Comparison of sensitivity of a Campy-Cefex dilution method and PCR in detecting Campylobacter in broilers

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ABSTRACT

The aim of the study was to compare the sensitivity of a Campy-Cefex dilution method and PCR for detecting *Campylobacter* in broilers and to see if any traces of the bacteria were to be found by these methods during winter when the bacteria is not detectable by conventional microbiological methods. The results of our studies done in 2004 and 2005 were compared with data from 2001-2003 soon after a national surveillance of *Campylobacter* spp. in broilers was initiated. Faecal samples from 607 broiler flocks were pooled, 10 samples per pool, and diluted in saline for the Campy-Cefex direct plating dilution method and PCR. The PCR amplification was performed in a Peltier Thermal Cycler and the primers used were C412F and C1288R. A total of 742 pooled caecal samples were collected at slaughter. Samples from each pool of 40 caeca were diluted in saline for the Campy-Cefex direct plating dilution method. The PCR method proved to be more sensitive than the Campy-Cefex method but still did not detect any traces of *Campylobacter* during winter. A comparison of the results from 2001-2003 with the results from 2004-2005 indicates that the percentage of positive flocks had diminished. The study underlines the importance of using sensitive methods for detecting *Campylobacter* spp. in order to minimize the risk of human exposure. The finding that the sensitive PCR method was not able to detect *Campylobacter* during winter suggests that the seasonal pattern of campylobacteriosis is due to a new vector in the spring which carries the bacterium.

Keywords: Broilers, Campylobacter seasonal variance, PCR.

YFIRLIT

Samanburður á næmi tveggja aðferða við greiningu á Campylobacter í kjúklingum, Campy-Cefex ræktunaraðferðar með raðþynningum og PCR aðferðar.

Tilgangur rannsóknarinnar var að bera saman næmi tveggja aðferða við greiningu *Campylobacter*, Campy-Cefex ræktunaraðferðar með raðþynningum og PCR aðferðar. Þessar aðferðir voru notaðar við greiningu á bakteríunni úr kjúklingasýnum yfir vetur þegar lítið smit greinist með hefðbundnum ræktunaraðferðum. Niðurstöður rannsóknarinnar sem stóð yfir frá árinu 2004 til ársins 2005 eru bornar saman við upplýsingar frá árinu 2000 þegar farið var út í fyrirbyggjandi aðgerðir gegn *Campylobacter* smiti í kjúklingaeldi. Ræktað var úr 607 safnsýnum frá kjúklingum í eldi, 10 saursýni saman í sýni. Gerð var þynning á sýnunum í saltvatni og þeim sáð í raðþynningum út á skálar með Campy-Cefex agar. Sömu sýni voru mögnuð upp með C412F og C1288R vísum í „Peltier Thermal Cycler“ PCR tæki. Ræktað var úr 742 safnsýnum frá kjúklingum úr slátrun,

40 botnlangasýni saman. Sýnin voru raðþynnt í saltvatni og þeim sáð út á skálar með Campy-Cefex agar. PCR aðferðin reyndist næmari en Campy-Cefex aðferðin en hún greindi þó ekki nein merki um *Campylobacter* í kjúklingasýnum yfir vetrarmánuðina. Samanburður niðurstaðna við stöðuna í kjúklingaeldi árin 2001-2003 bendir til þess að hlutfall *Campylobacter* jákvæðra kjúklingahópa hefur minnkað á tímabilinu. Niðurstöður rannsóknarinnar undirstrika mikilvægi þess að nota næmar aðferðir við greiningu á *Campylobacter* til að lágmarka hættu á smiti í fólk. PCR aðferðin greindi ekki Campylobacter að vetri til og bendi það til þess að hin ársbundna sveifla í *Campylobacter* eigi rætur sínar að rekja til nýsmits að vori.

INTRODUCTION

Thermophilic *Campylobacter* spp. is the major cause of human bacterial enteric infections in Iceland (Stern et al. 1992) as well as the most common cause of intestinal disorders in industrial countries (Anonymous 2000). Poultry is often incriminated as the main source of human infections, due to the high prevalence of *Campylobacter* in broilers.

From June 1998 to March 2000 Iceland experienced an epidemic of human campylobacteriosis, mostly due to consumption of fresh chicken (Hiett et al. 2001). Prior to 1996 only frozen products had been marketed in Iceland but after 1996 the consumers increasingly demanded fresh poultry. Since the fresh products contained higher levels of *Campylobacter* they caused a greater public exposure to the bacteria. In order to reduce the human exposure to *Campylobacter*, an assessment of the risk factors for *Campylobacter* infection in broiler flocks was carried out and in 2000 a national surveillance of *Campylobacter* spp. in broilers was initiated (Reiersen et al. 2003, Stern et al. 2003). Flocks which were detected as *Campylobacter* positive were slaughtered at the end of the week in order to minimize the risk of cross-contamination. The carcasses of positive flocks were also frozen before being put on the market because freezing was shown to cause a 10-100 fold reduction in the *Campylobacter* counts on carcasses (Stern et al. 1985, Georgsson et al. 2006). Lower prices paid to producers for frozen lots encouraged poultry farmers to improve hygiene as well as biosecurity measures in an attempt to reduce spread of the bacteria from the environment to broiler flocks. Improved hygienic poultry handling is believed to have resulted in reduced public exposure to campylobacteriosis (Stern et al. 2003).

The NMKL (Nordisk Metodik Kommitté för Livsmedel) method based on Preston preenrichment and using modified Charcoal Cefoperazone Deoxycholate agar (mCCD) (NMKL 119, 3rd Ed. 2007) has for years been the method mainly used for detecting *Campylobacter* in poultry in our laboratory. A comparison was made between the NMKL method and the less time consuming Campy-Cefex direct plating method (Stern et al. 1992). After concluding that the Campy-Cefex method was at least as sensitive as the NMKL method and required only 2 days compared to 3 days for the NMKL method (Hjartardottir et al. 2003) the Campy-Cefex method has been the sole method used. By comparing direct plating on Campy-Cefex and mCCD agar, Line et al. (2001) and Oyarzabal et al. (2005) have come to the same conclusion.

The conventional culture methods are time consuming and laborious. The bacterium is slow growing and fastidious and has specific requirements for an incubation atmosphere. A molecular technique, in contrast, is much faster. An investigation made in order to compare the sensitivity of a direct plating mCCD method and PCR has shown that the PCR method (Lund et al. 2003) is useful in detecting *Campylobacter* in pooled cloacal swabs from broilers. Similarily, a small survey made in 2004 to compare the Campy-Cefex test, the NMKL and PCR has indicated that PCR is the most sensitive method (unpublished data).

The main aim of this study was to further compare the sensitivity of PCR (Lund et al. 2003) and the Campy-Cefex test by using samples taken over a period of 15 months,

from July 2004 through September 2005. Another aim was to follow broiler flocks from rearing to slaughter through their ID numbers and determine if broilers, either positive or negative, had come in contact with the bacteria before slaughter and were detected as *Campylobacter* positive at the abattoir. In order to improve the sensitivity of the Campy-Cefex method the samples were serially diluted before direct plating. Regardless of media used, dilution of faecal samples before direct plating may improve isolation rates, reduce the need for subcultures and diminish competing flora (Nye et al. 2001). We also wanted to see if the survey done over a period of 15 months could give us information about the contamination status in the broiler houses during winter when the incidence of Campylobacter is much lower than in the summer months. Thus, a more sensitive PCR method might detect bacteria not detectable by culture.

In this study, samples for *Campylobacter* tests in broilers were taken according to the international surveillance programme which started in 2001 as an epidemiological study of *Campylobacter* in Iceland, called "Campy-on-Ice". Results made it possible to compare results from 2004 and 2005 with those of 2001-2003.

MATERIALS AND METHODS

Faecal samples

A total of 607 pooled faecal samples, 10 samples in a pool, were collected from every broiler flock as a part of the official Icelandic surveillance programme for Campylobacter in poultry.

PCR

The faecal sample pool was mixed well. Ten swabs were taken from each pool. The swabs were put into two 15 ml tubes with the transport medium containing 37 g l^{-1} brain heart infusion broth (Difco, Baltimore, MD, USA) with 5% (v/v) sheep blood and 0.5% agar. The tubes were tightly capped and kept in the refrigerator for approximately 24 h but no longer than 36 h. Then the 10 swabs were pooled in a tube containing 3 ml of sterile distilled water. The swabs were left for about 10 min at room temperature to release the bacteria. Eight hundred µl were transferred to a microfuge tube and centrifuged at 16000 g for 7 min. DNA was isolated from the pellet using a DNA isolation kit for blood/bonemarrow/tissue following the instruction of the supplier. (Roche, Mannheim, Germany). Three reference strains, *Campylobacter jejuni* subsp. *jejuni* (ATCC 33560), Campylobacter lari (ATCC 35221) and *Arcobacter butzleri* (ATCC 49616) were used as positive controls and sterile distilled UV radiated water served as a negative control. DNA which was not PCR amplified immediately was stored at -20°C.

The PCR mixture was prepared in a large volume and 47 µl distributed into PCR strips and kept at -20°C until used. Primers used for detection of *Campylobacter* spp. were C412F and C1288R (Linton et al. 1996).

The PCR amplification was performed in 50 µl volumes containing 3 µl of the DNA sample, 25 µl of a PCR Master Mix (Promega, Madison, WI, USA), 2 µl of a 25mM $MgCl_2$ solution 0.5 µl of a 100 mg ml^{-1} BSA solution and 20 pmol of each primer. The PCR was performed in a Peltier Thermal Cycler PTC-200 (MJ Research, Inc. Waltham, MA, USA). Cycling conditions were one cycle of 95°C for 2 min, 58°C for 1 min and 72°C for 1 min followed by 34 cycles of 95°C for 15 s, 58°C for 40 s and 72°C for 40 s. The last elongation step lasted 5 min. Fifteen microlitres of the PCR product were loaded onto a 2% SeaKem LE agarose gel (Cambrex Bio Science Inc. Rockland, ME, USA) containing 0.1 mg ml^{-1} ethidium bromide. The gel was visualized on a White/UV Transilluminator.

Campy-Cefex direct plating dilution method

The same faecal sample pool was used for PCR and Campy-Cefex direct plating. The faecal sample pool was mixed with sterile saline (¾) ratio faecal:saline. Briefly, 0.1 ml of the

diluted faecal sample was serially diluted in saline (10^1, 10^3 and 10^5). The dilutions, 100 µl each, were directly plated on Campy-Cefex agar and the plates incubated in a microaerobic atmosphere containing 6% CO_2, 6% O_2 and 4% H_2 in N_2 at 42°C for 48 h. *Campylobacter*-like colonies were picked up and *Campylobacter* spp. confirmed by microscopic examination.

Caecal samples

A total of 742 pooled caecal samples, 40 samples per pool, were collected at slaughter from the same broiler flocks which had previously been tested for been *Campylobacter* in faeces.. This was done as a part of the official Icelandic surveillance programme for *Campylobacter* in poultry. There were a larger number of pooled caecal samples because some broiler flocks had to be divided into more than one slaughter lot due to flock size.

Four pooled samples of 10 caeca each were taken from each broiler catch lot at slaughter, stomached and pooled. The 40 caeca pools were diluted in saline for the Campy-Cefex direct plating dilution method, in the same way as the faecal sample pools and incubated the same way.

RESULTS

A total of 607 faecal samples from broiler flocks and 742 slaughter caecal samples from the the same flocks were tested for the occurrence of *Campylobacter* spp. as a part of the Icelandic surveillance programme of *Campylobacter* in broilers. The investigation lasted from July 2004 through September 2005. Each flock had its own unique ID number.

The results show that there was a low prevalence of positive flocks during winter. From the 22 November 2004 till the 22 April 2005 only 3 broiler flocks were detected positive (Figure 1).

The data show that there is some difference in the sensitivity of the Campy-Cefex and PCR methods in detecting *Campylopbacter* in faecal samples, as the PCR method is slightly more sensitive. The results are summarised in Table 1. A total of 65 (11%) pooled faecal samles were found positive by Campy-Cefex direct plating, whereas 95 (16%) were found positive by PCR. Positive PCR samples were defined by the presence of distinct, white bands of a 816 bp PCR product in an agarose gel and given different marks ((+), +, +(+),++(+) and +++) according to the strength of the band. All other samples were considered negative. A picture of a typical agarose gel containing 25 samples together with three positive and one negative samples is shown in Figure 2.

There was a concordance of the methods in 95% or 575 out of 607 samples. A total of 64 samples were positive by both methods. There were 31 samples negative by culture but positive by PCR and one sample was positive only by the diluted direct plating Campy-Cefex method (Table 1).

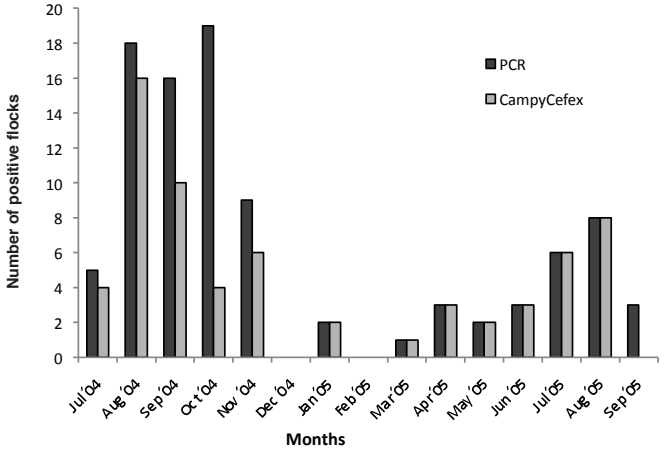

Figure 1. Monthly screening of *Campylobacter* spp. in broiler faecal samples. Diluted direct plating Campy-Cefex method compared with PCR.

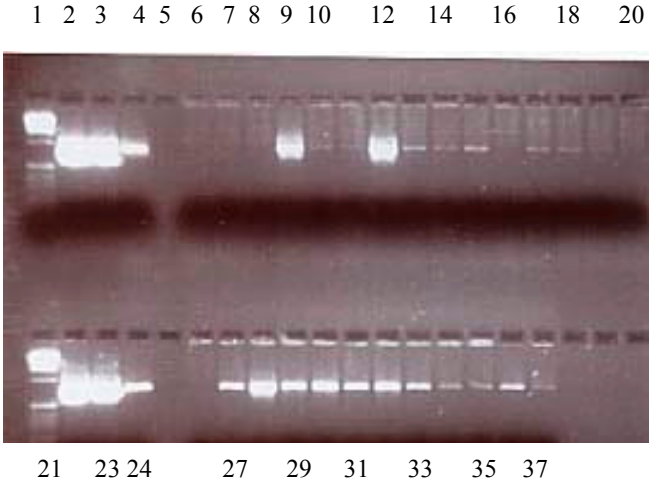

Figure 2. Gel showing typical results of Campylobacter PCR analysis. Lane 1 and 21 molecular size marker; lanes 2-4 and 22-24 positive controls (ATCC 33560, ATCC 35221, ATCC 49616); lanes 5 and 25 empty; lanes 6 and 26 negative control (distilled water). Lanes 7-20 and 27-37 containing 25 samples. PCR – samples lanes 7,8,16,20; PCR (+) samples lanes 10,11,19; PCR + samples lanes 13,14,15,17,18,34,35,36,37; PCR +(+) sample lane 33; PCR ++ samples lanes 27,29,31,32; PCR ++(+) sample lane 30; PCR +++ samples lanes 9,12,28.

A difference was found between producers as those with only one broiler house had proportionally fewer positive cases of *Campylobacter* than the bigger producers. At the farm level, at the time this investigation took place, some houses seemed to be completely free of Campylobacter (Figure 3). One faecal sample from broiler flocks, which was positive only by the Campy-Cefex method, was also positive at slaughter (Table 2). There were 11 out of 607 (1.8%) broiler samples positive only by PCR and also positive at slaughter. There were 47 out of 607 (or 7.7%) samples positive at slaughter but neither positive by the Campy-Cefex method nor PCR in broiler flocks. There were 20 out of 607 (3.3%) broiler samples positive by PCR only and negative at slaugter and 464 out of 607 (76%) negative by both methods and negative at slaughter. Only one sample was positive by direct plating only and positive at slaughter. These results

Table 1. Screening of *Campylobacter* spp. in broiler faecal samples. Diluted direct plating Campy-Cefex method compared with PCR.

	Direct plating +	Direct plating -	**Total**
PCR +	64	31	**95**
PCR -	1	511	**512**
Total	**65**	**542**	**607**

Table 2. Screening for *Campylobacter* spp. in broiler faecal samples. Diluted direct plating Campy-Cefex method and PCR compared with results from caecal slaughter samples.

	Broiler flocks; faecal samples				
	Direct plating + PCR +	Direct plating – PCR -	Direct plating + PCR +	Direct plating – PCR -	Total
Slaughter samples +	63	1	11	47	122
Slaughter samples -	1	0	20	464	485
Total	**64**	**1**	**31**	**511**	**607**

are shown in Table 2. Broiler flocks tested negative during winter by both methods, apart from two flocks in January and a single one in March (Figure 1).

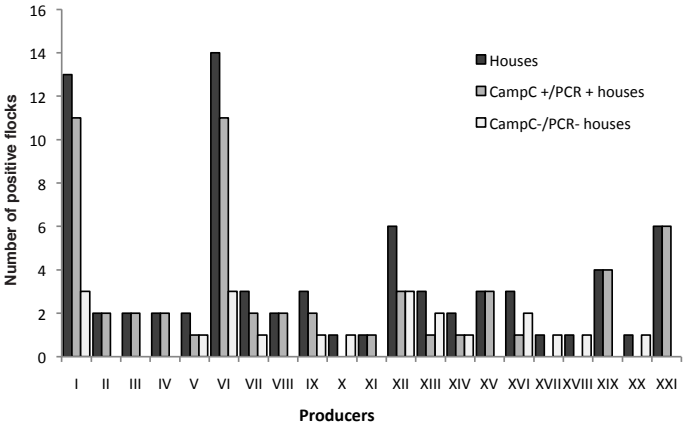

Figure 3. Screening of Campylobacter spp. in broiler faecal samples. Differences between producers.

DISCUSSION

The data, summarised in Table 2, show that 11 broiler flocks that tested negative by the Campy-Cefex method tested positive at slaughter by the same method. These samples tested positive by PCR before slaughter. These broiler flocks might thus have contaminated *Campylobacter* free flocks slaughtered later the same day. This indicates that a less time consuming and sensitive method like PCR is an important tool for planning slaughter to minimize the risk of cross-contamination.

Broiler flocks negative by both methods were in some cases found to be positive at slaughter. By reducing the time between sampling of broilers and slaughter, usually an average of 4.4 days, it might in most cases be possible to detect the bacteria before slaughter. The minimum time is 2 days for the diluted direct plating Campy-Cefex method since the test takes 2 days. The PCR method would be a far better choice since it only takes 8 hours. The time for the PCR method can even be reduced by further automation. The right timing of sampling is important as the prevalence of *Campylobacter* in a flock increases with the birds' age (Barrios et al. 2006, Stern et al. 2003).

Some of the PCR positive flocks were negative at slaugter by the Campy-Cefex method. Most of these samples were weakly (+) positive by the PCR method. A low contamination of *Campylobacter*, not intensive enough to accumulate in the birds might be the reason (Gregory et al. 1997). According to Siemer et al. (Siemer et al. 2003) a reduction of colonization in *Campylobacter* positive flocks is also possible even though the load of the organism is initially very high.

Arranging the samples by farmhouse ID showed that of the 20 broiler flocks positive by PCR but negative by the Campy-Cefex method at slaugther, 13 samples were taken in late October and were the last positive samples from those particular farmhouses that year. The positive cases in November were from different producers in another part of the country (Figure 1).

The broiler flocks weakly positive by PCR but negative at slaughter may indicate nonviable *Campylobacter* cells (Beumer et al. 1992, Medema et al. 1992) and it might be suggested that the weak positive samples were due to traces of *Campylobacter* from positive flocks raised earlier in the broiler houses or *Campylobacter* being present in very low numbers, close to the threshold of detection. It might also be due to the difference in targets detected by the two methods used whereas the Campy-Cefex agar is a selective media, which detects the heat tolerant *C. jejuni* and C. *coli* but the PCR method (Linton et al. 1996) appears to be genus specific for *Campylobacter* spp. (Stern et al. 1992).

This may be the case for flocks from certain houses tested in this study. Further testing like

multilocus sequence typing (MLST) are needed to give answeres to these speculations.

A comparison of broiler farms in this investigation shows a difference between producers as to the number of contaminated flocks whereas the smaller producers seem to have better control of biosecurity and hygiene at the farm level than the bigger ones. Some farmers have been more eager than others to improve biological security measures in an attempt to control spread of *Campylobacter* from the environment, resulting in flocks less likely to become colonized with *Campylobacter*. A complete changing every spring of the soil around the broiler houses has even been done on some farms (Personal Communication, Reiersen, 2005).

The number of human cases of campylobacteriosis in Iceland has diminished dramatically since 2000 even though data from 2000 (Reiersen et al. 2003) and 2001 through 2003 have shown uniform colonization rates of *Campylobacter* spp. in chickens (Stern et al. 2005). The prevalence rates for the slaughter years 2000, 2001, 2002 and 2003 were 16%, 17.6%, 17.3% and 12.7%, respectively, using conventional direct plating bacteriological methods. By comparing these rates from 2000 through 2003 with a 12.1% and 5.0% prevalence of positive broiler flocks in 2004 and 2005 (unpublished data) it can be seen that the percentage of positive flocks has diminished. These results are promising as it has been suggested that the most effective method to decrease *Campylobacter* prevalence in chickens is an intervention at the farm level (Newell et al. 2011, Herman 2003), although spread of the bacteria into broiler houses can probably not be completely prevented.

Campylobacter colonization in poultry follows a seasonal pattern in Iceland, as in other Nordic countries, with a peak in the warmer summer months (Jore et al. 2010, Guerin et al. 2008, Hofshagen and Kruse, 2005, Stern et al. 2005, Wedderkopp et al. 2000, Wedderkopp et al. 2001).

The reason for this seasonal effect is unknown but the role of migratory birds, insects and flies has been suggested (Hald et al. 2008, Jacobs-Reitsma, 1997). In a Danish study (Hald et al. 2008) flies, especially the house fly, that were captured from farm surroundings during summer months and trapped in ventilation vents were shown to be infected with *Campylobacter* spp. The house fly is known to be a transmitter of several diseases and their biology causes them to pick up *Campylobacter* spp. from fresh faeces. Furthermore, *Campylobacter* has been isolated from puddles and other water supplies (Hald et al. 2008). Fly screens that were used to prevent the influx of flies in broiler houses in Denmark in 2006 caused a dramatic decrease in *Campylobacter* spp.–positive flocks (Hald et al. 2007).

Broiler flock prevalence in Iceland has been lowered further since 2008 with the application of fly screening/netting on broiler house ventilation inlets on the largest farms. In winter months in Iceland there are no flies, and waterborne transmission to broiler flocks is a rare event.

From December to March only three flocks tested positive, by both diluted direct plating Campy-Cefex method and PCR, and all three proved positive at slaughter. Four other broiler flocks which were negative by both methods before slaughter tested positive at slaughter by the diluted direct plating Campy-Cefex method. These results indicate that a true change takes place in the spring that influences the *Campylobacter* burden in the broiler flocks. The oceanic climate in Iceland fluctuates with frequent changes of temperature. During winter the positive flocks are most likely due to contamination from the surroundings of the broiler houses, as is the case the summer. Gurin et al. (2008) have shown that flies at minimum ambient temperatures play a role in the epidemiology and seasonality of *Campylobacter* during summer. In winter the temperature can similarly become high enough to activate both flies and *Campylo-bacteria*.

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