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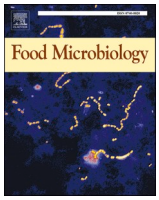

Rapid detection of *Campylobacter* spp. in chickens before slaughter

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ABSTRACT

Campylobacter continues to be the number one cause of bacterial gastroenteritis in Europe. Poultry, and especially broiler chickens, is considered an important reservoir for *Campylobacter* spp. Poultry producers prioritize to identify and reduce the number of *Campylobacter* contaminated chicken flocks by tightening biosecurity and mitigation actions at slaughter. *Campylobacter*-positive flocks must therefore be identified as close to slaughter as possible, and rapid detection methods are needed. Here we evaluated the applicability, sensitivity, and specificity of four commercially available rapid methods to detect *Campylobacter* in naturally contaminated chicken cecal droppings on-farm before slaughter against an established qPCR method. The Biofire® FilmArray® Gastrointestinal Panel assay, the VIDAS *Campylobacter* assay, the Singlepath® *Campylobacter* test, and OptiGenes' Genie *Campylobacter* isothermal DNA amplification were assessed in a pilot-study. The OptiGenes' Genie *Campylobacter* isothermal DNA amplification was also tested under field conditions. The Biofire® FilmArray® showed superior sensitivity and specificity compared to the three other rapid tests but had a lower throughput and a higher cost. While the VIDAS *Campylobacter*, Singlepath® *Campylobacter* and the isothermal DNA amplification were affordable, their unsatisfactory sensitivity (10%–71%) left these unsuitable to monitor *Campylobacter* carriage in chickens. An additional finding of this study is that 38% of flocks positive for *Campylobacter* at slaughter became contaminated during the last week of rearing. Therefore, increased efforts to develop suitable methods to detect *Campylobacter* rapidly and reliably in chickens close to slaughter are needed.

1. Introduction

Campylobacter is the most common cause of human bacterial gastroenteritis worldwide, including Norway (Jørgensen, 2020), with an estimated cost of 2.4 billion € annually in the EU alone. Reducing the number of campylobacteriosis cases is of high priority for stakeholders ((BIOHAZ), 2010). Chicken is considered an important reservoir for human disease, and the most efficient way to reduce the number of campylobacteriosis in the population is to limit the number of *Campylobacter* positive chicken flocks. The food industry and public health authorities, therefore, spend large resources on reducing *Campylobacter* spp. colonization in poultry, for instance through actions taken in The Norwegian Action Plan against *Campylobacter* in broilers (APaC) (Torp, 2018, 2019, 2020). In the APaC, all chicken flocks slaughtered between May and October are tested for the presence of *Campylobacter* a maximum of six days before harvest, and if positive, the meat is frozen to avoid contaminated chicken meat entering the market. However, a considerable number of chicken flocks turn *Campylobacter* positive the last week of rearing and is therefore at high risk of being missed in the APaC (Torp, 2018). Knowledge about the flocks' *Campylobacter* status closer to slaughter would therefore improve control of *Campylobacter-positive* flocks and the efficacy of APaC.

Numerous rapid detection methods are commercially available, such as immunoassays (Singlepath®, mini VIDAS®) or nucleic acid amplification methods (FilmArray®, Genie II ®). The Singlepath® *Campylobacter* is a lateral flow immunochromatographic test, in which the sample is mixed with gold-colloid particles coated with capture antibodies specific to the target antigen and drawn across a nitrocellulose membrane. If the antigen is present in the sample, it is captured and bound to the colored particles to form a visually apparent line. The

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method is rapid (~20 min), easy to use, but intended for enriched culture (Sigmaaldrich, 2021). The mini VIDAS *Campylobacter* kit (VIDAS CAM assay) is an enzyme-linked immunoassay for the detection of *Campylobacter* antigens, although after enrichment. VIDAS CAM assay is sensitive and specific enough to detect low levels of *Campylobacter* in broiler meat after enrichment (Liu et al., 2009). It is fast (<1 h) and performed in a closed automated system, minimizing the risk of cross-contamination between samples and interpretation challenges (BiomerieuxIndustry). The BioFire FilmArray Gastrointestinal Panel (FilmArray GI Panel) is a fully automated DNA extraction and multiplex-PCR analysis apparatus. The FilmArray GI Panel can detect 22 different enteric pathogens simultaneously, among these *Campylobacter* spp., in a closed system and reports each targets presence qualitatively. It is developed for the rapid detection and identification of multiple pathogens in clinical samples (Buss et al., 2015), but the panel also has high sensitivity and specificity in other matrixes such as water (Davidson, 2018). OptiGene's Genie® II is an instrument that performs real-time loop-mediated isothermal amplification (LAMP) for rapid detection of *Campylobacter* spp. with a specific *Campylobacter* (CAM LAMP) detection assay. CAM LAMP is simple, requires minimal sample preparation, and provides results within an hour, highly applicable under field conditions. LAMP has been used to detect *Campylobacter* directly from poultry carcass swabs and poultry litter collected on socks with other primers and reagents, with relatively good results (Romero and Cook, 2018; Romero et al., 2016).

The Norwegian system for monitoring *Campylobacter* on chicken farms (APaC) will not detect chicken flocks that turn positive during the last week of rearing due to long transportation time of the samples by mail to the central laboratory. Therefore, rapid, robust on-site testing is desirable to separate the flocks contaminated in the last week prior to harvest from negative flocks. Here we investigated the sensitivity, specificity, user-suitability, and applicability of Singlepath® *Campylobacter*, VIDAS CAM assay, FilmArray® GI Panel, and CAM LAMP to detect *Campylobacter* in broiler cecal droppings and compare their results with those of the qPCR method applied in the APaC (Lund et al., 2004). Further, the best performing method all aspects considered was compared in sensitivity, specificity, and applicability under field conditions during the seasonal peak of *Campylobacter* in chickens and compared with the established qPCR. We also estimated how frequently chicken flocks contracted *Campylobacter* during the last week of rearing by comparing qPCR results of farmer-collected cecal droppings taken six days prior to slaughter with those taken as close to slaughter as possible.

2. Material and methods

2.1. Samples

Sixty and 30 samples positive and negative for *Campylobacter,* respectively, were included in the **pilot study**. The samples were collected and analyzed through the APaC six or seven days before slaughter, and each sample consisted of ten pooled swabs from fresh cecal droppings collected on dry, sterile swabs by the farmer and sent by mail for *Campylobacter* analysis by qPCR (Lund et al., 2004; Torp, 2018). Six ml of saline was added to the pooled swabs. Eight µl was subjected to DNA extraction and qPCR for *Campylobacter* detection as described earlier (Lund et al., 2004), while required volumes (see "Rapid detection methods and the APaC pipeline" for details) of the remaining saline solution were used as material for one or more of the four rapid *Campylobacter* detection methods (Fig. 1): Singlepath® *Campylobacter,* VIDAS CAM assay, FilmArray® GI Panel, and CAM LAMP. The number of samples tested by each rapid method varied (Fig. 1), mainly due to practical constraints, and time from collection to analysis ranged between one and 17 days, on average six days for all four methods.

The **field study** was performed on-site at a slaughterhouse between June and late September 2020, coinciding with the increase in the prevalence of *Campylobacter* in broilers (summer peak) (Jonsson et al., 2012; Torp, 2018). As close to slaughter as possible, the farmers collected ten fresh cecal droppings from each flock (n = 122) using dry swabs, pooled them, and sent these with the slaughter truck. On arrival at the slaughterhouse, the samples were kept refrigerated (~4 °C) or frozen (~20 °C) until analysis using CAM LAMP and the gold standard qPCR (Lund et al., 2004), respectively (see "Rapid detection methods and the APaC pipeline" for details). Samples were analyzed by CAM LAMP twice weekly, while the qPCR was done in one run after the sampling was completed.

2.2. Rapid detection methods – pilot study

In the pilot, the sensitivity, specificity, and usability of Singlepath® *Campylobacter,* VIDAS CAM, CAM LAMP and FilmArray® GI Panel were tested against the qPCR results (Lund et al., 2004).

We tested 39 samples (34 positive and 5 negatives, Fig. 1) with the Singlepath® *Campylobacter* detection method (Merck Life Science AS, Oslo, Norway) using a modified version of the manufacturers' procedure for detection of *Campylobacter* directly from chicken cecal samples (Singlepath® Direct Campy Poultry Kit, Merck KGaA, Darmstadt, Germany, personal communication from Merck). Briefly, 1 mL sample was heat-inactivated for 15 min at 95 °C, cooled and 140 µl added to the

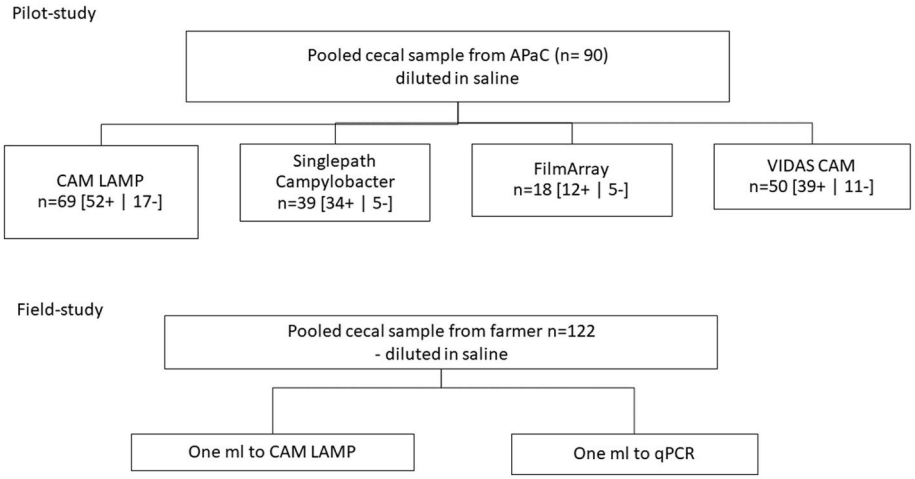

Fig. 1. Overview of samples in the pilot and field-study. The number of positive and negative samples as defined by the qPCR are indicated with a plus and minus, respectively, within brackets. APaC: The Norwegian Action Plan against *Campylobacter* in broilers.

Singlepath® device.

A total of 50 samples (39 positive and 11 negatives) was tested with the VIDAS CAM following the instructions from the manufacturer; the miniVIDAS apparatus was warmed for 30 min and calibrated prior to analysis. Samples (1 ml each) were heated for 15 min at 95 °C, cooled and 500 μl added to the VIDAS® CAMPYLOBACTER (CAM) test strip (BioMérieux). The strip was analyzed using the miniVIDAS instrument (BioMérieux), and test values above 0.1 were considered positive.

Sixty-nine samples (52 positive and 17 negatives) were analyzed using the CAM LAMP rapid detection method using the manufacturer's instructions (Optigene, 2019) with minor modifications. Shortly, one ml of the sample was added to a lysis tube containing 6M KOH (OptiGene Limited, Horsham, UK) and heated for 5 min at 80 °C before cooling. Five μl lysed sample was mixed with *Campylobacter* isothermal master mix (OptiGene) and run on the Genie® II instrument (OptiGene). Each run included a positive (OptiGene) and negative control as described by the manufacturer. Samples were reported as negative, positive (high), positive (medium) or positive (low) depending on the fluorescent signal.

Finally, 18 samples (12 positive and five negatives) were analyzed using the Biofire FilmArray® system according to the manufacturer's instructions. Briefly, analysis buffer and sample buffer were added to the FilmArray® GI pouch (Biomerieux) before 200 μl sample was added and the sample was run on an automated analysis program (~1h) on the FilmArray machine.

2.3. Rapid detection methods – field study

In the field study, farmers submitted one pooled sample of 10 swabs with cecal droppings (see samples) to the slaughterhouse. The pooled sample was prepared as follows: 10 ml sterile sodium chloride solution (0.9%) (VWR International AS, Oslo, Norway) was added to the pooled swabs, and shaken manually for 15s. The mix was left to sediment for 10 min at room temperature. Two aliquots of one ml each was made; one ml was used as substrate in the CAM LAMP reaction as described above for the pilot, while one ml was stored at −20 °C prior to analysis by the qPCR used as gold standard (Lund et al., 2004). In addition, results from the sampling done in the APaC program was made available for comparison with the results achieved at slaughter by CAM LAMP and qPCR.

2.4. Data analysis

The database was established in ExcelR (Microsoft), while data analysis was performed in STATA® Special Edition v 15.1 (Stata/SE 15.1. for Windows, College Station, TX). Test properties as sensitivity, and specificity were described using the diagt procedure using qPCR as the reference gold standard. The positive (PPV) and negative prediction value (NPV) were calculated based on the expected prevalence ~14% during summer, as described earlier (Tenny, 2020).

3. Results and discussion

Table 1 gives the basic test properties of the tests used in the pilot. As can be seen, the sensitivity of FilmArray, CAM LAMP, Singlepath *Campylobacter* and VIDAS CAM was high (91%), moderate (71%, 53%), and low (10%), respectively. The false positive rate was approximating nil for all rapid test methods except CAM LAMP that had a false positive rate of 6% (one false positive (Table 1). Except for FilmArray, all rapid tests frequently resulted in false negatives (Fig. 2, Table 2); the Singlepath *Campylobacter* detected one of two (47.0%) positive flocks, while the VIDAS CAM detected one of ten positive flocks. In a chicken population with a low *Campylobacter* prevalence, as seen in Norway (Torp, 2018), this poor sensitivity did not affect PPV, but NPV dropped (Table 1). Indeed, the probability of a sample being truly positive for *Campylobacter* when the test was positive was 66% for CAM LAMP, 97% for FilmArray, and 100% for VIDAS CAM and Singlepath *Campylobacter*. The probability of a flock being truly negative when the test was negative was 87–98% (Table 1, Fig. 2).

Table 1
Characteristics of four rapid detection methods for *Campylobacter* measured against a gold standard - the qPCR by Lund et al. (2004).

Rapid test	Sensitivity (95% CI)	Specificity	PPV	NPV
CAM LAMP[P]	0.71 (0.60–0.83)	0.94 (0.72–1.0)	0.66[a]	0.95 [a]
CAM LAMP[F]	0.65 (0.44–0.83)	0.94 (0.88–0.98)	0.77[b]	0.91[b]
Singlepath®	0.53 (0.35–0.70)	1.0 (0.48–1.0)*	1.0[a]	0.93 [a]
VIDAS® CAM	0.10 (0.03–0.24)	1.0 (0.72–1.0)*	1.0[a]	0.87 [a]
FilmArray®	0.91 (0.62–0.99)	0.83 (0.36–0.99)	0.97[a]	0.98 [a]

Positive prediction value: PPV, Negative prediction value: NPV.
[P]: pilot study.
[F]: field study.
*One-sided, 97.5% confidence interval.
[a] Using a prevalence of 14% positive flocks during the summer, i.e. PPV+ is valid under Norwegian circumstances, as described by Tenny et al. (2020).
[b] Field-study, the tested flocks are representative of the population and therefore the true prevalence.

Whatever test applied must reliably rule out that flocks carry *Campylobacter,* i.e. have few false negatives and a high NPV. This conservative approach might lead to negative flocks being treated as positives but will reduce the risk to the consumer. Further, it is desirable that the method chosen is rapid, user friendly and affordable. Based on these demands and the results from the pilot, the Singlepath *Campylobacter* and VIDAS CAM were ruled out for testing during the field-study due to an unacceptable high fraction of false negatives. These immune based methods are intended to be used after an enrichment step, and both have high detection limits; Singlepath *Campylobacter* can detect 10^4-10^7 bacteria per ml (Ripolles-Avila et al., 2020), while VIDAS CAM's detection limit is reported as low as 4.4 CFU/25g prior to enrichment (BiomerieuxIndustry). Indeed, the VIDAS CAM has successfully detected *Campylobacter* in chicken meat (Liu et al., 2009), porcine lymph nodes (Nesbakken et al., 2003), chicken carcass, and slaughter house environment (Reiter et al., 2005), all after enrichment. Chickens harbor 10^8-10^9 *Campylobacter* per gram of cecal content, and it was therefore expected that both immune based methods would perform better. Other factors, such as possible lower carriage loads in Norwegian chickens, bacterial cell lysis and death during transport and storage and dilution of *Campylobacter* due to pooling of swabs would lower the levels of intact antigens and result in frequent false negatives.

Of the two nucleic acid amplification methods, FilmArray® correctly identified 11 out of 12 positive samples and all negatives tested, well in line with high specificity and sensitivity achieved in earlier studies analyzing fecal and water samples (Buss et al., 2015; Davidson, 2018). The FilmArray® is simple and reliable, performed in a closed system, reducing hands-on time and risk of human error. While the FilmArray® gave superior results compared to the other tests, the cost of this test (~120$/sample) was prohibitive for use in mass screening. Further, the FilmArray® only runs a single sample at a time, making high throughput difficult without investment in a larger instrument park. CAM LAMP has an affordable price (10$/sample), and a rapid and simple analysis procedure, and runs 12 samples simultaneously on the Genie II instrument. Albeit CAM LAMP achieved a lower sensitivity (71%) compared to the FilmArray® in the pilot, CAM LAMP was assessed as the most suitable candidate rapid detection method all aspects considered. The CAM LAMP was therefore chosen for a large-scale field-study to compare its performance against the qPCR and assess its user-friendliness in the hands of the slaughter-house workers. Fecal samples from 122 chicken flocks were tested for the presence of *Campylobacter* using CAM LAMP and qPCR in parallel, resulting in 22 positives and 100 negatives with the CAM LAMP and 26 positive and 96 negatives with the qPCR. CAM LAMP sensitivity was lower in the field-study (65.4%, Table 1) compared to the pilot, but achieved an equivalent specificity (95.8%). Our CAM LAMP results contrasts those of Romero et al. (2016) (Romero and Cook, 2018; Romero et al., 2016) and Sabike et al. (2016) (Sabike

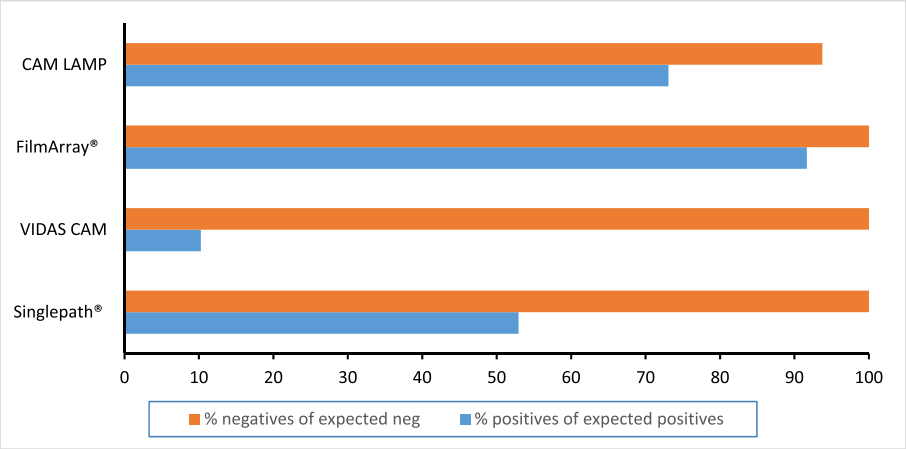

Fig. 2. Results from the pilot-study. The relative proportion of the samples with a positive and negative result in both the rapid detection method and the qPCR used as gold standard, is represented by blue and orange bars, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Results from the pilot-study. Absolute number of samples tested with the rapid detection method and correspondence with results achieved with qPCR is given.

Rapid method	Positive/qPCR positive	Negative/qPCR negative	Total (n)
Singlepath®	18/34	5/5	39
VIDAS CAM	4/39	11/11	50
CAM LAMP	38/52	15/16	69
FilmArray®	11/12	5/5	17

et al., 2016), who applied three different LAMP assays to detect *Campylobacter* in spiked poultry boot socks, chicken carcasses and fecal samples. A higher sensitivity was achieved in their studies, possibly due to the inclusion of a DNA purification step (centrifugation (Sabike et al., 2016), immunomagnetic separation (IMS) (Romero et al., 2016)), the use of in-house assays and different suspension volume (Romero and Cook, 2018). A purification step may concentrate *Campylobacter* and reduce the presence of inhibitors; indeed, when Romero and colleagues used suspensions of turkey boot samples spiked with *Campylobacter* as template without IMS, amplification was inhibited due to matrix inference. The performance of a DNA amplification method can also vary with the amplification target, primase and master mix used, and both Romero (2016, 2018) and Sabike (2016) used in-house primers while we used a commercialized Optigene CAM LAMP assay. Romero and Cook (2018) suspended the boot socks tested in 100 ml, thereby diluting not only the *Campylobacter,* but also the inhibitors. However, distinguishing false and true negatives is impossible without the presence of an internal amplification control (IAC), simply because we do not know if the lacking signal is due to lack of *Campylobacter* or amplification. The absence of an IAC is a critical inadequacy of the commercial CAM LAMP, as the sample preparation step is rapid, but crude. It is therefore unlikely that all inhibitors are removed from a complex fecal matrix. In addition, the LAMP as a method will never be as sensitive as a qPCR (which was used as our gold standard), as a qPCR can detect down to a few DNA molecules and commonly have detection limits of five target copies, while CAM LAMP will only amplify from 100 copies and up (personal communication, Optigene). Indeed, the application of a direct LAMP assay in place of a traditional qPCR to detect SARS-CoV-2 in nose and throat swabs has raised controversy in the UK (Wilson-Davies et al., 2021). The consumable cost of the CAM LAMP is, however, favorable (~12$/sample) and CAM LAMP has been applied by poultry processors in the UK for several years (personal message, Optigene). Also, similar LAMP assays have been designed and/or applied to detect foodborne and animal pathogens such as *Salmonella* serovar Enteritidis (Yang et al., 2010) *et* Typhimurium (Techathuvanan et al., 2010), *Brucella* spp. (Ohtsuki et al., 2008), and *Flavobacterium columnare* (Yeh et al., 2006).

During our study we also had a chance to follow the development of the *Campylobacter* status of 122 flocks during the last week of rearing. The APaC program uses the same sampling procedure and qPCR (Lund et al., 2004) to test the presence of *Campylobacter* up to six days prior to slaughter as we did at time of slaughter. Nearly forty percent of the flocks positive for *Campylobacter* at slaughter (38.5%) was negative six days prior, demonstrating that sampling as close as possible to slaughter is of paramount importance and that reliable cost-efficient rapid detection methods is urgently needed. These flocks are not detected in the APaC program and will be treated as *Campylobacter* negative flocks; the flock will not be subjected to sequential slaughter, nor will the meat be frozen prior to consumption. This lack of action for a substantial part of *Campylobacter* positive chicken flocks represents a risk for cross-contamination to other flocks in the slaughterhouse and will result in *Campylobacter* contaminated chicken meat to the consumer (Elvers et al., 2011). Although the CAM LAMP was not a robust option for a rapid detection method as used here, other developed alternative LAMP assays combined with better sample preparation steps could be an alternative.

In conclusion, of the four rapid detection methods tested, only FilmArray® was of sufficient applicability and performance, albeit with high costs and low throughput. Our study also shows that a considerable portion of broilers slaughtered during the summer is contaminated by *Campylobacter* during the last week of rearing, emphasizing the need for a rapid detection method that will provide a reliable *Campylobacter* status as close to slaughter as possible. Therefore, alternative detection methods should be sought to aid in this work.

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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