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Real-time PCR detection of *Campylobacter* spp.: A comparison to classic culturing and enrichment

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ABSTRACT

The major disadvantage of the current gold standard for detection of the food pathogen *Campylobacter*, *i.e.* culturing, is the lengthy procedure. In this study we assessed the use of real-time PCR for detection of *Campylobacter*. To this end, 926 poultry samples, taken from transport containers and broiler caeca in The Netherlands in 2007, were subjected to three different real-time PCR detection methods: one targeting the *Campylobacter jejuni hipO* gene, one targeting the *Campylobacter coli glyA* gene, and one generically targeting *Campylobacter* spp. 16S rDNA sequence. The PCR results from the three different PCR protocols were compared to the work of Nauta et al. (2009) who analyzed the same set of samples collected from 62 broiler flocks by means of enrichment culturing.

The results indicate that the generic 16S campylobacter PCR detection is equally reliable but much faster (4 h instead of ≥2 days) than detection by means of culturing. Moreover, PCR detection targeting the *hip*O and the *gly*A gene provide the possibility of *C. jejuni* and *C. coli* species discrimination. The generic *Campylobacter* spp. PCR analysis also confirmed the high incidence of *Campylobacter* spp. in poultry samples (~90%) and the species specific PCR showed the simultaneous presence of *C. jejuni* and *C. coli* in ~24% of the samples. Furthermore, the results from the three PCR analyses suggested the occurrence of alternative *Campylobacter* species in almost 10% of the samples. The campylobacter PCR detection methods reported here can replace traditional culturing because of being quicker and more reliable.

1. Introduction

Campylobacter spp., especially *Campylobacter jejuni*, are the most common cause of bacterial gastro-enteritis in the European Union (EU), including The Netherlands. The incidence of campylobacteriosis in 24 EU member countries is over 51.6 reported cases per 100,000 inhabitants in 2005 (EFSA, 2006). In The Netherlands, approximately 6200 laboratory-confirmed cases of campylobacteriosis caused by *C. jejuni* were reported in 2004 (Janssen et al., 2006). Since most patients undergoing campylobacteriosis recover without consulting their physician, the actual number of campylobacteriosis is much higher, being approximately 59,000 cases per year (Janssen et al., 2006). Occasionally, campylobacter infection is also associated with rare post-infection symptoms such as reactive arthritis and Guillain-Barré syndrome. In a small number of cases Campylobacter may contribute among other factors to death of the patient (Kemmeren et al., 2005).

Campylobacter spp. are frequently found as commensals in many animal hosts, ranging from mammals to birds, including many farm animals such as cattle, pigs, sheep, goats, and poultry such as broilers and turkey (Evans, 1992; Humphrey et al., 2007; Petersen et al., 2001; Shane, 1992). In these natural hosts, especially in poultry, campylobacter occurs in the intestinal tract, often in large numbers (Mead et al., 1995). Due to the efficient fecal-oral infection route, once a campylobacter infection reaches a broiler flock, the whole flock is usually infected within several days (Jacobs-Reitsma et al., 1995; van Gerwe et al., 2009). During the slaughter process, contamination of the carcass can occur, leading to contaminated poultry products at the retail level. These contaminated poultry products are considered as the major cause of human campylobacteriosis (Humphrey et al., 2007; Wilson et al., 2008).

In order to prevent cross-contamination of campylobacter-free broiler flocks by infected broiler flocks at the slaughterhouse, logistic measures have been implemented, with slaughter of campylobacter-negative flocks planned before slaughter of flocks positive for campylobacter (Nauta and Havelaar, 2008). Such requires

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a rapid campylobacter detection test. The current culture based detection method of campylobacter (ISO-10272), being the gold standard of detection takes at least two days. In this way, campylobacter testing prior to transport of the flocks is not informative as infection may occur in the time between sampling and obtaining the result (Jacobs-Reitsma and Bolder, 1998). Therefore, several alternatives have been developed, such as PCR methods and immunoassays such as ELISA and LFA (Nauta et al., 2009), as well as real-time PCR detection methods (LaGier et al., 2004; Lund et al., 2004).

In the current study we have developed, adapted and improved three different real-time PCR detection methods for campylobacter. One real-time PCR detection method was based on 16S rDNA sequences of *Campylobacter* spp. as developed previously by Lund et al. (2004). The two other real-time PCR detection methods are targeting *C. jejuni* (newly developed primer probe set, targeting the *hipO* gene) and *Campylobacter coli* (adapted from LaGier et al., 2004). The unique aspect of our approach is the large set of poultry samples (n = 926), which has previously been described and tested for presence of campylobacter by culturing methods (Nauta et al., 2009) and subsequently used to assess the reliability of three different PCR detection methods for *Campylobacter* spp. These poultry samples as described by Nauta et al. (2009) originated from a sampling of 62 flocks in the autumn of 2007 in The Netherlands. The results show that the PCR detection method targeting the 16S rDNA sequence is at least as reliable as the gold standard: classic culturing with or without enrichment prior to culturing. Similarly, the combination of the *C. jejuni* and *C. coli* targeting PCR methods perform well and allow an insight into the distribution of these most prevalent *Campylobacter* species in poultry birds. Moreover, the automated DNA extraction procedure in combination with the fast delivery time makes the real-time PCR methods promising for fast detection of campylobacter within broiler flocks.

2. Materials and methods

2.1. Sampling of broiler flocks

Samples were collected by quality control officers at two broiler processing plants one day a week during a three month period in the autumn of 2007 as described by Nauta et al. (2009). For each of the 62 broiler flocks five samples of feces from transportation containers and ten samples of cecal contents were obtained. Each of the five fecal samples was a 25 g pool of feces taken at three levels of the container with a volume of 1 m^3 and 4 or 5 shelves to accommodate 30 broilers for transportation (Tinker et al., 2005). These samples were taken by swabbing the floors with a sterile plastic bag reverted over the hand. Caeca were collected from the birds individually after evisceration. Fecal and cecal material was frozen at −80 °C after testing material by culturing techniques. Subsequently, these samples were transported in frozen state for molecular analysis by real-time PCR. Prior to DNA isolation for PCR, the frozen samples were stored for a maximum period of 4 month at −80 °C.

2.2. DNA isolation

DNA for real-time PCR analysis was extracted from 0.1 g of the different caeca and fecal samples simultaneously in 96-well 1 ml microtiter plates (Axygen, USA) containing 0.3 ml zirconium-silica beads (0.1 mm bead size, Biospec Products, USA) and 0.25 ml of lysis buffer (AGOWA mag Mini DNA Isolation Kit, AGOWA, Germany). $CaCl_2$ was added to a final concentration of 5 mM. Next, 0.4 ml of phenol (Sigma–Aldrich, USA) was added and the samples were homogenized with a Mini beadbeater-96 (Biospec Products, USA) for 2 min and centrifuged (10 min, 3,900 g). The aqueous phase containing the DNA was subsequently purified with the AGOWA DNA Isolation Kit (AGOWA, Germany) according to the manufacturer's instructions. DNA was eluted in 63 μl AGOWA EB-buffer (AGOWA, Germany). For optimal efficiency and reduced handling time, the DNA isolation was automated. To this end, a Perkin Elmer automated workstation (JANUS) was used in combination with the Agowa sep 9600 magnetic particle manipulator, enabling the simultaneous DNA isolation of 2 × 96 samples and subsequent quantitative PCRs in a total of 4 h with only 1.5–2 h handling time. Cross contamination as a consequence of simultaneous DNA extraction in 96 well format was excluded by checking with pure culture processing in combination with negative controls in which target bacteria are absent (results not shown).

2.3. Real-time PCR

Three different real-time PCR protocols were applied. For detection of *C. jejuni*, primers and the corresponding probe were newly designed targetting the *hipO* gene of *C. jejuni* (Table 1). For detection of *C. coli*, the quantitative PCR protocol of LaGier et al. (LaGier et al., 2004) was adapted to the fast real-time PCR method by minor adaptation (omitting two 'G's at the 3′-end) of the forward primer (Table 1). Finally, for detection of *Campylobacter* spp. (*e.g. C. jejuni*, *C. coli*, *C. lari*, and *C. hyointestinalis*) a genus specific 16S rRNA encoding DNA region is targeted. To this purpose the method of Lund et al. (Lund et al., 2004) was adapted to the fast real-time PCR method. To this end, the forward primer was elongated with three bases, a new reverse primer was designed (Table 1), and the TaqMan probe was redesigned to contain the minor groove binding (MGB) quencher dye (Table 1).

Real-time PCR was performed on an Applied Biosystems 7500 thermal cycler, using the TaqMan® Fast Universal PCR Master Mix. For the real-time PCR, 5 μl DNA, 10 μl TaqMan® Fast Universal PCR Master Mix, 1 μl (10 pmol) of forward and reverse primers (Table 1), 1 μl (5 pmol) TaqMan probe (Table 1) were mixed, and 2 μl DNase-free water was added to a final volume of 20 μl. The cycling conditions consisted of 3 min at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Real-time data were analyzed with Applied

Table 1
Nucleotide sequences of the primers used for real time detection of *Campylobacter*. Indicated in bold are the 5′- and 3′-reporter dyes of the different probes.

Name	Sequence (5′ → 3′)	Target
Cj-F2	ATGAAGCTGTGGATTTTGCTAGTG	*hipO* gene
Cj-R3	AAATCCAAAATCCTCACTTGCCA	*hipO* gene
Cj probe	**FAM**-TTGTGAATTTAATCATCGTCC-**MGB**	*hipO* gene
Ccoli-F2	CATATTGTAAAACCAAAGCTTATC	*glyA* gene
Ccoli-R	AGTCCAGCAATGTGTGCAATG	*glyA* gene
Ccoli probe	**VIC**-TAAGCTCCAACTTCATCCGCAATCTCTCTAAATTT-**TAMRA**	*glyA* gene
16S-CampyF1	CACGTGCTACAATGGCATATACAA	16S rDNA *Campylobacter*
16S-CampyR1	CCGAACTGGGACATATTTTATAGATTT	16S rDNA *Campylobacter*
16S-CampyP1	**FAM**-AGACGCAATACCGTGAGGT-**MGB**	16S rDNA *Campylobacter*

Biosystems 7500 software (version 1.4). Upon completion of the run, a cycle threshold (Ct) was calculated and plotted against the log input DNA to provide standard curves for the quantification of unknown samples.

3. Results

3.1. Detection of Campylobacter spp., C. jejuni and C. coli by real-time PCR

A total of 926 samples derived from 62 broiler flocks, consisting of 308 container samples and 618 caeca samples, were tested for the presence of *C. jejuni* using our *C. jejuni*-specific PCR. A positive result, indicating the presence of *C. jejuni* was obtained for 178 out of the 308 container samples and for 260 out of the 618 caeca samples, totalling 438 positive samples out of the 926 samples (Table 2).

A similar analysis using our *C. coli* specific PCR resulted in positive results for 178 out of 308 container samples and 257 out of 618 caeca samples, totalling 435 positive samples out of the 926 total samples, almost exactly the same number as found for *C. jejuni*. Combining the results of the *C. jejuni* and *C. coli* specific PCR resulted in 246/308 container samples testing positive for *C. jejuni* and/or *C. coli* as well as 406/618 caeca samples (Table 2). *C. jejuni* and *C. coli* spike experiments using campylobacter-negative fecal samples demonstrated the detection limit of both PCR protocols being between 10^2 and 10^3 cells per gram feces (data not shown).

When applying the generic PCR detection method for *Campylobacter* spp. the presence of *Campylobacter* spp. was detected in 692 out of the total 926 samples (269/308 container samples and in 423/618 caeca samples, Table 2). This indicated that the generic 16S PCR detection is slightly more sensitive than the *C. jejuni* and *C. coli* specific PCRs combined since it tested positive for 40 more samples than the *C. jejuni* and *C. coli* specific PCRs combined (Table 2).

From the 308 container samples, 23 tested negative for all three PCR methods. Likewise, 137 caeca samples tested negative for the three PCR methods, suggesting the absence of campylobacter in 160 of the total 926 samples or at least present under the detection limit of 10^2–10^3 cells/g of sample.

3.2. Detailed comparison between the campylobacter PCR detection methods

A detailed comparison between the results obtained with the three campylobacter PCR detection protocols showed both overlaps and differences for the container and caecal samples (Table 2/Fig. 1). Even though the 16S PCR tested positive for a total of 40 more samples than the combined results of the *C. coli* and *C. jejuni* PCR (Fig. 1, Table 2), the number of samples uniquely testing positive in the 16S PCR is much higher at 114 (39 in the container samples and 75 in the caeca samples (Fig. 1, Table 2).

The *C. jejuni* specific PCR detected only seven positive samples (three in the container samples and four in the caeca samples) that were not detected by the 16S PCR (Fig. 1 and Table 2), indicating that the 16S PCR is a robust method for detection of *C. jejuni*. In contrast, the *C. coli* specific PCR uniquely detected a total of 70 (15 in the container samples and 55 in the caeca samples) samples, suggesting that the 16S PCR is less robust for detection of *C. coli* than for *C. jejuni*. In addition, three samples were positive for both the *C. jejuni* and *C. coli* PCR, but negative for the 16S PCR.

A striking result from the combined analysis of the PCR detection results is the simultaneous presence of *C. jejuni* and *C. coli* in over one-third (110/308) of the container samples and in over one-sixth (111/618) in the caecal samples (Table 2, Fig. 1). In fact, the

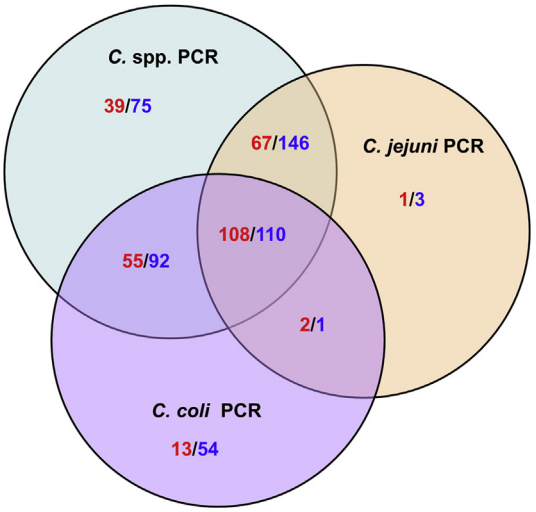

Fig. 1. Venn-diagram summarizing the results of the three PCR detection methods. The red numbers represent the number of positive samples in the container samples, whereas the dark blue numbers represent the number of positive samples in the caeca samples.

Table 2
Summary of all detection results.

	Container	Caeca	Total
Total samples	**308**	**618**	**926**
16S positive	269	423	692
C. jejuni positive	178	260	438
C. coli positive	178	257	435
PCR positive (3 PCRs combined)	285	481	766
Culture positive after enrichment	239	367	606
C. jejuni and *C. coli* positive	110	111	221
C. jejuni and/or *C. coli* positive	246	406	652
16S and/or *C. jejuni* and/or *C. coli* positive	285	481	766
Positive in 16S but not in *C. jejuni* or *C. coli* specific PCR	39	75	114
Positive in *C. coli* specific PCR but not in 16S PCR	15	55	70
Positive in *C. jejuni* specific PCR but not in 16S PCR	3	4	7
Culture positive/PCR positive	292	529	821
Culture positive/PCR negative	7	48	55
Culture negative/PCR positive	53	162	215
Negative in all three PCRs	23	137	160
Negative for all detection methods	16	89	105

number of container samples containing only *C. jejuni* (68, Fig. 1) is smaller than the number of container samples containing both *C. jejuni* and *C. coli* (110, Table 2, Fig. 1). The same is true for *C. coli*, with the number of container samples containing only *C. coli* (68, Fig. 1) being smaller than the number of container samples containing both species (110, Table 2, Fig. 1).

3.3. Comparison of campylobacter detection by culturing and real-time PCR

When comparing the PCR detection results of all samples (n = 926) to the culturing results, the detection power of real-time PCR is shown by a total of 215 samples scoring positive by at least one PCR method whereas they are negative in culturing (Table 2, Fig. 2). In contrast, culturing only scored 55 samples positive that were negative in all three PCR detection methods (Table 2, Fig. 2). Of the 926 samples, a total of 105 samples scored negative for all detection methods (16 in the container samples and 89 in the caeca samples, Table 2), showing an incidence of campylobacter in 88.7% (821/926*100%) of the tested samples, taken during the autumn of 2007 in The Netherlands.

4. Discussion

In this study we have adapted and developed three real-time PCRs for detection of campylobacter. One PCR was a more generic PCR targeting the 16S rDNA region of at least the four thermotolerant *Campylobacter* species *C. jejuni*, *C. coli*, *C. upsaliensis*, and *C. lari*, whereas the two other PCRs were species-specific for *C. jejuni* or *C. coli*, detecting the presence of the *hipO* gene or the *glyA* gene respectively. A thorough comparison of these three PCR approaches for the use of campylobacter detection in poultry samples was done based on the same set of samples previously described in the study by Nauta et al. (Nauta et al., 2009). This offers a unique opportunity to compare our real-time PCR detection methods to the current gold standard (*i.e.* culturing, including enrichment) for detection of campylobacter in a large number of samples from the actual food chain. Based on the results, it is concluded that PCR detection of campylobacter is a reliable and much faster alternative for culturing.

A clear advantage of the PCR methods is their speed and limited handling time due to the deployment of robotics for high-throughput DNA isolation. In comparison to culturing, including enrichment, PCR results are available within 4 h, much faster than the minimally required 72 h for enrichment and culturing. Besides speed, robustness is an obvious requirement for a detection method.

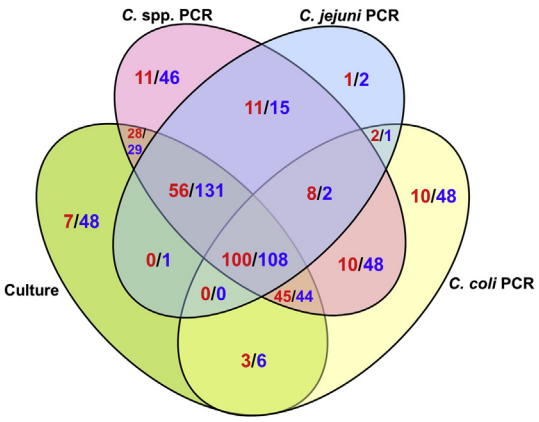

Fig. 2. Venn-diagram summarizing the results of the culturing and the three PCR detection methods. The red numbers represent the number of positive samples in the container samples, whereas the dark blue numbers represent the number of positive samples in the caeca samples.

Both culturing and the PCR detection methods score unique positive samples, although the PCR approach has the edge here as it detects almost four times more unique positive samples than culturing (55 samples are uniquely detected by culturing, whereas PCR detected 215 unique samples, Table 2). Sixty percent of the samples uniquely detected by culturing (33/55) were only detected after enrichment (which theoretically allows detection of 1 cfu/sample), suggesting that the numbers of campylobacters in the original samples were too low for detection by PCR with its detection limit of 10^2 to 10^3 cells per gram feces. Although enrichment prior to PCR detection will most likely turn these false-negatives into positives, the extra time required does not warrant this approach.

The 22 remaining samples uniquely detected by culturing however, contained campylobacters in numbers that should be easily detected by PCR (data not shown). The exact reason why these samples were negative in the PCR detection is unclear, but inefficient DNA extraction and the remains of PCR inhibitors could explain these results. Introduction of internal controls for DNA extraction and PCR amplification could solve these false-negatives. Another, explanation for the lack of PCR detection could be the incorrect scoring of colonies as campylobacters, thus creating false-positives in the culturing results. This is more difficult to solve, but suspected colonies could be checked by PCR or another identification method.

Samples uniquely positive for campylobacter by PCR could be due to various reasons: the campylobacters could be dead or in a non-culturable state, undetectable in culturing due to other organisms overgrowing the campylobacters, or the PCR could produce false-positive results. PCR detection is capable of detecting DNA of dead or unculturable bacteria. One might argue that dead campylobacters of which the DNA is detected by PCR, are not capable of causing disease and should therefore be considered as false-positive samples. However, any sample detecting live or dead bacteria is indicative for the presence of a once or still viable population of campylobacters. Therefore, even detection of dead bacteria is indicative of campylobacter presence in animals or the slaughter line and thus cause for concern. Moreover, viable but non-culturable (VNBC) campylobacters (Jackson et al., 2009) would be scored as campylobacter-negative by culturing even though under suitable conditions, *e.g.* the human intestinal system, they might revive from their VNBC state and cause disease. PCR detection on the other hand would detect VNBC campylobacters.

False-positives in the PCR detection could theoretically explain (part of) the higher sensitivity of PCR detection. Based on previous work however, the incidence of false-positives due to cross-contamination is very low (<1%, results not shown). Moreover, known campylobacter-free caecum material tested negative by culturing techniques as well as by PCR detection (data not shown), supporting evidence for the low risk of false positive signals using PCR.

In this discussion, PCR detection has been dealt with as one approach, whereas three PCRs have been described in this study. The generic 16S PCR is the method that detects the most campylobacters on its own (692/926) and is therefore advised if only one method is to be employed. However, addition of the two species-specific PCRs is beneficial since it results in more positive samples (766/926). This means that the generic 16S PCR misses 74 samples that are additionally detected by using the C. jejuni/C. coli real-time PCR. Looking in more detail, the phenomenon does coincide with low detection levels in the species specific PCR. Such implies that the generic 16S PCR is less sensitive than the dedicated

species specific PCRs despite the fact that the primer probe combination is 100% complementary to the *C. coli* and *C. jejuni* target sequences.

Another advantage of the use of the two species-specific PCRs is the identification of the two most common causes of human campylobacteriosis in poultry samples (Blaser, 1997; Meinersmann et al., 2002). In this study, a total of 221 samples tested positive for the simultaneous presence of *C. jejuni* and *C. coli*. This is a surprisingly high incidence, because it is generally assumed that in poultry samples *C. jejuni* is far more prevalent than *C. coli* (Deckert et al., 2010; Moran et al., 2009; Pepe et al., 2009), although other studies also suggest *C. coli* to be more abundant than generally assumed (Schnider et al., 2010).

Another striking observation when the three PCR detection methods are compared is the high presence of 'other' *Campylobacter* species. Since neither culturing nor the 16S PCR distinguishes between the thermotolerant *Campylobacter* species, the 166 samples testing uniquely positive in culturing and/or the 16S PCR detection (Fig. 2) are suggestive to contain *Campylobacter* species other than *C. jejuni* or *C. coli.* The generic 16S PCR detects also *Campylobacter* spp. other than *C. jejuni* and *C. coli*, a.o. *C. lari*, *C. upsaliensis*, *C. sputorum*, *C. gracilis*, *C. curvus*, and *C. mucosalis* (data not shown). Even when considering that a number of samples have been missed by the *C. jejuni* and *C. coli* PCR (*e.g.* the same number of samples that were not detected by the 16S PCR whereas they were detected by at least one of the species specific PCRs, *i.e.* 73, Fig. 2), this would still suggest the presence of *Campylobacter* spp. other than *C. jejuni* or *C. coli* in approximately 10% ((166−73)/926*100%) of all samples. It is beyond the scope of this paper to look into more detail what *Campylobacter* species are present in these samples but it certainly warrants further research.

In total, 105 (11.3%) samples were negative by both culturing and PCR detection (Table 2). This means that in the other 88.7% of the samples campylobacter has been detected by at least one method. This study therefore confirms the findings of Nauta et al. (Nauta et al., 2009).

In comparison to the other two fast detection methods described in (Nauta et al., 2009), our PCR approach offers far improved sensitivity over LFA and ELISA. LFA only tested positive for 138/304 = 45.4% of the tested container samples (Nauta et al., 2009), whereas ELISA tested positive for 205/297 = 69.0% of the tested container samples (Nauta et al., 2009). In contrast, the 16S PCR tested positive for 269/308 = 87.3% of the container samples in this study (Table 2). Clearly, our PCR approach has the advantage of being fast and reliable and has a higher sensitivity than the other fast detection methods such as LFA and ELISA.

In conclusion, by comparing real-time PCR as a method for detection of *Campylobacter* spp. to culturing, we have shown that real-time PCR is a fast and reliable alternative for culturing. If only one PCR method is to be implemented in daily routine, the generic 16S PCR is most suitable since it covers a wide range of *Campylobacter* species. If multiple PCRs fit with the daily routine, the species specific PCRs add to the rate of detection and identification of the most dominant *Campylobacter* species causing human campylobacteriosis. Implementation of the generic PCR detection as described in this manuscript could enable logistic slaughtering where it is first determined which poultry flocks are negative for campylobacter in order to prioritize the campylobacter-free flocks in the slaughter process to prevent cross-contamination by campylobacter-positive flocks.

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