Campylobacter on processed chicken skin.

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Abstract:

Due to concerns over the prevalence of *Campylobacter* in chicken meat, member countries in the European Union (EU) undertook a surveillance program enumerating *Campylobacter* on chicken carcasses. A sample size of 25g of principally composed of neck skin was used, although breast skin could also be used if there was insufficient neck skin to meet the required sample mass. The aim was to establish a baseline for *Campylobacter* contamination of carcasses, against which future interventions could be assessed. However, in the United Kingdom (UK), it was considered that the differing ratios of neck to breast skin in samples could affect the results obtained. Accordingly, a comparison of the numbers of *Campylobacter* enumerated on neck and breast skin samples obtained from the same chilled chicken carcasses was undertaken at four different chicken slaughterhouses. It was determined that the neck skins were significantly ($P<0.05$) more heavily contaminated with *Campylobacter* compared with breast skin. Statistical analyses found that there was no relationship that would allow a conversion between counts obtained on the two skin types. Ongoing surveillance of *Campylobacter* over a period of six years was funded by UK poultry processors using samples consisting solely of neck skin and the results of this surveillance, undertaken between 2011 and 2016, are reported. Given the higher *Campylobacter* counts on a sample exclusively consisting of neck skins, this protocol would yield results whereby the industry would find it more difficult to achieve the contamination reduction target based on the EU baseline surveillance. This study found that the contamination reduction target for the UK of not more than 10% of chicken carcasses exceeding 1,000 CFU *Campylobacter*/g neck skin was not met by the UK government's target date of the end of 2015.

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Highlights:

- A comparison of *Campylobacter* on chicken neck and breast skin was undertaken
- Neck skin was significantly ($P<0.05$) more contaminated compared with breast skin
- No relationship between *Campylobacter* counts for the two skin types was found
- A UK government reduction target for highly contaminated chicken was not achieved

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Introduction

In 2008, the European Union (EU) undertook a survey of *Campylobacter* numbers in chicken broiler meat in 26-member states, plus Norway and Switzerland. This showed that the UK produced more broilers than any other country in the EU *(4)* and was ranked tenth worst in terms of *Campylobacter* prevalence of broiler carcasses with around 75% of samples testing positive. Around 67% of the UK samples had contamination more than 10 CFU *Campylobacter*/g skin sample. The European food safety authority has attributed 80% of human campylobacteriosis cases in the EU to poultry *(4)* and a British study, focusing on England and Wales, estimated that over 300,000 cases of campylobacteriosis and around 60 deaths were caused annually as a likely consequence of the *Campylobacter* associated with chicken meat *(1)*.

In response to the EU survey findings, a UK working group was established with members drawn from the Food Standards Agency (FSA, a UK government department), the British Poultry Council (BPC; a trade poultry processor association) and the British Retail Consortium (BRC; a trade association for larger retailers). The primary purpose of the working group was to identify and implement interventions aimed at reducing the numbers of *Campylobacter* on British poultry meat *(5)*. Consequently, the group pledged to undertake regular monitoring based on samples taken from broilers immediately post-chill in BPC-member slaughterhouses in order to monitor progress towards a reduction target agreed with the government regulator. Three bands for *Campylobacter* numbers (lowest <100, medium 100-1000 and highest >1000 CFU/g) were set in accordance with the EU baseline surveillance, along with target reductions in the percentages of the test samples that fell within each banding. The target for the highest band was a reduction from 27% of samples tested to 10% before the end of 2015 *(3)*.

A slaughterhouse-based continuous monitoring program was established by the UK poultry processing industry who donated the results of examinations of three pooled poultry neck skins processed as a single sample. *Campylobacter* enumeration was based on ISO-10272-2 *(13)* and plants collected at

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least five test samples from each of their processing lines on a weekly basis. The test sample was chosen because it is widely used in the EU for the statutory testing of poultry carcasses for *Salmonella (2)*.

For the EU *Campylobacter* survey *(3)*, the testing protocol was also based on ISO-10272-2. However, the sample collected was to comprise 25 g of neck skin, and should this weight not be achieved then the weight was to be made up by adding skin taken from between the neck and breast region (called the "neck extension region") of the same chicken carcass. Thus, a significant barrier to using the EU survey results as a baseline in the UK, and measuring changes against it, was the use of different test sample material. Consequently, this study was undertaken to determine if a statistical comparison of *Campylobacter* enumerated on both sample types collected from the same flock would show a relationship which would permit the results from the two sample types to be transformed from one type to the other.

Finally, we report the progress of the UK Poultry Industry over a six-year period, from 2011 to 2016, towards reducing the numbers of *Campylobacter* present on chicken broiler meat samples.

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Materials and Methods

Sample collections. Excision-based sample collection of neck skins was undertaken on moving lines during normal commercial processing immediately after the chilling phase of processing. Neck skin sampling involved turning a sterile 304 mm x 177 mm stomacher bag (Seward, Thetford, England) inside-out over a gloved hand and excising a carcass neck skin to provide a sample mass of at least 10 g without removing the carcass from the processing line. For the investigation of the relationship between individual and pooled neck skin counts, individual neck skins were stored in their own bags.

A comparison of counts of *Campylobacter* from neck and neck extension skin samples was also undertaken. Skin was excised from both regions of a single carcass that was removed from the processing line to facilitate the sample collections. To simplify the description of the two sample types 'neck extension skin' samples will subsequently be referred to as 'breast skin' samples. Each neck or breast skin sample was excised using a separate pair of sterile scissors and stored in separate stomacher bags on a layer of bubble wrap over crushed ice until the commencement of microbiological examination. Broiler skin samples for neck and breast comparisons were collected from four different processing plants and tested individually.

Microbiological examination. Maximum recovery diluent (MRD, Oxoid, Basingstoke, UK) was added to each sample (9:1, w/w) before homogenization for 1 min using a stomacher (Model number BA 6021, Seward, UK). Portions of diluent were removed from the sample for immediate quantitative enumeration of *Campylobacter*, or after mixing with other samples to form a combination sample. In both cases, the portions were vortex mixed (Genie, Fountain Valley CA USA; vortex mixer 2) for 10 s to ensure a homogeneous distribution of bacteria. *Campylobacter* were enumerated using the ISO-10272-2 protocol *(13)*. Decimal dilutions were made using MRD and plating was onto modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid). Incubation was under microaerobic conditions (CampyGen sachets, Oxoid) at 41.5°C for 48 h. Confirmation of *Campylobacter* spp. was by phase contrast microscopic

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examination of five colonies picked from Columbia blood agar subculture to confirm corkscrew motility, in addition to a lack of visible growth at 25°C under microaerobic conditions and at 41.5°C under aerobic incubation after 48 h. Presumptive colonies were also confirmed by positive oxidase activity.

Collection and reporting of UK poultry processing industry test results. A relational database (SQL Server 2008, Microsoft Corp. Redmond WA. USA) was used to store the microbiological test results. Three methods for the collection of industry test results were used:

- Copies of laboratory test result certificates were collected from participating plants. The laboratory test method was checked to ensure compliance with the ISO-10272-2 reference method, and the data were entered into the database by manual entry. Data entry errors were identified by periodic double entry of about 10% of the test results and comparison of the two datasets. An inputting error of >0.01% (1 error in 100 entered fields) triggered re-entry of all data from an entire session by a different person and subsequent re-comparison of both data sets.
- Results were also collected as electronic documents (e.g., spreadsheets (MS Excel 2010; Microsoft) or comma-separated value files) directly from testing laboratories. Electronic result submissions were electronically transformed (Excel) if required (i.e. to convert test results into a standard reporting format of CFU/g) and directly pasted into the database. All transformations were independently checked to ensure electronic submission was free from data manipulation errors.
- Three slaughterhouses entered their own test results into the database using a web interface; an approach that was not widely adopted. Basic validation of dates, bacterial numbers and sample types that were entered into the web database was undertaken to ensure sensible and appropriate inputs and to prevent the introduction of malicious computer code designed to disrupt the database. No independent verification of test results was undertaken for web-based results entry.

Processors were provided with an anonymised identity code that was used for all reporting. Results were reported in a manner agreed with the poultry processors supplying the test results. A range of reports was

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constructed, which included summaries for individual plants, a comparison between individual plants and the national dataset that contained all participating plants and group summaries for larger processors with multiple lines and processing plants.

Statistical Analyses. The numbers of *Campylobacter* colonies counted from breast skin or neck skin samples obtained from the same carcass were compared using the method of Bland and Altman *(7)*. In brief, the range between the two sample types was compared by evaluating bias, assessed as the mean log difference between the two sets of counts and ± twice the standard deviation of the differences, and bias tested using a paired t-test. Chi Squared or Fisher's exact tests were used as appropriate to test for any significant differences in the distribution of test results grouped as scores derived from counts into histogram-style bins. For all tests, the threshold for significance was $P<0.05$ unless otherwise stated.

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Results and Discussion

Since the routine enumeration of *Campylobacter* used three pooled samples, and detailed analysis required individual samples it was necessary to compare the results of enumeration undertaken by both methods, Table 1. When the calculated $\log_{10}$ mean of three randomly-selected, independently-tested chicken neck skin samples were compared with the $\log_{10}$ result of the same three samples physically combined into a single pooled test sample statistical comparison by t-test showed that there were no significant differences between the calculated mean and physically-combined results. The finding was consistent for the analysis of samples collected from four individual plants and when the results for all four plants were compared *en bloc*. When the statistical analysis was treated as two different methods for measuring the same parameter *(7)*, there was also no significant difference between the two sets of measurements. This means that it is possible to test chicken skin samples individually for *Campylobacter* numbers and use those results to predict the test results for a physically-combined test. A similar result has been shown previously for bacterial enumeration of individual and pooled swab samples taken from red meat carcasses *(11)*; although we believe this is the first time a similar finding has been reported for excised chicken skin.

Comparing the numbers of *Campylobacter* on the breast skin with the neck skin sample when both sample types were excised from the same carcass, Table 2, it was found that counts for the breast skin were significantly lower than those on the neck skin (paired t-test, $P < 0.05$, Table 2) for all four individual plants where samples were collected, and also when the test results from all four plants were compared *en bloc*. To visualize the relationship between the results of the two sample types a Bland and Altman *(7)* plot was prepared, showing the mean of the paired counts against their differences (Figure 1). There was a significant positive slope in the relationship between mean and difference. However, this was largely due to the two values on the right-hand side of the plot and significance was lost when they were discarded as outliers ($P = 0.77$), thus we can only assume a constant offset when converting from one sample type to

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the other. The finely dashed lines in Figure 1 show the 95% 'limits of agreement' (the mean difference ± 2 x standard deviations of the mean difference) between the two measures as being from -0.44 to 2.27 log CFU/g, that is the variability that could be expected when converting from one measure to another. Given the poor limits of agreement, it is obviously not practicable or useful to convert between two individual measurements.

A calculated approach was used to further investigate the real-world implications of using the EU surveillance data as a baseline that progress towards the reduction target could be measured against. Consideration was made that counts were higher on neck skin compared with breast skin. The baseline used a combination of breast and neck skin to ensure a minimum sample weight of 25 g, whereas industry-supplied results were derived solely from neck skin. This study used the results of individually-tested sample components and the consequent calculated combined (pooled) result. The comparisons were based on the calculated combined neck and breast skin taken from one carcass compared with that neck skin combined with two neck skins taken from near-adjacent birds on the processing line, to form a standard pooled sample. The results (Table 3) show that for three out of the four plants where samples were collected, the counts from the two sample types were significantly different. For the remaining plant, P=0.05; which was on the cusp of significance. When the results from all four plants were analyzed *en bloc*, the two sample types were strongly significantly different. It was more cost-effective for the UK poultry processing industry to take one sample for statutory *Salmonella* testing and to use a portion of that same sample for the voluntary *Campylobacter* testing. However, changing the skin type tested from a combination of neck and breast skin to pooled neck skin would make it more difficult to achieve the UK target for *Campylobacter* reductions on broiler carcasses if compared with the EU surveillance as a baseline, due to the greater numbers on the latter samples. In January 2018, the EU introduced process criteria for broilers of ≤1000 CFU *Campylobacter*/g in ≤20 out of 50 pooled neck skin samples. The criteria became law in all 28 EU member states and will become progressively stricter in 2020 (15/50) and 2025

(10/50). Our findings are strongly indicative that the sample change to pooled neck skins means comparisons should not be made with historical baseline surveillance.

Table 4 contains the information required to allow an assessment of how much of an impact the sample change would cause. Table 4 sorted researcher-collected and tested results into the histogram-style bins that were used to determine if the *Campylobacter* reduction target for poultry meat had been met. Based on the test results of samples collected in four high-throughput chicken processing plants, it was apparent that the three-pooled neck skin sample had higher numbers of test results in the highest band compared with the sample type (neck and breast) used for the baseline survey. We also noted that 63.3% of single neck skins (Table 4) were in the highest contamination banding. However, when the single skins were pooled into groups of three, the percentage of highly contaminated samples increased to 85%. A possible explanation is that some of the neck skins were highly contaminated and so increased the average count after pooling. However, without further investigation such an explanation is speculative.

An exact Chi square test using the UK baseline survey bin values as the expected range showed the differences in sample numbers assigned to each bin were significantly elevated ($P<0.001$) for the pooled neck skins compared with the EU baseline survey sample type.

Progress towards the 2015 *Campylobacter* reduction target *(3)* was also assessed over an almost six-year period using test results donated by the UK poultry processing industry. An anonymous, percentile-based overview of the distribution of the donated test results by year is shown as Figure 2 and progress towards the performance target is shown as Table 5. The shapes of the graphs between the 30th and 95th percentiles shown in Figure 2 were similar across all six years. However, for years 2015 and 2014, there were small numbers of exceptionally highly contaminated neck skins which contained more than 10^7 CFU/g neck skin. In general, most neck skin samples were contaminated below 10^4 CFU/g. The results depicted in Table 5 are from a survey representing more than 95% of the UK national throughput over a period of several years. A Chi Square test of the table showed a highly significant difference between banding

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between years. The 2015 dataset had elevated numbers of neck skins in the most contaminated band and reduced numbers of neck skins in the least contaminated band compared with the other years (Table 5). We note that parts of the UK were subject to abnormally high rainfall in 2015 and that 2014 was also wet with elevated numbers of highly contaminated neck skins compared with the other years (Table 5). However, it has been reported previously that there is no obvious relationship between rainfall and colonization of broiler caeca by *Campylobacter (6)*. Using the industry-supplied test results and an expanded dataset of around 15,000 results for 2015 as the deadline loomed, the JWG reduction target that the highest level of contamination, (>1,000 CFU/g skin) would fall to 10% by the end 2015 was not met. However, the least contaminated chickens (<100 CFU/g), was no worse than the measured baseline of around 42% (Table 5).

Our recent observations in UK poultry slaughterhouses are that over the last few years there have been widespread alterations of processes to include additional chilling capacity and automated neck skin trimmers. The trimmers are a deliberate strategy by poultry processors to remove a heavily contaminated section of the carcass as a way of reducing the total carcass load and the risks of *Campylobacter* infection associated with consumption of fresh poultry meat *(14, 15)*. Over the same period there have been reports of less contaminated whole chicken in the UK, assessed by the testing of carcass neck skins purchased at retail. A great deal of effort has been expended by the poultry industry internationally to reduce contamination incidence and numbers of *Campylobacter* on broiler carcasses, particularly on farms *(8-10, 16)*. Although the farm biosecurity efforts are commendable, it is not clear at present why the reported reductions occurred and if the removal of a significant portion of neck skin has resulted in a greater proportion of a skin test sample being composed of less contaminated breast skin. In the UK it may be that the introduction of automated neck skin trimmers could be used to re-evaluate the sample used for routine surveillance. Although it is more time consuming, previously we have reported favorably on the use of whole carcass rinsing as a sample collection method that provides test results with quite low variance between birds for a variety of indicators *(12)*.

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Using industry-donated test results, this study found that the reduction target for the UK of not more than 10% of chicken carcasses being highly contaminated was not met by the end of 2015. Higher numbers of *Campylobacter* are found on neck skin compared with breast skin and so the change of the test sample made it more difficult to achieve the contamination reduction target when assessed against the EU baseline surveillance.

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Figure legends

Figure 1 Bland and Altman plot showing the relationship between the mean and the difference of individual, paired neck skin, and breast skin, samples excised from chicken broilers after the chilling stage of processing. The solid horizontal line shows the average difference between the two types of sample and the lightly dashed lines the 'limits of agreement' (equal to the mean difference ± 2.SD).

Figure 2 Annual percentile summaries of the numbers of *Campylobacter* supplied by the UK broiler processing industry. Isolations were from post-chill chicken broiler neck skins on 23 UK processing lines representing more than 95% of the national throughput. Summaries are for January to December for the years shown in the top right of each graph. The numbers of samples collected for each year from 2016 to 2011 respectively were; n=2912, n=15100, n=8265, n=5684, n=5279 and n=3291.

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Table 1 The relationship between a calculated log_{10} mean of three individual chicken broiler neck skin *Campylobacter* examinations and the test result of the same three samples physically combined into a single sample. The results were analyzed by paired t-test and the difference and standard error (SE) of the difference between samples is reported. The SD reported is the standard deviation of the log mean count of the individual and combination neck skin samples.

Plant identifier	Mean log mean count of batches of three randomly-selected neck skins tested individually (CFU/g) ± SD	Number of results (samples tested)	Mean log *Campylobacter* numbers of the same three samples physically combined into a single sample (CFU/g) ± SD	Number of samples	P value (paired t-test)	Difference between physically-combined and calculated mean (CFU/g)	SE (CFU/g)
A	3.45 ± 0.31	10 (30)	3.46 ± 0.45	10	0.960	0.01	0.11
B	3.28 ± 0.46	10 (30)	3.31 ± 0.40	10	0.903	0.03	0.21
C	3.33 ± 0.75	10 (30)	3.37 ± 0.80	10	0.432	0.04	0.05
D	4.07 ± 0.61	10 (30)	4.10 ± 0.72	10	0.557	0.03	0.05
Combined	3.53 ± 0.62	40 (120)	3.56 ± 0.68	40	0.676	0.03	0.06

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Table 2 Difference between the log numbers of *Campylobacter* counted on chicken broiler neck skin and breast skin taken from the same carcass. SD is the standard deviation and SE is the standard error of the difference between results.

	Mean log *Campylobacter* numbers (CFU/g)		
Plant identifier	Mean difference between log neck skin and breast skin counts ± 2SD of the difference	Number of samples compared	SE of the difference between sample types
A	1.14 ± 1.24	20	0.14
B	0.77 ± 0.94	20	0.11
C	0.91 ± 1.92	20	0.22
D	0.84 ± 1.04	20	0.12
Combined	0.92 ± 1.36	80	0.08

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Table 3 A summary of *Campylobacter* test results from researcher-collected and tested samples of chicken broiler skin and the results of t-tests for significant differences. Significantly different comparisons are denoted *. Mean log numbers of *Campylobacter* are shown ± the standard deviation (SD).

Plant identifier	Mean counts of batches of three randomly-selected neck skins tested individually ± SD		Mean counts of neck skin and neck skin extension tested individually ± SD		P value
	Mean log *Campylobacter* numbers (CFU/g) ± SD	Number of results (samples tested)	Mean log *Campylobacter* numbers (CFU/g) ± SD	Number of samples	(t-Test)
A	3.45 ± 0.31	10 (30)	3.05 ± 0.54	20	0.050
B	3.28 ± 0.46	10 (30)	2.78 ± 0.73	20	0.045*
C	3.33 ± 0.75	10 (30)	2.60 ± 0.82	20	0.022*
D	4.07 ± 0.61	10 (30)	3.45 ± 0.71	20	0.027*
Combined	3.53 ± 0.62	40 (120)	2.97 ± 0.76	80	<0.001*

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Table 4 A summary of *Campylobacter* test results for researcher-collected and tested chicken skin samples. Test results were sorted into the contamination ranges used for monitoring progress towards the UK *Campylobacter* reduction target for poultry meat.

Skin sample type (number of samples)	Number of samples in each banding (percentage %)		
	<100 CFU g^{-1}	100-1000 CFU g^{-1}	>1000 CFU g^{-1}
Single breast (80)	32 (40.00)	37 (46.25)	11 (13.75)
Single neck (120)	80 (6.67)	36 (30.00)	76 (63.33)
Neck and breast (80)	9 (11.25)	31 (38.75)	40 (50)
Three pooled neck (40)	0 (0)	6 (15)	34 (85)

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Table 5 A summary of progress towards the *Campylobacter* reduction target in the UK between 2011 and 2016. Pooled neck skin samples were tested to determine the numbers of *Campylobacter* by 23 UK poultry processors.

Year	Percentage (%) of samples in each band (number of test results in each band)		
	<100 CFU/g	100-1000 CFU/g	>1000 CFU/g
2016	54.60 (1577)	28.13 (785)	17.27 (550)
2015	42.53 (6636)	25.49 (3799)	31.98 (4989)
2014	50.54 (4165)	22.53 (1857)	26.93 (2219)
2013	60.56 (3548)	21.97 (1287)	17.48 (1024)
2012	54.37 (2887)	24.14 (1282)	21.49 (1141)
2011	60.31 (1969)	22.82 (745)	16.88 (551)