pipetted on the different selective media. The plates were air-dried and incubated overnight at 37°C under anaerobic conditions. Plates containing between 30 and 300 colonies in 6 × 20 μL droplets were counted and numbers of CFU/g faeces were calculated. All plates with droplets consisting of more than 300 colonies were determined as uncountable. For each growth medium the recovery of C. perfringens from the spiked faeces was calculated relative to the growth of the starting inoculum on the same growth medium. Furthermore, to assess the specificity of the four tested media, both C. perfringens colonies as well as the total amount of colonies (contaminant microbiota at the countable dilution (30-300 colonies)) were recorded.

2.4. Identification of bacterial isolates using MALDI-TOF MS

From each of the selective media, at least 4 different colonies visually identified as C. perfringens, as well as a selection of
3.2. Identification of C. perfringens in faecal samples from all tested media

Reliable and preferably easy identification of C. perfringens in samples with a complex microbial composition is important to avoid unnecessary additional purification and identification steps. Therefore, the visual appearance of C. perfringens, as well as the concomitant microbiota on the different plates was assessed. On CBA, C. perfringens can be recognized due the typical double haemolytic zone around the colonies. However, also growth of non-haemolytic colonies was observed, which were identified as Enterococcus faecium and Enterococcus hirae, whereas haemolytic colonies were confirmed to be C. perfringens (Fig. 1(e) and (f)). On both TSC and SFP, C. perfringens colonies appear black due to reduction of the sulphite incorporated in the medium. On TSC, white colonies with black spots were confirmed to be C. perfringens, whereas growth of other faecal microbiota could be observed as translucent or white colonies, which both failed to be identified via MALDI-TOF, since the organism did not seem to be included in the database (Fig. 1(c) and (d)). However, it was observed that after an additional 24 h of incubation, more colonies developed black spots, which were confirmed to be C. perfringens as well. On SFP, C. perfringens was identified as white colonies with black spot, surrounded by an opaque halo which is due to its lecinthinase activity on the egg yolk incorporated in the medium. Other faecal microbiota was present as white colonies without an opaque zone, some of which were identified as Enterococcus faecalis (Fig. 1(a) and (b)). On CHCP, C. perfringens produced orange colonies (Fig. 1(g) and (h)). On some plates, blue/green colonies could be observed which were identified as Clostridium disporicum (Fig. 1(i)).

3.3. C. perfringens recovery rates from faecal samples are similar on CBA, TSC, SFP and CHCP

To determine the limit of detection and limit of quantification of C. perfringens from samples containing complex microbial mixtures, fresh chicken faeces were spiked with known concentrations of C. perfringens, ranging from $10^1$ to $10^7$ CFU/mL, after which the recovery rate was determined on each selective medium (Fig. 2, Table S2). Although C. perfringens could be detected on all selective media, for all tested inoculum levels, reliable quantification was only possible at an inoculum level of at least $10^3$ CFU/mL. At the lowest tested inoculum level ($10^1$ CFU/mL) high variability of recovery was observed for all four tested selective media, with recovery levels ranging from 0% (false negatives) to 270% in one replicate. Furthermore, the recovery rate from samples containing low C. perfringens load ($10^1$ CFU/mL inoculum) was clearly different from all other inoculum levels for all tested selective media (P-values ranging from P = 0.16 to P = 0.0092, Table 1). However, at inoculation levels where C. perfringens could be accurately enumerated (above the limit of reliable quantification; $>10^3$ CFU/mL) no difference in C. perfringens recovery between the different inoculum levels could be observed, on all selective media (Table 1).

Furthermore, no significant differences in C. perfringens recovery were detected between the media at the inoculum levels of $10^7$, $10^5$ and $10^3$ CFU/mL, whereas on CHCP recovery was significantly lower at the inoculum level of $10^3$ CFU/mL as compared to SFP (P = 0.0132, Tables 1 and 2).

3.4. CHCP shows significantly higher specificity for isolation of C. perfringens from faecal samples compared to the other media

For accurate enumeration of C. perfringens from samples with mixed faecal microbiota, not only the recovery rate but also the specificity of the selective media is important. In order to objectively assess the specificity of the four tested selective media, the number of C. perfringens counts relative to the total colony count (including contaminant microflora) was recorded (Fig. 3).

At highest inoculation levels ($10^7$ CFU/mL) C. perfringens outgrew other contaminants on all tested media, with highest specificity observed on CHCP and TSC, which performed equally well (P = 0.83) and significantly better than CBA and SFP (Table 2). At lower C. perfringens inoculation levels, increased growth of contaminants was observed on CBA, TSC and SFP, but not on CHCP, which showed significantly higher specificity for C. perfringens (still 95.05% specificity at the lowest C. perfringens inoculation level ($10^5$ CFU/mL, Fig. 3(d)). TSC has good specificity at both $10^2$ and $10^4$ CFU/mL inoculum (respectively 95.05% and 85.55% C. perfringens specificity), but growth of contaminants significantly increased when lower C. perfringens levels were present in the faeces (P < 0.0001, Table 1,
No difference in specificity towards \textit{C. perfringens} could be observed between CBA and SFP medium, with acceptable background growth only when high \textit{C. perfringens} load was present in the faeces (>80% background at 10^5 CFU/mL inoculum) (Fig. 3(a) and (c)).

4. Discussion

Isolation and fast detection of \textit{C. perfringens} is essential in veterinary medical diagnostics and veterinary research, as it allows to choose antimicrobial therapies, either or not after antimicrobial resistance determination. The isolation of \textit{C. perfringens} from complex microbiota as a pure culture can, however, be challenging, as to date, no selective culture medium is described that allows maximal recovery of \textit{C. perfringens} while preventing all growth of other bacterial types present in the sample.

In this study, the use of a novel chromogenic selective medium (CHROMagar™ \textit{C. perfringens}) was described. As the inclusion of antibiotics in the selective media used to date can also hamper the ability of certain \textit{C. perfringens} strains to grow on the plates [12], the recovery of pure cultures of \textit{C. perfringens} on CHCP was assessed. All tested \textit{C. perfringens} strains were easily recovered from the plates, indicating that the supplements used to suppress the growth of other bacteria had no influence on the viability of \textit{C. perfringens}. Furthermore, all 28 \textit{C. perfringens} strains appeared as orange colonies, while all the strains that were not \textit{C. perfringens}, appeared as blue/green colonies. In fact, all \textit{Bacillus}, \textit{Lactobacillus} and \textit{E. coli} strains that have the ability to grow on CBA, did not grow on CHCP plates at all, which underlines the selectivity of this novel medium.

In a second part of the study, CHCP was compared to three traditionally used selective media (CBA, TSC and SFP) in its ability to isolate and enumerate a known concentration of \textit{C. perfringens} cells from spiked chicken faeces. Overall, no difference in \textit{C. perfringens} recovery could be observed between the different media. However, at the inoculation level of 10^5 CFU/mL, CHCP had significantly lower recovery than SFP. Nevertheless, this difference in recovery was not observed when both higher or lower \textit{C. perfringens} levels were present in the faeces, suggesting that all media performed equally well. This is in accordance with a study conducted by Harmon et al. [24], where similar \textit{C. perfringens} recovery of pure cultures of \textit{C. perfringens} on SFP and TSC was observed. However, some of the
**Table 1**

P-values for the comparison of different inoculum levels for *C. perfringens* recovery from faeces (CP recovery) and *C. perfringens* counts relative to total colony count (CP specificity). P-values were assessed for each selective medium.

<table>
<thead>
<tr>
<th>CFU inoculum</th>
<th>CBA</th>
<th>TSC</th>
<th>SFP</th>
<th>CHCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CP recovery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CP specificity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CP recovery</td>
<td>CP specificity</td>
</tr>
<tr>
<td>10&lt;sup&gt;-1&lt;/sup&gt;–10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.192&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.0975</td>
<td>0.0628</td>
<td>0.9645</td>
</tr>
<tr>
<td>10&lt;sup&gt;-1&lt;/sup&gt;–10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.0272&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.0088&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.0865</td>
<td>0.0001&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-1&lt;/sup&gt;–10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.0411&lt;sup&gt;**&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.1558</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-1&lt;/sup&gt;–10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.9991</td>
<td>0.6988</td>
<td>0.9991</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-1&lt;/sup&gt;–10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.9094</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.9762</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P-values of the comparison of *C. perfringens* recovery rates from faeces between different inoculum levels.

<sup>b</sup>P-values of the comparison of the difference of *C. perfringens* counts relative to total colony count between different inoculum levels (Colonies were counted at the same dilution that was used to enumerate *C. perfringens* (30-300 colonies in 6 × 20 μl droplets)).

<sup>*</sup>P < 0.05, <sup>**</sup>P < 0.01, <sup>***</sup>P < 0.001.

**Table 2**

P-values for the comparison of different media for *C. perfringens* recovery from faeces (CP recovery) and *C. perfringens* counts relative to total colony count (CP specificity). P-values were assessed for each inoculum level.

<table>
<thead>
<tr>
<th>Compared media</th>
<th>CFU inoculum</th>
<th>CBA</th>
<th>TSC</th>
<th>SFP</th>
<th>CHCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CP recovery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CP specificity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CP recovery</td>
<td>CP specificity</td>
<td>CP recovery</td>
</tr>
<tr>
<td>10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.9778</td>
<td>0.0071**</td>
<td>0.0771</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.9746</td>
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<tr>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.9262</td>
<td>0.9995</td>
<td>0.8917</td>
<td>0.6984</td>
<td>0.9199</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.9999</td>
<td>0.0488&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.9651</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.9914</td>
</tr>
<tr>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.9965</td>
<td>0.0093&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.0122&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.7142</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.9678</td>
<td>0.8316</td>
<td>0.1982</td>
<td>0.0246&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.9992</td>
</tr>
<tr>
<td>SFP - TSC</td>
<td>0.9068</td>
<td>0.062</td>
<td>0.6388</td>
<td>&lt;0.001&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.7888</td>
</tr>
</tbody>
</table>

<sup>a</sup>P-values of the comparison of *C. perfringens* recovery rates from faeces between media.

<sup>b</sup>P-values of the comparison of the difference of *C. perfringens* counts relative to total colony count between media (Colonies were counted at the same dilution that was used to enumerate *C. perfringens* (30-300 colonies in 6 × 20 μl droplets)).

<sup>*</sup>P < 0.05, <sup>**</sup>P < 0.01, <sup>***</sup>P < 0.001.
of non-\textit{C. perfringens} colonies. This is in line with previous reports, describing that SFP medium was not sufficiently selective to limit the growth of facultative anaerobes such as enterococci [26], which were also part of the contaminant microbiota on SFP in this work. Although not specific, the visual identification of \textit{C. perfringens} on SFP is quite clear due to the opaque halo and black colour of colonies on the medium. On CBA, \textit{C. perfringens} grows as haemolytic colonies, but, conclusive identification can be more challenging than on SFP. Indeed, it occurred that the haemolytic zone around one colony was so large that other, non-\textit{C. perfringens} colonies fell into this zone and could therefore lead to false positives. Moreover, although not identified in this study, other haemolytic bacteria such as \textit{Bacillus sp.}, \textit{Enterococcus sp.}, and some \textit{E. coli} strains could also give false positive results [14,15].

In conclusion, all tested selective media (CBA, TSC, SFP and the novel CHCP) had a limit of quantification at the inoculation level of 10^7 CFU/mL. However, CHCP outperformed the other media when it came to specificity, especially when low \textit{C. perfringens} loads were present in the sample. Therefore, CHROMagar™ for \textit{C. perfringens} can be recommended for easy and fast detection of \textit{C. perfringens} in samples containing complex bacterial mixtures, such as faeces.

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\section*{Declaration of competing interest}

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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\section*{Appendix A. Supplementary data}

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2020.102205.

\section*{References}


