Prevalence of *Campylobacter* spp. in feces of chickens at children's farms and social care farms in the province of Utrecht

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Abstract

Campylobacteriosis is the most commonly reported zoonosis in humans in the European Union. While most campylobacteriosis cases are foodborne related, direct contact with (farm) animals, especially with poultry which are a natural host for Campylobacter spp., are thought to be another important route of infection. In the Netherlands, children's farms and social care farms are considered the largest interface between the general public and live (farm) animals. Whereas research on the prevalence of Campylobacter spp. is mostly performed on commercial poultry farms, this study aims to quantify the prevalence of Campylobacter spp. in chickens at children's farms and socials care farms in the Netherlands.

At six-teen children's farms and nine social care farms feces samples from chickens were collected in the province of Utrecht. Thereafter, the samples were examined in the laboratory for identification of Campylobacter spp. using the ISO 10272-1:2017(E) method, confirmed with the Thermo Scientific^{TM} Campylobacter Test (TSC-test). The outcome of the TSC-test is used to examine the difference between the prevalence at the children's farms and social care farms, using a chi-squared test of independence.

The prevalence of Campylobacter at all 25 farms is 56% with 50% at the children's farms and 67% at social care farms. The chi-squared test of independence showed that there was no significant difference in prevalence between the children's farms and social care farms ($p-value = 0.42$ ($p > .05$)). These results indicate that at least half of the children's farms and socials care farms Campylobacter is present in feces of chickens.

Conclusion: little is known about the prevalence of Campylobacter at children's farms and social care farms. While this was a small-scale research and plenty more (large-scale) research should be performed to confirm these results, it demonstrates that Campylobacter is easily found on half of the farms. Therefore, owners and managers of these farms should take proper hygienic measures to reduce this potential zoonotic risk. Better understanding of this potential risk is needed to provide appropriate options for prevention of campylobacteriosis due to direct contact with farm animals at children's farms and social care farms.

I. Introduction

In the Netherlands, there are about 450 children's farms and 700 farms that offer farm work as a means of therapy for the mentally or physically disabled, so called social care farms. The yearly number of unique visitors these farms get is estimated at 1.5 million and they visit these farms an estimated 9 to 11 million times. This makes these farms probably the largest interface in The Netherlands between the general public and live farm animals and thus the most important pathway with respect to (potential) transmission of zoonoses through contacts with livestock

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and their fomites (Hassink, Hulsink, and Grin 2014).

The main purposes of the children's farms are educational and recreational, whereas these of the social care farms are much more commercially driven, since they have to provide an income for the farmer and his or her family. In both cases the protection of employees, visitors and pupils is regulated with the Dutch law on Health and Safety at work (*Arbeidsomstandighedenwet* 2019). Knowledge about zoonotic diseases and their ways of transmission are thus a requirement for abiding the rules and regulations, because this knowledge is crucial for devising measures to prevent the transmission of pathogens through frequent and close animal – human contacts. That is to say, transmission, through direct (physical) contacts with the animals and their fomites or indirect contacts via water, air or soil (Klous et al. 2016). However, in the case of social care farms and children's farms, thorough knowledge about even the presence or absence of most zoonotic pathogens is often lacking (Hassink, Hulsink, and Grin 2014).

With regard to the total burden of disease of the Dutch population, *Campylobacter* spp. is considered one of the most relevant zoonotic pathogens with a conservatively estimated incidence of 67.260 human cases a year. At a broader level, *Campylobacter* is even so considered an arguably relevant bacterial zoonotic pathogen in the European Union (EU) as approximatately 1% of the Europeans suffers from campylobacteriosis (Humphrey, O'Brien, and Madsen 2007) and is therefore called the most commonly reported zoonosis in humans in the EU since 2005 (*The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017* 2018).

Campylobacter spp. are gram negative, microaerophilic bacteria and are commonly found in the gastrointestinal tract of domestic and wild birds and mammals (Bolton 2015). Fernandez et al., suggested that the *Campylobacter* genus comprises 20 species and subspecies in humans (Fernández et al. 2008). Yet, the thermophilic instestinal species *Campylobacter jejuni* and *C. coli* provides for the majority of the human campylobacteriosis cases (Moore et al. 2005). Poultry are the natural host for *Campylobacter* species and broilers are often colonised in the ceca, in particular with *Campylobacter jejuni* (Hermans et al. 2011)(*The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017* 2018). Thus, *Campylobacter* is mainly found in feces, and when present the highest load is detected in cecal feces. Even though *Campylobacter* is unable to grow outside the host, they are able to survive for days and possibly a week in specific environmental conditions (Shreeve, Toszeghy, Ridley, et al. 2002). Although the attribution of campylobacteriosis cases are mostly through contaminated broiler meat, other important sources of campylobacteriosis include other contaminated food, contaminated water, environmental sources and direct contact with (farm) animals (Natsos et al. 2016). The latter is thought to be a relevant factor with pets (mainly cats and dogs) and city farm animals, however numerous uncertainties about the impact of campylobacteriosis cases through direct animal contact is still prevalent (Nauta et al. 2005). In many cases the direct link between the source and the human cases are missing *e.g.* due to the genetic instability of *Campylobacter* spp. epidemiological studies are often not able to locate the specific origin (Duarte et al. 2014).

Data from Smid et al. show that chickens are the most common reservoir/source (mostly due handling, preparation and consumption of broiler meat) of *Campylobacter* infection in the Netherlands with an attribution of 68% (worldwide varying from 38% to 77%) (Smid et al. 2013) (Skarp, Hänninen, and Rautelin 2016). On the other hand, the National Institute for Public Health and the Environment in the Netherlands demonstrated that 82% of the laying farms were found positive in the Netherlands (*State of Zoonotic Diseases* 2017). Further, *Campylobacter* infections in humans are more frequently reported during spring and summer in countries with a more temperate cli-

mate (Kovats et al. 2005). This may explain the wide range of Campylobacteriosis cases worldwide (as mentioned before, prevalence among countries varies between 38% and 77%). Yet, the seasonality difference is unexplained, but vectors like flies has been suggested (Nichols 2005). Worldwide, a lot of research has been done at poultry farms on the prevalence of *Campylobacter* spp., but there are only a few studies done on the prevalence of the most common zoonotic agent in the EU at children's and social care farms.

For example, a Dutch research in 2007 reported that the percentage of samples taken at *Campylobacter* spp.-positive children's farms and social care farms were 56,5 and 50,5, respectively (Heuvelink et al. 2007). In North America, various studies on zoonotic pathogens in feces at petting zoos and county fairs have been performed. Unfortunately, these studies had no valuable data due to the low number of samples taken from chicken feces (Roug et al. 2012) (Conrad et al. 2018).

With the lack of data on the prevalence *Campylobacter* spp. at children's and social care farms, the objective of this study is to investigate the prevalence of *Campylobacter* spp. in chickens at children's farms and social care farms in the province of Utrecht, the Netherlands. Additionally, differences in prevalence between children's farms and social care farms are compared, considering that the farms differ in structure and public purpose.

II. Materials & Methods

i. Farm visits

During the period from October to January 2019-2020, a total of 25 children's (16) and social care farms (9) throughout the provinces of Utrecht and North Holland in the Netherlands were visited. The number of farms included in the study were calculated using *Sample Size Calculator* (Kane 2016) using the known prevalence of *Campylobacter* spp. of poultry farms in the Netherlands (prevalence 2017: 82% (*State of Zoonotic Diseases* 2017)) as well as the known prevalence of the children's farms and social care farms (prevalence 2007: 56,5% and 50,5 %, respectively) (Heuvelink et al. 2007). The *Sample Size Calculation* analyzed that in total 30 children's and social care farms should be visited. Due to time and costs, only a total of 25 farms could be visited. The children's farm were selected using the *vSKBN* website (*vSKBN.nl* 2019) and *Google* (searching terms: *kinderboerderij*; *Utrecht*; *kippen*). Social care farms were obtained using the *Zorgboeren* website (*Zorgboeren.nl* 2019) which most of the social care farms are associated with. The selection of farms was based on the presence of poultry animals, more specifically: chickens. As mentioned before, the reason for this is because *Campylobacter* spp. is a natural host in poultry animals and, more importantly, they are responsible for an estimated 80% of human campylobacteriosis cases (*Analysis of the baseline survey on the prevalence of Campylobacter in broiler batches and of Campylobacter and Salmonella on broiler carcasses, in the EU, 2008-Part B* 2010b). Two to five farms were visited on a Monday or Tuesday each week for a total of seven weeks. The farms visited each week were relatively close to each other to reduce time and costs. When selected, the farms were called to ask if they were interested to participate in the study. It was explained what was needed from the farms (chicken feces), that participation is anonymous and that they would, individually, receive the result. Samples of mostly fresh chicken feces were collected in pens and paddocks between 8 a.m. and 13 p.m. at the farms. When sampling on the farm, also the number of chickens were counted and written down. A total of 25 g feces samples should be taken at each farm, with a minimum of 5 different feces samples, preferably fresh cecal feces. This is because *Campylobacter* spp., when present, is a habitant of the ceca of the chickens. The samples were stored in a cool box during transport to the laboratory and processed within 5 h after collection. The fecal en cecal samples were examined for the presence of *Campylobacter* spp.

ii. Bacteriological examination of *Campylobacter* spp.

For identification of *Campylobacter* spp., first the *ISO 10272-1:2017(E)* method was used (ISO.org 2017) for all samples. From each fecal sample (from each farm), 25 g was taken and enriched in 225 ml Buffered Pepton Water in a Stomacher® bag to achieve a 10^{-1} dilution. The dilution was mixed by hand for 2 minutes to form a smooth suspension. 1 ml of the dilution was deposited in a *Campylobacter* selective broth (CCDB tube (code C)) and again mixed. Thereafter, the CCDB tube was incubated under microaerophilic atmosphere (6% O_2) at 42°C for 24 h. Next day, the suspension was mixed and subsequently a small sample was taken using a inoculation loop to transfer the inoculum on a *Campylobacter* selective agar plate (CCDA Preston). As quickly as possible, all CCDA plates were incubated under microaerophilic atmosphere (6% O_2) at 37°C for 48 h. On the last day, the CCDA plates were macroscopically inspected and the typical *Campylobacter*-like colonies (spreading grey/white colonies) were used for further analysis. First, a so called *catalase/oxidase* test was performed. The catalase reagent consists of a solution of 10 ml hydrogen peroxide (30% H_2O_2) in 90 ml distilled water. For the oxidase test, 100 mg of N,N,N',N'Tetramethyl-1,4-phenyleendiamine-dihydrochloride was dissolved in 10 ml distilled water. The *catalase* test was defined as positive when small gas bubbles appeared when the catalase solution was dropped on a small sample of suspected *Campylobacter* colonies. The *oxidase* test was confirmed when a cotton bud with a small sample of colonies immediately turned blue/purple after it came in contact with the oxidase solution. Only when both positive, the Thermo Scientific^{TM} Campylobacter Test (*TSCT*) (ThermoFisher 2019), a rapid latex agglutination test, was performed as final test for identification of enteropathogenic, thermophilic campylobacters cultured on the CCDA plates. Only positive results from the *TSC-test* are reckoned as *Campylobacter* spp. positive farms (sensitivity: 98.6%, specificity: 99,7%). As the manual of the *TSC-test* specify, differences can be seen in degree of agglutination. However, a positive results indicates, even if there is just a minor agglutination observed, that the feces contains *Campylobacter* spp.

iii. Data analysis

The outcome of the *TSC-test* will be used to examine the difference between the prevalence of *Campylobacter* spp. at children's farms and social care farms using a chi-squared test of independence (Petrie and Watson 2013). A 'One-at-a-time' sensitivity analysis will be performed when not enough farms are sampled. A OAT sensitivity analysis changes one-factor-at-a-time (in this case: changing one number of the positive or negative children's or social care farms) to see what the effect is on the output of the chi-squared test (Saltelli et al. 2008).

III. Results

i. Farm visits

In total 43 children's farms (28) and social care farms (15) were contacted. The 28 children's farms were selected for possible partaking of which 20 were actually called for invitation. They were selected on their location and presence of chickens. Seventeen of the 20 farms answered the call, of which all farms agreed to participate. Unfortunately, one children's farm could not be visited. Due to costs it would be not feasible to visit the farm, as it would be the only farm visit that day. As mentioned before, the other 16 children's farms all agreed to participate. Nine of the 15 social care farms participated in the study. Of the six which didn't participate, three could not be reached and the other three refused to join due to different (personal) reasons (*e.g.*: being afraid the results would have compulsory consequences for their farm, even when told that it would be not).
Five to 15 different feces samples had been collected at each children's farm and social care

Table 1: *Overview of the farms sampled including the number of chickens present at the farm. From each farm five to 15 fecal samples (including cecal samples) were taken. The number of cecal samples taken are showed separately. Last column shows if the farm is tested positive for Campylobacter spp..*

Children's farms #	**No. Chickens**	**No. Cecal samples** [a]	***Campylobacter* spp.**
Farm 1	10	2 (1x fresh)	Negative
Farm 2	10	2 (0x fresh)	Positive
Farm 3	10	1 (0x fresh)	Positive
Farm 4	3	1 (0x fresh)	Negative
Farm 5	12	2 (2x fresh)	Negative
Farm 6	10	5 (5x fresh)	Negative
Farm 7	34	4 (4x fresh)	Positive
Farm 8	28	1 (0x fresh)	Positive
Farm 9	9	3 (3x fresh)	Negative
Farm 10	15	2 (2x fresh)	Negative
Farm 11	7	1 (1x fresh)	Positive
Farm 12	11	3 (1x fresh)	Negative
Farm 13	15	3 (3x fresh)	Negative
Farm 14	30	4 (1x fresh)	Positive
Farm 15	10	2 (2x fresh)	Positive
Farm 16	7	2 (0x fresh)	Positive
Social care farms #	**No. Chickens**	**No. Cecal samples**	**Campylobacter**
Farm 17	14	2 (0x fresh)	Negative
Farm 18	10	2 (2x fresh)	Positive
Farm 19	250	4 (3x fresh)	Positive
Farm 20	18	2 (2x fresh)	Positive
Farm 21	10	2 (1x fresh)	Negative
Farm 22	40	3 (3x fresh)	Positive
Farm 23	50	2 (1x fresh)	Positive
Farm 24	30	2 (0x fresh)	Positive
Farm 25	10	2 (1x fresh)	Negative

[a] In addition, the number of fresh cecal samples are noted considering the fact that fresh cecal samples are preferred for optimal results (ISO.org 2017) (Hermans et al. 2011).

farm. At each farm it was possible to retrieve 25 g of feces and at least one cecal sample (see Table 1). However, not all of the samples were fresh fecal and/or cecal samples. As mentioned before, at all farms the number of chickens present were counted. The smallest farm (in number of chickens) had three chickens and the biggest 250. The average number of chickens at children's and social care farms was 14 and 48, respectively. Both combined the average was 26 chickens per farm. The median of children's farms and social care farms was 10 and 18, respectively. Both combined the median was 11 chickens per farm (Table 2).

ii. Bacteriological examination of *Campylobacter* spp.

The amount of CCDA positive samples on children's farms and social care farms were 10/16 (63%) and 7/9 (78%), respectively. From those 10 and seven CCDA positive samples, all but one were found positive on the *catalase/oxidase* test. The one, from a children's farm that was not found positive, had not been immediately tested after found positive on the CCDA plate. Therefore, nine samples from children's farms and seven from social care farms were tested with the *TSC-test*. Eight out of the nine children's farms tested positive with this test and

all social care farms tested positive (7/7). Thus, with both farm types combined, 17/25 (68%) were found CCDA positive, 16/25 (64%) were positive on the *catalase/oxidase* test and 14/25 (54%) were eventually confirmed with the presence of *Campylobacter* spp. using the TSC-test (Table 2).

iii. Data analysis

A chi-squared test of independence showed that there was no significant difference in prevalence of *Campylobacter* spp. between the children's farms and the social care farms, $X^2 = 0.6494$, $p-$value $= .420345$ ($p > .05$). Because only 25 of the 30 farms were sampled, a OAT sensitivity analysis was performed. In spite of this analysis, no significant differences were seen between the children's farms and social care farms (the $p-$value varied between 0.1531 and 2.6247 ($p > .05$)).

IV. Discussion

With 50 % and 67 % prevalence of *Campylobacter* spp. at the children's farms and social care farms, respectively, the results are roughly the same as in the earlier Dutch study performed, with 56,5 % and 50,5 % at children's farms and social care farms, respectively (Heuvelink et al. 2007). Thus, only a higher percentage has been found on the social care farms, however nine farms participated in the study in contrast to 131 farms from Heuvelink et al.. In spite of the substantial difference in farms participated, this study only looked at poultry feces instead of Heuvelink et al., where they combined their results from feces from different animals (*e.g.*: cows, pigs and goats). At farm level, the same amount of chickens housed are desired. Unfortunately, but unavoidable, considerable difference are seen between the number of chickens per farm, especially at the social care farms (Table 1). The number of chickens on social care farms varied from 10 to 50 (with a mean of 26), except for one farm that housed 250 chickens (the mean alters to 48). The difficulty with these numbers is that no national data are available on mean number of chickens per children's or social care farm. Yet, 250 chickens is probably the maximum kept at the farms because of the identification and registration obligation for farms with more than 250 chickens, resulting in more labor and responsibility for the farms (*Identificatie en Registratie RVO* 2019). In addition, the farms were not randomly selected, but selected on their location for logistics reasons and preparedness to participate, not looking at the number of chickens per farm. Thus, no evident conclusion can be made if the size of the sampled farms are comparable to the rest of the children's farms and social care farms in the Netherlands.

The chi-squared test of independence showed no significant differences in prevalence between the children's farms and social care farms ($X^2 = 0.6494$, $p-$value $= .420345$ ($p > .05$)). Furthermore, only 25 of the calculated 30 farms were visited due to time and costs. To analyze if it would make a difference when 30 farms were sampled, a OAT sensitivity analysis was performed (Saltelli et al. 2008). One number of the positive or negative children's or social care farms was changed with $+/-$ five to see what the effect is on the output of the chi-squared test. For example, if five extra positive samples were found on the social care farms, the prevalence would shift from 67% to 78 %. However, still no significant differences were seen in prevalence between farms when all possible variable factors were altered. Although the chance of a false positive or false negative sample is very unlikely due to the high the sensitivity (98.6%) and specificity (99,7%), a couple of other factors could have affected the results. For instance, the number of feces/cecal sampled was not standardized and varied among the farms. Also, not all feces/cecal samples were fresh, which is preferred for optimal results (ISO.org 2017). Furthermore, the highest concentration and the biggest chance of detecting *Campylobacter* spp. is found in fresh cecal samples (Hermans et al. 2011). Only one cecal sample could be collected at four farms. Interestingly, three of these four farms turned out positive for *Campylobacter*

Table 2: *Summary of the results from the farm visits and the different laboratory tests for Campylobacter spp. from chicken fecal samples collected on children's farms and social care farms.*

		No. positive/Total no. (%)[v]		
Farms (No. farms)	**$\bar{X}-$M No. Chickens[w]**	**CCDA+[x]**	**Cat/Oxi+[y]**	**TSC-T+[z]**
Children's farms (16)	14−10	10/16 (63%)	9/10 (56%)	8/9 (50%)
Social care farms (9)	48−18 †	7/9 (78%)	7/7 (78%) *	6/7 (67%)
Total (25)	26 − 11	68%	64%	**56%**

[v] % based on total number of farms.
[w] The mean ($\bar{X}$) & median (M) number of chickens at the farms.
[x] Found positive when *Campylobacter* spp. like colonies were found on the plates.
[y] Found positive when the colonies tested from the positive CCDA plates were positive for both the *catalase* and *oxidase* test.
[z] Found positive when the Thermo Scientific™ Campylobacter Test tested positive from colonies which were positive for both *CCDA* and *Cat/Oxi*.
† The wide difference between the mean and the median is a result of one large farm (250 chickens) in the group. When left out, the mean and median are 24 and 16, respectively.
* One sample was not immediately tested for the *catalase/oxidase* test. The sample turned out to be negative for both, but was positive for CCDA.

spp.. So, it is arguable if the number of cecal samples taken at a farm are important for finding *Campylobacter* spp.. Experimental studies at poultry farms show that chickens are highly susceptible to infection and can be colonized with doses as low as 10 colony-forming units (cfu). Within three days after colonization the animals become maximum colonized up to 10^9 cfu per gram of cecal content and are life long colonized (D.G. Newell and Wagenaar 2000). In addition, other studies demonstrate once the first chicken is detected with *Campylobacter*, the infection is transmitted rapidly throughout the whole flock with 100% positive chickens within days. Even when direct contact between animals is impossible (Shreeve, Toszeghy, Pattison, et al. 2000). In summary, when one chicken is infected it is most likely the whole flock is infected, which can explain the positive farms that only had one cecal sample.

Looking at the results of the study, *Campylobacter* spp. has been detected at least on 50% of the farms. But the question remains: are the farms that tested negative out of danger? Many studies have been done on prevention and elimination of *Campylobacter*, especially at commercial broiler farms and laying farms (DG Newell et al. 2011). The review of Newell at al. indicates that an adequate biosecurity is highly important for reducing *Campylobacter* spp, but sustaining such measures on the farms appears to be extremely difficult. If even commercial farms with intensive hygienic measures and biosecurity-based interventions are not able to prevent or eliminate *Campylobacter* spp., it can not be expected from children's farms en social care farms that they are capable of managing such infection. With this in mind, not a single farm can guarantee that they are 100% free of *Campylobacter* and should assume their chickens spread *Campylobacter* through their feces. On top of that, as long as the farms are obliged by the law to protect their employees, visitors and pupils on Health and Safety at work, appropriate hygienic measures should be taken to reduce the risk to a minimum (*Arbeidsomstandighedenwet* 2019). Examples of hygienic measures that are feasible: all visitors and employees should clean their hands regularly on the farm, especially when they come in direct contact with the animals. Furthermore, often chickens move freely at the farms, so locking them up should reduce the amount of direct contact with people. Minimize direct contact

between very young children, elderly, pregnant women or immunocompromised persons and animals, as they are more sensitive to a more serious infection with *Campylobacter*. Systematically cleaning and/or changing clothes and boots when leaving a pasture or pen by chancing overalls and using boot disinfection shows a decrease of 50% of flock colonization in commercial farms (Gibbens et al. 2001). These hygienic measures are easily applicable, however, the compliance of the staff and visitors are mandatory for a successful result (DG Newell et al. 2011).

In conclusion, the prevalence found was as expected looking at earlier studies along with no significant differences in prevalence between children's farms and social care farms. But, the exact understanding of *Campylobacter* spp. and the perfect strategy to control the infection in chickens and other animals, continues to be a mystery. Still, preventive hygienic measures should be taken to reduce the possible transmission of *Campylobacter* spp. and ultimately decreasing the *Campylobacter* infections in humans.

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