Methods

4.1 Data sources and recovery methods for isolates

The analysis presented was based on collated data from eight studies based on phenotypic AMR results and data from two studies based on predicted AMR profiles from genome sequence data. The databases with AMR data have been included in an excel file detailing all isolates from these studies (Appendix 1). An overview of the source datasets is presented in Table 1a and Table 1b.

The so-called PHE (now known as UKHSA) 1, 2 and 3 datasets represented datasets from the earliest FSA projects investigating AMR in Campylobacter spp. recovered from retail chicken that included portions and frozen chicken meat samples. The PHE 4 dataset represented Campylobacter spp. isolates from retail chicken during the FSA funded project FS102121 (PHE, 2015; PHE, 2017; PHE, 2018; PHE 2019; all under the project title of “A microbiological survey of Campylobacter contamination in fresh whole UK produced chilled chickens at retail sale”); all were speciated and AMR tested at the UKHSA Gastrointestinal Bacteria Reference Unit (GBRU).

Table 1a. Overview of datasets used to analyse trends in AMR in C. jejuni and C. coli detected in chicken in the UK.

Data set	Sampling	Type of samples	Isolate recovery(a)	Total num selection
APHA 1, 2007 to 2008	Broiler (chicken) flocks at slaughter, sampling weighted to sample proportionally based on slaughterhouse throughput. More than 85% of UK broiler production reflected in the sampling frame, stratified to ensure year round coverage.	Caecal contents, pooled from 10 broiler chickens collected at slaughter. Each batch of broilers originated from a single farm house.	From direct culture (mCCDA agar) or enrichment followed by culture (mCCDA)	In 2007, fr positive ba 55 C. coli In 2008 fro positive ba 45 C. coli

Data set	Sampling	Type of samples	Isolate recovery(a)	Total num selection
APHA 2, 2012 to 2016	Broiler flocks at slaughter, weighted to sample proportionally based on abattoir throughput. More than 85% of UK broiler production reflected in the sampling frame, stratified to ensure year round coverage.	A) Caecal contents, pooled from 10 broilers. Each batch of broilers originated from a single farm house. B) Neck-skin sample from a single carcase chicken sampled post-chill.	A and B: From direct culture (on mCCDA)	A) C. jejur at random (n=61), 20 (n=180). I selected fo B) WGS w jejuni and 2012 (ran isolates bu round cov 2015 all av selected w coli in 201 in 2014, 2 2015
APHA 3, 2018	Broiler flocks at slaughter weighted to sample proportionally based on throughput. More than 60% of UK broiler production reflected in the sampling frame, stratified to ensure year round coverage.	Single broiler caecal content.Each broiler was from a single chicken house.	Direct from mCCDA	170 C. jeju but stratifi coverage.

Table 1b. Overview of datasets used to analyse trends in AMR in C. jejuni and C. coli detected in chicken in the UK.

Data set	Sampling	Type of samples	Isolate recovery(a)	Total number of samples, selection of isolates	AMR method

PHE1, FSA 2001 survey	Retail – designed to reflect market share	Fresh/frozen, whole/portions, skin/meat, UK retail (with a small % non-UK origin)	Direct from mCCDA or via enumeration on mCCDA	In total 4866 samples; of 2697 campylobacter-positive samples 1208 C. jejuni and 421 C. coli were tested (aiming for one isolate per sample; limited random drop-out due to isolate die-off). 982 isolates from enrichment; 647 isolates direct from mCCDA	Break-point
PHE2, FSA 2007-2008 survey	Retail – designed to reflect market share	Fresh/frozen, whole/portions, skin/meat, UK retail (with a small % non-UK origin)	Detection (enrichment then mCCDA)	In total 3274 samples were tested; 1358 were campylobacter-positive; from these 803 C. jejuni and 714 C. coli were tested for AMR (aiming for one isolate per sample; limited random drop-out)	Break-point

PHE3, CLA SSP 2004-2007 survey	Retail – random sampling from retail stores	Fresh/ frozen, whole only, UK retail (with a small % non-UK origin)	Enrichment (Bolton broth and mCCDA)	In total 2264 samples were tested; 1804 were campylobacter-positive; from these 800 C. jejuni and 389 C. coli were tested for AMR ; (aiming for one isolate per sample – limited random drop-out due to isolate die-off)	Break-point
PHE4, FSA 2014-2018 survey	Retail – probably reflecting market share; neck-skin	Fresh, whole only, UK	Direct enumeration (mCCDA)	~13000. Every nth(b) isolate (organic and free-range bias)	Break-point
PHE5, FSA-MIC 2017 survey	Retail – reflecting market share; meat/skin	Fresh/frozen, whole/ portions (with a small % non-UK origin)	Direct enumeration (mCCDA)	Several picks per sample. All attempted (random drop-outs due to isolate die off); one isolate of each C. jejuni and C. coli used from each sample.	MIC
PHE 6WGS, 2018-2020 survey	Retail – only from non-major retail stores; neck-skin	Fresh, whole chicken of UK origin	Direct enumeration (mCCDA)	One per sample; random drop-out due to isolate die-off	WGS predicted

(a) ISO 10272-1:2006 and ISO 10272-2:2006; (b) Random non-recoverable isolates replaced by next available isolate.

In the PHE 4 dataset isolates were obtained from fresh, raw, whole UK produced chicken collected from retail stores across the UK. Samples were collected from different types of stores including both major as well as minor retailers and details of samples can be found elsewhere (PHE, 2015; PHE, 2017a; PHE, 2018a; PHE, 2019). A proportion of isolates were tested for their

antimicrobial susceptibility properties; every tenth isolate (or next viable isolate) were included, although selection was adjusted to ensure representation of producer premises and retailers as deduced from market share data. In an attempt to improve representation from free range and organic chicken, all recoverable isolates (i.e. able to grow after frozen storage) from organic and a high proportion of isolates from free range chicken were included. The PHE samples were collected from the point of retail and consisted of whole carcases or portions, and in some studies frozen samples were included. All PHE testing was carried out on skin or meat samples.

The source of the Campylobacter isolates in each dataset was from (broiler) chicken. The APHA datasets contained isolates from samples collected from structured slaughterhouse surveys representative of UK broiler production at the time, accounting for > 60% of UK broiler throughput (APHA dataset 1: Lawes et al., 2012; APHA dataset 2: Lawes, 2017; APHA dataset 3: VARSS, 2019). For these datasets caecal samples were collected from flocks at slaughter and carcases were collected after chilling for neck-skin sampling. Within each APHA dataset the isolates selected for MIC testing were representative of the wider selection of isolates collected from the parent survey.

The detailed description of laboratory methodology used in each dataset was published previously (APHA dataset 1, Lawes et al., 2012; APHA dataset 2, Lawes, 2017; APHA dataset 3, VARSS, 2019; PHE, 2016; PHE, 2017b; PHE, 2018b; PHE, 2020; PHE, 2021). In general, for all studies, Campylobacter spp. were isolated using methodology based on ISO-10272. This was mainly by direct culture on mCCDA agar (as described in ISO-10272 part 2) or for a subset of samples via enrichment broth and culture onto mCCDA or Preston agars (as described in ISO-10272 part 1). While we cannot rule out sampling bias in the source datasets there is no evidence to suggest they would present a biased sample of Campylobacter isolates from chicken over the time frame studied (from 2001 to 2020), although coverage was not continuous. All samples were collected by trained personnel and all testing laboratories participate in External Quality Assurance schemes and operate comprehensive internal quality assurance schemes as part of the requirements of their accreditation to ISO 17025/2005 and were assessed annually by the United Kingdom Accreditation Service (UKAS). All analyses were performed by trained and competent staff in UKAS accredited laboratories operating an appropriate quality management system. The UKAS accreditation pertaining to the phenotypic antimicrobial susceptibility testing at PHE was according to the ISO 1518:2012 standard. Phenotypic AMR testing at APHA was compliant with the EU decisions and technical guidance at the time of testing, with laboratories participating in external quality assurance exercises to verify assay performance.

Two additional datasets with genome sequence-based AMR data contained isolates not already present in the phenotypic datasets. One dataset represented isolates (773 C. jejuni and 255 C. coli) from chicken sampled between August 2018 and October 2020 from retail stores not part of major chains (as part of project FS102121; PHE, 2021). The other dataset represented isolates (863 C. jejuni and 209 C. coli) obtained from chicken neck-skin samples at slaughter and were sampled between 2012 and 2015 (as part of FSA project FS241051 and subsequently genome sequenced as part of FSA project FS101013; University of Oxford, 2021). For both these datasets genome sequencing of one isolate from every sample testing positive for Campylobacter spp. was attempted (for a limited number of the samples testing positive for campylobacters initially, no isolate was tested for AMR due to loss of viability after frozen storage).

In total AMR profiles were predicted by genome sequencing for 1,636 C. jejuni and 464 C. coli isolates.

4.2 MIC and breakpoint harmonisation and adjustments

All minimum inhibitory concentration (MIC) testing was by the microbroth dilution method, using the sensititre system. MIC testing at APHA laboratories was in compliance with the relevant EU

Commission Decision and EFSA technical specifications, in place at the time of sample collection. All breakpoint testing carried out at GBRU was done using Muller Hinton Agar containing specified breakpoint concentrations of antimicrobials to determine resistance. Briefly this was performed as follows: preparation of a suspension of each isolate in sterile saline to McFarland 0.5 turbidity and inoculation onto the surface of each of the antimicrobial containing agars. An isolate was considered resistant when growth was detected on the agar containing the antimicrobial, but scored sensitive if no growth was observed and the corresponding antimicrobial-free plate showed pure growth from the suspension applied.

The prediction of antimicrobial resistance by analysis of whole genome sequence data, generated by standard methods, was via the UKHSA pipeline (Painset et al., 2020). In general the antimicrobial resistance profiles created within the collated datasets were as recommended in the ECDC and EFSA protocol for harmonised monitoring of antimicrobial resistance in human Salmonella and Campylobacter isolates (EFSA and ECDC, 2016) and the EU Commission decision (Decision 2013/652/EU). However, across the datasets there was some variation in the criterium to define a resistant organism. To allow comparison of resistance rates between the different datasets, it was necessary to adopt a harmonised approach for the determination of a resistant C. jejuni or C. coli isolate. In this study the ECOFF thresholds defined by ECDC for CIP, ERY, GEN and TET were used to define a resistant isolate. These did match the thresholds defined in EU Commission decision with the exception of the C. jejuni threshold for TET; the difference was very minor (1 mg/l to 2 mg/l) and extremely unlikely to affect determinations of trends on TET resistance. For STR and NAL the thresholds were defined by the EU decision, as there are no thresholds specified by ECDC. This approach aligns with thresholds used to validate the calling of AMR genetic determinants via the UKHSA pipeline for these antimicrobials (Painset et al., 2020; although resistance to NAL was not included in validation for the pipeline calling of genetic determinants for AMR). The harmonised MIC thresholds and threshold used in this study are presented in Table 2.

Table 2. Harmonised MIC and break point (BP) thresholds used in this study in mg/l.

Antimicrobial	C. jejuni MIC	C. jejuni BP	C. coli MIC	C. coli BP
Ciprofloxacin	>0.5	0.5	>0.5	0.5
Nalidixic acid	>16	16	>16	16
Erythromycin	>4	4	>8	8
Tetracycline	>2*	2	>2	2
Gentamicin	>2	2	>2	2
Streptomycin	>4	4	>4	4

*EFSA interpretative threshold is >1 = resistant

In some of the earlier PHE datasets that were based on breakpoint testing, different thresholds applied compared to the current harmonised thresholds shown in Table 2. To allow comparison between earlier and more recent datasets, an adjustment factor for the earlier data was calculated. In summary, when an original threshold was higher than the harmonised threshold, there would be an underestimate of resistance occurrence in the original data set. As an example, the effect of changing the threshold for GEN and C. jejuni is outlined in Table 3. If the harmonised threshold was 2 but in an earlier dataset a threshold of 4 was used, any isolate with an MIC of 4 would change from being sensitive to resistant with the harmonised threshold. Therefore, the adjustment needs to account for the proportion of isolates determined sensitive by the original threshold (equivalent to an MIC of 4 or less) that could have an MIC value of 4.

Table 3. The effect of changing the threshold for gentamicin resistance (expressed as the break point (BP)) on a population of C. jejuni.

MIC	Number of isolates (a)	Susceptible (S) or Resistant (R) if MIC is >2)	Expected outcome with harmonised BP (2)
?0.016	0	S	No growth (S_)
0.03	7	S	No growth (S_)
0.06	20	S	No growth (S_)
0.12	625	S	No growth (S_)
0.25	3593	S	No growth (S_)
0.5	6264	S	No growth (S_)
1	708	S	No growth (S_)
2	35	S	No growth (S_)
4	**1**	**R**	**Growth (R_)**

MIC	Number of isolates (a)	Susceptible (S) or Resistant (R) if MIC is >2)	Expected outcome with harmonised BP (2)
8	2	R	Growth (R_)
16	0	R	Growth (R_)
32	2	R	Growth (R_)
64	41	R	Growth (R_)
128	0	R	Growth (R_)
256	0	R	Growth (R_)
>512	0	R	Growth (R_)

(a) MIC distribution source (EUCAST, 2012) (Eucast MIC data 2012)

EUCAST provide distributions of MIC values for C. jejuni and C. coli, and these were used to estimate the proportion of these isolates in a population (Table 3). A plausible assumption was made that the distribution generated by EUCAST was representative of the distributions of Campylobacter in the datasets in this study. In this example of 11,298 C. jejuni isolates and resistance to GEN, 11,253 isolates had an MIC value of 4 or lower, whilst only one isolate had an MIC value of four. Hence the percentage of sensitive isolates (original threshold) that should be re-classified to resistant is 0.01% and 99.99% of the original sensitive isolates would remain sensitive. In this example the change from original to adjusted will be minor (Table 3). A similar process is followed when the original threshold is lower than the harmonised threshold, which leads to an overestimation of resistance in the original dataset. This process was repeated for each antimicrobial for C. jejuni and for C. coli. A summary of the estimated adjustments required is presented in Table 4, and it is apparent that in most cases the adjustments were neglible. The one exception is for C. coli and ERY, whereby the adjustment results in the number of resistant isolates being reduced by approximately 25% for some of the earlier PHE datasets.

Table 4. Adjustment to the percentage of resistant Campylobacter jejuni and C. coli isolates taking into account changes in threshold concentrations to ensure harmonisation.

Antimicrobial	Species	Original threshold	Harmonised threshold	Adjustment direction
Ciprofloxacin	C. jejuni	1	0.5	Sensitive decrease
Ciprofloxacin	C. coli	1	0.5	Sensitive decrease
Erythromycin	C. coli	4	8	Resistant decrease
Tetracycline	C. jejuni	8	2	Sensitive decrease
Tetracycline	C. coli	8	2	Sensitive decrease
Gentamicin	C. jejuni	4	2	Sensitive decrease
Gentamicin	C. coli	4	2	Sensitive decrease

In this study the definition of multidrug resistance (MDR) was defined in accordance with that used in the 2014 antimicrobial resistance report for the EU (EFSA and ECDC, 2016), specifically this is organisms that display resistance to at least three different classes of antimicrobial.

In summary data analysis was performed to:

1. Explore the trends in resistance to six antimicrobials for C. coli and C. jejuni isolates from chicken sampled in the UK from 2001 to 2020.
2. Adjust the percentages of resistant isolates for the analysis to explore the impact of changes to the antimicrobial thresholds over time.
3. Investigate the relationship between antimicrobial resistance and other variables, for example chicken production type and season.
4. Also analyse WGS derived resistance data to determine any impact of including those on trends.

Data
A file from the database was up-loaded with the following fields:

- Unique sample ID
- Sample dataset name
- Sampling year

- Sampling month
- Sample type (caecal/slaughterhouse carcass/retail fresh carcass/retail fresh portion/retail frozen carcass/retail frozen portion)
- Sample production type (Standard/Free range/Organic)
- Sample origin (UK/Other)
- Campylobacter species (C. jejuni/C. coli)
- Ciprofloxacin (S/R)
- Nalidixic acid (S/R)
- Erythromycin (S/R)
- Tetracycline (S/R)
- Gentamicin (S/R)
- Streptomycin (S/R) (not available for all isolates)

Descriptive analysis
AMR trends for C. jejuni and C. coli (separately), were plotted for samples (regardless of sample type) from the UK. Confidence intervals in figures show the likely range of the results allowing for the number of samples taken. The 95% confidence intervals mean that we would expect the true prevalence to fall within the lower and upper confidence limits 95% of the time.

4.3 Statistical analysis

The percentage of resistant isolates (using both phenotypic and genome-sequenced based AMR data) over time were presented in figures created in MS Excel 2013. All other statistical analyses were performed in STATA 15.

Pearsons chi squared tests were used to investigate the relationship between eight categorical exposure variables and the antimicrobial resistance outcomes based on phenotypic data. Exposure variables included: sampling year, sampling year category (derived from sampling year, considered as an alternative to sampling year), sample category (caeca, whole bird, portions), production category (conventional, free range, organic), origin category (UK, non-UK), sampling month, season (derived from sampling month (December, January and February = Winter), considered as an alternative to sampling month). Processing plant origin was not included in the analysis as there was insufficient data available to analyse this factor.

Season was available for all except for seven isolates; a small proportion of samples were frozen -these were all from retail in the years from 2001- 2008.

Univariate analysis was performed to explore the unadjusted odds ratio for antimicrobial resistance in each risk factor category against a baseline category. The strength of association between each risk factor and the antimicrobial resistance in question was used to determine the stepwise order in which variables were included in the multivariable model. Only variables with a p value of < 0.25 were tested in the multi variable model. Mantel-Hanzel odds were used look for evidence that the sampling year acted as an effect modifier on the other variables included in the model. The final multivariable logistic regression model for each antimicrobial was created in a stepwise fashion, testing the strength of the model with each new exposure variable against the preceding model using a likelihood-ratio test. Where the inclusion of a variable significantly improved the fit of the model to the data, it was included, and the next variable was tested. Where no significant improvement was made it was rejected from the final model. The final model was tested using a Goodness of fit test and by calculating the area under the ROC curve.

Cross tabulations were analysed by the calculation of Clopper-Pearson exact 95% confidence intervals for the proportion in each category. In addition, the Pearson chi square test of association has been used to test the null hypothesis of no association between the measured variable and AMR in Campylobacter. Fisher’s exact test was used for individual comparisons when sample sizes were small.