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Surveillance Study of Antimicrobial Resistance in Bacteria Isolated from Chicken and Pork Sampled on Retail Sale in the United Kingdom.

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Abbreviations

AFBI	Agri-Food and Biosciences Institute
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
APHA	Animal and Plant Health Authority
BPW	Buffered Peptone Water
BSAC	British Society for Antimicrobial Chemotherapy
ºC	Degrees Celsius
Cfu	Colony forming units
CI	Confidence Interval
CTX	Cefotaxime
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off value
EFSA	European Food Safety Authority
EQA	External Quality Assurance
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FSA/FSS	Food Standards Agency/Food Standards Scotland
FW&E	Food Water and Environmental
g	Gram
GBRU	Gastrointestinal Bacteria Reference Unit
h	Hour(s)
ISO	International Organisation for Standardisation
l	Litre
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LIMS	Laboratory Information Management System
mCCDA	modified Charcoal Cefoperazone Deoxycholate Agar
mg	Milligram
ml	Millilitre
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
MIC	Minimum inhibitory concentration
MDR	Multi-drug resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
N	Number
PCR	Polymerase chain reaction
PHE	Public Health England
SOP	Standard Operating Procedure
spp.	Species (plural)
UKAS	United Kingdom Accreditation Service
VRE	Vancomycin-resistant enterococci
WGS	Whole Genome Sequencing

1. Executive Summary

A systematic review of antimicrobial resistance (AMR) in the food chain by the Food Standards Agency (2016) concluded that there was a lack of data on AMR prevalence in British-produced food, leading to difficulty in monitoring trends or producing risk assessments for the exposure of consumers. A key recommendation from this review was to address these gaps in evidence by developing research and surveillance to monitor AMR levels in foodborne pathogens and commensal bacteria in poultry and pork meat. As a result, a short-term, cross-sectional surveillance study was carried out over a two month period to determine the prevalence of AMR in pathogenic and indicator bacteria isolated from fresh/frozen chicken (whole or portions) and fresh pork mince on retail sale in the United Kingdom.

Between the beginning of September and end of October 2017, a representative survey based on market share data collected 339 samples of raw chicken (whole or portions) and 342 samples of raw pork mince from retailers in England, Wales, Scotland and Northern Ireland. Samples included both domestically produced and imported meats. These were tested for *Escherichia coli* (including ESBL-producing *E. coli), Klebsiella* spp. and enterococci, as well as *Campylobacter* spp. in the case of chicken samples, and *Salmonella* spp. in pork. One isolate of each bacterial type from each sample was randomly selected for additional testing to determine the minimum inhibitory concentration (MIC) for a range of antimicrobials. The selection of a single isolate from each sample removed the risk of introducing bias in the results through the analysis of multiple identical isolates from the same sample. For some samples, additional *Campylobacter* isolates were examined in order to assess the variation in resistance between multiple isolates from the same sample of chicken. Whole Genome Sequencing was used to further characterise all *Salmonella* isolates and a selection of *E. coli* and *Klebsiella* isolates that showed resistance to multiple antibiotic groups.

Salmonella spp. were only detected in 5/342 (1.5%) of pork mince samples, of which four were identified as *Salmonella enterica* serovar Typhimurium and one as *Salmonella enterica* serovar Derby. All four *S.* Typhimurium isolates were resistant to ampicillin and tetracycline (as well as having reduced susceptibility to sulfamethoxazole), with one also showing resistance to chloramphenicol, whilst the *S.* Derby isolate was susceptible to all antimicrobials except sulfamethoxazole. None of the salmonellae had phenotypes consistent with the production of ESBL or AmpC enzymes.

Campylobacter spp. were detected in 85/339 (25%) of all chicken samples (fresh and frozen). Freezing has substantial effect on the number of *Campylobacter* cells. In this study all 34 frozen samples out of the 85 total were found to be negative for *Campylobacter*. Determination of MICs was performed for a total of 157 *C. jejuni* and 45 *C. coli* isolates from 79 samples. Isolates from the remaining 6 samples were not recoverable and no MIC determination was undertaken for these. Of the *C. coli* isolates 46.7% (21/45) were resistant to ciprofloxacin, 6.7% (3/45) resistant to erythromycin and 60% (27/45) resistant to tetracycline. For the *C. jejuni* isolates 38.9% (61/157) were resistant to ciprofloxacin, 7.6% (12/157) resistant to erythromycin and 61.8% (97/157) resistant to tetracycline. All isolates were sensitive to gentamicin and only one *C. coli* isolate was resistant to streptomycin. Multidrug resistance was found in 8.9% *C. coli* (4/45) and 0.6% *C. jejuni* (1/157). Of 66 samples from which *C. jejuni* was detected, ciprofloxacin resistant *C. jejuni* were detected in 25 samples (38%), erythromycin resistant in 6 samples (9%) and tetracycline resistant in 39 samples (59%). Of 21 samples from which *C. coli* was detected, ciprofloxacin resistant *C.*

coli were detected in 8 samples (38%), erythromycin resistant in 3 samples (14%) and tetracycline resistant in 11 samples (52%).

E. coli were detected more frequently in chicken samples (165/339; 49%) than in pork mince (35/342; 10%). Of isolates examined for MIC determination, a higher percentage of those from chicken had resistance to ciprofloxacin (34/131; 26%), nalidixic acid (33/131; 25%) and gentamicin (9/131; 7%) compared to those from pork (12/94 [13%]; 3/94 [3%] and 0/94 [0%] respectively). In contrast, resistance to chloramphenicol and tetracycline occurred more often in isolates from pork (68/94 [72%] and 22/94 [23%], respectively) than from chicken (48/131 [37%] and 9/131 [7%]). *E. coli* organisms demonstrating the ESBL (but not AmpC) phenotype were detected in 44/681 (6.5%) of meat samples tested, including 16/342 (4.7%) of pork and 28/339 (8.3%) of chicken samples. Furthermore, the AmpC phenotype alone was detected in 39/339 (11.5%) of chicken samples but not in any pork samples, while the ESBL+AmpC phenotype was detected in chicken samples only (6/339; 1.8%). The difference in prevalence of the ESBL phenotype (including those that were both ESBL and AmpC) between chicken (34/339; 10.0%) and pork (16/342; 4.7%) samples was statistically significant. Results from retail chicken showed a decrease in the proportion of samples positive for ESBL-producing *E. coli* compared to other recent UK studies, which reported that 29.7% in 2016 and 65.4% in 2013/2014 of samples were positive for ESBL-producing *E. coli*.

Enterococci were isolated more frequently from chicken samples (180/339; 53%) than from pork mince samples (103/342; 30%). In the 298 isolates tested, resistance was rare, with only three (1%) exhibiting vancomycin resistance and one (0.3%) showing resistance to teicoplanin.

In contrast to *E. coli* and enterococci, *Klebsiella* species were detected more frequently in pork mince (127/342; 37%) than chicken (22/339; 6.5%). Of 85 *Klebsiella* isolates examined for MIC determination, rates of resistance were lower than observed in *E. coli* isolates for all antimicrobials tested, with the exception of ampicillin, to which *Klebsiella* species are intrinsically resistant.

This survey provides a baseline of the prevalence, types and levels of AMR bacteria found in UK retail chicken and pork mince which the FSA can use to monitor its progress in reducing AMR in these foods and inform UK AMR strategy. The data generated from this study will provide a baseline dataset for longitudinal comparisons within country as well as comparisons with data from other countries, and will provide a useful benchmark against which to monitor the impact of future interventions.

Antimicrobial resistance was detected in a proportion of all the types of bacteria examined, with resistance to the most clinically important drugs[1] generally appearing to be more prevalent in chicken isolates than pork. However, the risk of acquiring AMR related bacterial infections from these foods is very low provided that they are cooked and handled hygienically. Due to the strategy of sampling in relation to market share, there were insufficient samples from non-UK countries to allow statistical analysis of differences between UK and non-UK produced meat. However, this may be a focus for a future study.

[1] Critically Important Antimicrobials as defined by WHO
http://www.who.int/foodsafety/publications/cia2017.pdf?ua=1

2. Introduction

Antimicrobial resistance (AMR) is a significant threat to public health as well as having a major global economic impact (O'Neil, 2016) and has been identified as a key priority, not only by the UK government but by countries throughout the world. In 2016, 193 countries agreed a United Nations Declaration on AMR (World Health Organisation, 2016), and leaders at the G20 summit agreed on the need to tackle the economic impact of this issue (Anon, 2016). The EU One Health Action Plan Against Antimicrobial Resistance (European Commission, 2017) sets out objectives based around three main pillars: making the EU a best practice region; boosting research, development and innovation; and shaping the global agenda. This includes the need to strengthen surveillance and reporting regarding AMR and antimicrobial use in both humans and food-producing animals. This One Health approach is a key part of the UK Five Year Antimicrobial Resistance Strategy 2013-2018 (Department of Health and Department for Environment Food and Rural Affairs, 2013). A systematic review of AMR in the food chain funded by the Food Standards Agency (2016) concluded that there was a lack of data on AMR prevalence in British-produced food, leading to difficulty in monitoring trends or producing risk assessments for exposure of consumers. A key recommendation from this review was to address these gaps in evidence by developing research and surveillance to monitor AMR levels in foodborne pathogens and commensal bacteria in poultry and pork meat.

Antibiotics may be used in agriculture, either to treat or prevent disease. This usage may increase the risk of antimicrobial resistance developing, either in pathogenic bacteria or commensal organisms in the animal gut or the farm environment. Thus, meat may become contaminated with antimicrobial-resistant pathogens from the animal gut during slaughter, or through cross-contamination from the environment, whilst fruit and vegetables may become contaminated from the environment or from irrigation water. Moreover, contamination of food products with antimicrobial-resistant commensal organisms may result in the subsequent transfer of resistance genes to pathogens within the human gut (Salyers et al, 2004; Karami et al, 2007).

In 2013, an estimated 531 tonnes of antibiotics were dispensed in the UK for human use (Public Health England, 2015). Data for 2016 indicated that 337 tonnes of active antibiotic ingredient were sold for animal use, representing a 17% decrease on the previous year (VARSS, 2017). The impact of antibiotic use in both humans and animals on the development of AMR is well documented. A Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) report (ECDC, EFSA and EMA, 2017) identified significant associations between the use of the third- and fourth-generation cephalosporins in humans and increased resistance to these antibiotics in *E. coli* isolates from human infections. Similarly, an association was shown between the use of carbapenems and polymyxins in human medicine and increased resistance of *Klebsiella pneumoniae* isolates to these antimicrobials. Meanwhile, the use of tetracyclines and polymyxins in animals was linked with increased resistance to these groups of drugs in *E. coli*. Also, an increase in fluoroquinolone resistance in *Salmonella* and *Campylobacter* isolates from human infections (which are zoonotic in nature) was linked to consumption of fluoroquinolones in animals (ECDC, EFSA and EMA, 2017).

The use of antimicrobials as growth promoters has been banned in EU countries since 2006, but may be less well controlled in other areas of the world. Therefore, it is important to consider the contribution of imported foods, as well as those produced within Europe, to the overall issue of AMR in the food chain.

Surveillance of AMR in animals, humans and food has been carried out within the EU by the European Food Safety Authority (European Food Safety Authority, 2016), according to the requirements set down in Commission Implementing Decision 2013/652/EU (European Commission, 2013). This assessed the prevalence of resistance to key antimicrobials in *Salmonella, Campylobacter, E. coli* and *Staphylococcus aureus*. Data submitted from 28 EU Member States indicated that a relatively high proportion of isolates of *Salmonella, E. coli* and *Campylobacter* from broilers were resistant to fluoroquinolones and tetracyclines, whereas the proportion of *Salmonella* isolates from humans with resistance to fluoroquinolones remained generally low. Methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in meat from broilers, turkeys and pigs from three countries, with the highest prevalence rates being observed in turkey meat. A recent study of *Campylobacter* in raw chicken on retail sale in the UK showed that approximately half of *C. jejuni* and *C. coli* isolates were resistant to ciprofloxacin, whilst the proportion of isolates with resistance to erythromycin was low (PHE, 2017).

Randall et al. (2017) examined various raw meats, fruits and vegetables purchased in the UK in 2013/14 for the presence of extended-spectrum β-lactamase (ESBL)-producing and carbapenem-resistant *E. coli*. They found that 1.9% and 2.5% of beef and pork samples, respectively, were positive for ESBL-producing *E. coli* compared with 65.4% of chicken samples. None of 400 fruit and vegetable samples yielded ESBL-producing *E. coli* and none of the meat, fruit or vegetable samples yielded carbapenem-resistant *E. coli*. None of the foodstuffs yielded *E. coli* with CTX-M-15 ESBL, which dominates in human clinical isolates in the UK.

A further study focussed on the presence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in food on retail sale in England (Fox et al, 2017). MRSA was recovered from 7.3% of samples. Isolates were shown to belong to the livestock-associated clade of clonal complex (CC) 398. LA-MRSA is a public health concern worldwide, but has only been reported sporadically in the UK.

In response to the recommendations from the systematic review of AMR in the food chain (Food Standards Agency, 2016) that evidence should be gathered on AMR levels in foodborne pathogens and commensal bacteria in poultry and pork meat, this study aimed to collect data on the prevalence of AMR in retail chicken and pork mince to monitor trends in emerging AMR issues in the food chain, and to allow tracking of progress with interventions aimed at tackling AMR.

3. Methods

3.1 Sample collection at retail and transportation to the testing laboratory

A provisional design for the survey was completed and approved by the FSA before commencement of sample collection and can be found in the FSA protocol of the survey (https://www.food.gov.uk/research/foodborne-diseases/surveillance-study-of-antimicrobial-resistance-in-uk-retail-chicken-and-pork).

Between the beginning of September and end of October 2017, 339 samples of fresh/frozen raw chicken (whole and portioned) and 342 samples of fresh raw pork mince were collected by Hallmark Veterinary and Compliance Services. To note that frozen pork mince was not sampled due to low availability. Chicken and pork samples were taken randomly and a portion of samples were of non-UK origin. Whilst a small number of samples (approximately 40) in this study had a non-UK approval code, only five chicken samples (and no pork mince) were from outside the EU. Samples were collected according to market share, and therefore it appears that imported meat does not currently constitute a significant proportion of the retail chicken and pork mince market in the UK.

Sample collectors were instructed to sample from pre-determined retail outlets in Northern Ireland and within 10 geographic regions of Great Britain based on regional spend index and market share data. Within a retail outlet, sample collectors were allowed to select any suitable product of the appropriate category as if they were a typical shopper. The product categories were well defined to ensure consistency between samplers.

After collection, samples were packed into cold boxes with sufficient ice packs to achieve a temperature of < 8°C during transit. The samples were dispatched by overnight courier to one of four microbiology laboratories for examination (PHE Porton FW&E Laboratory, PHE London FW&E Laboratory, Animal and Plant Health Authority laboratory [APHA] at Weybridge or Agri-Food and Biosciences Institute [AFBI], Belfast). Where samples were received at the laboratory at a temperature above 8 °C, an assessment was made of the potential impact on microbiology results (in practice, this affected 23 samples that arrived at a temperature between 9 and 11 °C. Due to the short transit times in these cases, the conditions on receipt were considered to be acceptable and unlikely to have a significant effect on the results observed).

3.2 Examination of meat samples for the presence of pathogens and hygiene indicator bacteria

A 10^{-1} homogenate of each meat sample was prepared by diluting a 27 g aliquot of meat in Buffered Peptone Water (BPW), according to ISO 6887-1:1999 (International Organisation for Standardisation 1999). A portion of this homogenate (20 ml) was retained and used to enumerate *E. coli*, enterococci and (in the case of chicken samples) *Campylobacter* spp., according to the methods described in Table 1. The remaining 250 ml of homogenate was incubated at 37 °C for 18 h and then sub-cultured for the detection of *Salmonella* spp. (in the case of pork samples), *Klebsiella* spp. and presumptive ESBL-producing *E. coli* (Table 1).

During a pilot phase (the first two weeks of testing), enterococci and *E. coli* in pork samples were also sought by means of sub-culture to selective agar plates (as described in Table 1)

from the 250 ml enrichment broth (above). This was to ensure that a suitably sensitive method was being used to obtain sufficient numbers of isolates for AMR testing. At the end of the pilot phase, a decision was made to revert to enumeration testing only for these organisms, since preliminary data demonstrated that a sufficient detection rate was observed using enumeration only, and this technique was likely to provide a more representative picture of the range of strains present in the samples than an enrichment method that may lead to overgrowth by one or a few more robust strains.

Where target bacteria were isolated, five isolates selected at random (or all isolates if less than five were available) were sub-cultured onto transport media and sent by overnight delivery to the Animal and Plant Health Authority laboratory at Weybridge (for *E. coli* and *Salmonella*) or PHE Colindale (for *Campylobacter,* enterococci and *Klebsiella*) for MIC determination.

Table 1. Summary of methods used for enumeration and detection of target organisms

Test Parameter	Method	Reference
Sample preparation	1 in 10 dilution in BPW; ISO 6887-1:1999	ISO, 1999
Escherichia coli	Surface spread technique using TBX agar and incubation at 30°C for 4 h followed by 44°C for 21 h.	Roberts and Greenwood, 2003
Enterococci	Surface spread technique using Slanetz and Bartley agar and incubation at 37°C for 48 h. Confirmation on Bile Aesculin Agar.	In-house method
Campylobacter species	Surface spread technique using Campylobacter Selective Agar; ISO 10272-2:2006	ISO, 2006
Klebsiella species	Enrichment in BPW and subsequent sub-culture onto Klebsiella Selective HiCrome™ Agar. Incubation at 37°C for 24 h. Confirmation of identity by biochemical testing or MALDI-ToF.	In-house method
Salmonella	Pre-enrichment in BPW and subsequent selective enrichment followed by sub-culture onto selective agar plates; ISO 6579:2002	ISO, 2002
Presumptive ESBL-producing *E. coli*	Enrichment in BPW and subsequent sub-culture onto MacConkey Agar containing 1mg/l cefotaxime (MacConkey CTX), and incubation of plates at 44°C for 20 h. Confirmation of identity by biochemical testing or MALDI-ToF.	In-house method

3.3 Determination of minimum inhibitory concentrations for bacterial isolates

3.3.1 *E. coli* and *Salmonella*

For *E. coli* and *Salmonella* strains, one isolate of each type from each sample was selected to determine minimum inhibitory concentration (MIC). The remaining four isolates from each

sample were retained in storage at ambient temperature. Where a sample gave positive results for both the enumeration of *E. coli* per gram and the presence of extended-spectrum beta-lactamase- (ESBL) producing *E. coli* in 25 g, one isolate of each type was subjected to MIC determination.

Table 2. Antimicrobials included in AST testing, interpretative thresholds for resistance in *Salmonella* and indicator *E. coli* (first panel) as provided for in Decision 2013/652/EU[2]. *Klebsiella* were tested against antimicrobials according to BSAC (3.2.3)

Antimicrobial	**Interpretative thresholds of resistance (ECOFF[a]) (mg/l)**		
	Salmonella	***E. coli***	***Klebsiella***
Ampicillin	> 8	> 8	> 8
Cefotaxime	> 0.5	> 0.25	> 0.25
Ceftazidime	> 2	> 0.5	> 0.5
Meropenem	> 0.125	> 0.125	> 0.125
Nalidixic acid	> 16	> 16	NA, >16[c]
Ciprofloxacin	> 0.064	> 0.064	> 0.125
Tetracycline	> 8	> 8	> 8
Colistin	> 2	> 2	> 2
Gentamicin	> 2	> 2	> 2
Trimethoprim	> 2	> 2	> 2
Sulfamethoxazole	NA; > 256[b]	> 64	NT
Chloramphenicol	> 16	> 16	NA, >8[c]
Azithromycin	NA; > 16[b]	NA; > 16[b]	NA, >16[c]
Tigecycline	> 1	> 1	NA, >2[c]
Amoxicillin-clavulanic acid	NT	NT	NA, >8[c]
Piperacillin/tazobactam	NA; > 8[d]	NA; > 8[d]	NA; >8[d]
Ertapenem	See Table 3	See Table 3	> 0.064
Amikacin	NT	NT	>8
Tobramycin	NT	NT	>2
Cefepime	See Table 3	See Table 3	NA, >4[c]
Ceftazidime avibactam	NT	NT	NA, >8[c]
Cefoxitin	See Table 3	See Table 3	NA, >8[c]

NA: Not available; NT: Not tested; a) EUCAST epidemiological cut-off (ECOFF) values unless otherwise specified; b) ECOFF value not currently established – complementary threshold missing from Decision 2013/652/EU used; c) values used for analysis according to EUCAST clinical breakpoints or screening cut-offs; d) EUCAST ECOFF value not currently established; piperacillin threshold in the presence of a fixed tazobactam level of 4 mg/l according to BSAC (http://bsac.org.uk/wp-content/uploads/2012/02/BSAC-Susceptibility-testing-version-14.pdf))

[2]https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/sp.efsa.2017.EN-1176

For determination of MICs of most antibiotics against *E. coli* and *Salmonella*, isolates were inoculated into Mueller Hinton broth at a suitable dilution for application to commercially prepared plates containing two-fold dilution series of antimicrobials in accordance with European Decision 2013/652/EU. After incubation at 37 ºC for 18-24 h the plates were examined and growth end-points established for each antimicrobial to provide MICs.

Microbiologically resistant and susceptible interpretation for the MICs were obtained by comparison with epidemiological cut-off values (ECOFFs) published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) if available (Table 2).

MICs of piperacillin with a fixed concentration of 4 mg/l tazobactam were determined by an agar dilution method based on the method of the British Society of Antimicrobial Chemotherapy (BSAC) (Andrews, 2001; BSAC, 2015). For piperacillin/tazobactam, no ECOFF was available, so the recommended BSAC cut-off to denote resistance was used.

The presence of carbapenemase, ESBL or AmpC enzyme producers was determined initially by assessing isolate MICs against the microbiological breakpoints for meropenem, cefotaxime and ceftazidime. Any isolates from the MacConkey CTX plates showing meropenem, cefotaxime or ceftazidime MICs greater than the respective cut-offs for either *E. coli* or *Salmonella* were tested against a further panel of antimicrobials containing meropenem, imipenem, ertapenem, temocillin, cefoxitin, cefepime, cefotaxime with and without clavulanate and ceftazidime with and without clavulanate (Table 3).

Table 3. Panel of antimicrobials and interpretative thresholds for resistance used for testing only *Salmonella* spp. and indicator *E. coli* isolates resistant to cefotaxime, ceftazidime or meropenem (second panel).

Antimicrobial	Interpretative thresholds of AMR (ECOFF[a]) (mg/l)	
	Salmonella	***E. coli***
Cefoxitin	> 8	> 8
Cefepime	NA[b]; > 0.125	> 0.125
Cefotaxime + clavulanic acid	Not applicable[c]	Not applicable[c]
Ceftazidime + clavulanic acid	Not applicable[c]	Not applicable[c]
Meropenem	> 0.125	> 0.125
Temocillin	NA[b] > 32	NA[b] > 32
Imipenem	> 1	> 0.5
Ertapenem	> 0.06	> 0.06
Cefotaxime	> 0.5	> 0.25
Ceftazidime	> 2	> 0.5

NA: Not available; a) EUCAST epidemiological cut-off (ECOFF) values unless otherwise specified; b) ECOFF value not currently established – complementary threshold used; c) interpretation (yes or no) based on synergy or no synergy (between the two antimicrobials).

The interpretation of isolates as having an ESBL phenotype was as follows:

Isolates that were susceptible to meropenem and cefoxitin, but that showed resistance to one or both of cefotaxime and cetazidime and also showed a reduction (synergy) in MIC of ≥

8-fold against combined cefotaxime / clavulanate and/or ceftazidime / clavulanate when compared with the cephalosporin alone were classed as ESBL producers.

Isolates that were susceptible to meropenem but that showed resistance to one or both of cefotaxime and cetazidime, that also had an MIC of greater than 8 mg/l against cefoxitin and showed no reduction to MICs or a reduction of less than three dilution steps for cefotaxime or ceftazidime in the presence of clavulanate were considered to have an AmpC phenotype.

Isolates that were susceptible to meropenem but resistant to one or both of cefotaxime and cetazidime and resistance to cefoxitin and also showed a reduction (synergy) in MIC of ≥ 8-fold against combined cefotaxime / clavulanate and/or ceftazidime / clavulanate when compared with the cephalosporin alone were considered to have an ESBL + AmpC phenotype.

The single cefotaxime-resistant *Klebsiella* isolate was tested against cefotaxime and ceftazidime.

3.3.2 *Campylobacter* spp.

Confirmed *Campylobacter* isolates were sent to the PHE Gastrointestinal Bacteria Reference Unit (GBRU) for species identification and archiving. A PCR assay was first performed to determine the species of *Campylobacter* prior to MIC testing (Best et al, 2003). If more than one species was detected amongst isolates from a single sample, both were subjected to MIC testing. For a subset of samples, more than one isolate of each species was tested to determine if the susceptibility profiles for *C. coli* or *C. jejuni* isolates from the same sample were similar or not.

Antimicrobial susceptibility testing was determined by using an agar dilution method as this was the accredited method. Mueller Hinton Agar with the addition of 5 % horse blood containing specified breakpoint concentrations of antimicrobials was used to determine susceptibility. Agar quality was monitored using control strains with known MIC results. A standard procedure was used, briefly described 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 antibiotic but scored susceptible if no growth was observed and the corresponding antimicrobial free plate showed pure growth from the suspension applied.

3.3.3 *Klebsiella* spp.

Minimum inhibitory concentrations were determined using a standard agar dilution procedure (BSAC, 2015). *Klebsiella* isolates were tested against a panel of 21 antibiotics (see below), including third-generation cephalosporins (cefotaxime and ceftazidime), carbapenems and colistin. Results were interpreted using current EUCAST epidemiological cut-offs. Where ECOFFs were not available the clinical breakpoints were used. For cefoxitin the screening cut-off of 8 mg/l was used. Values used were obtained from the EUCAST website: https://mic.eucast.org/Eucast2/. The antimicrobials included in the main testing panel were: ampicillin, amoxicillin-clavulanic acid, cefepime, cefotaxime, ceftazidime, ceftazidime-avibactam, cefoxitin, piperacillin-tazobactam, ertapenem, meropenem, nalidixic acid, ciprofloxacin, azithromycin, amikacin, gentamicin, tobramycin, chloramphenicol, tetracycline, tigecycline, trimethoprim and colistin. For a single cefotaxime resistant isolate inhibitor

combinations of ceftoxime-clavulanic acid and cetazidime-clavulanic acid were tested to determine the ESBL status of the isolate.

Table 4. EUCAST interpretative thresholds for antimicrobial susceptibility testing in *C. jejuni* and *C. coli*

Antimicrobial	Species	ECOFF threshold (mg/l)
Erythromycin (Ery)	*C. jejuni*	> 4
	C. coli	> 8
Ciprofloxacin (Cp)	*C. jejuni*	> 0.5
	C. coli	> 0.5
Tetracycline (Tet)	*C. jejuni*	> 1
	C. coli	> 2
Gentamicin (G)	*C. jejuni*	> 2
	C. coli	> 2
Nalidixic acid (Nal)	*C. jejuni*	> 16
	C. coli	> 16
Streptomycin (S)	*C. jejuni*	> 4
	C. coli	> 4

Antimicrobial susceptibility profiles were determined using the ECOFF values, which separate the naive, susceptible bacterial populations from isolates that have developed reduced susceptibility to a given antimicrobial agent (Table 4) as recommended in the ECDC EU protocol for harmonised monitoring of antimicrobial resistance in human *Salmonella* and *Campylobacter* isolates (ECDC, 2016). The ECOFFs may differ from breakpoints used for clinical purposes, which are defined against a background of clinically-relevant data.

3.3.4 Enterococci

Minimum inhibitory concentrations were determined using a standard agar dilution procedure (BSAC, 2015). Results were interpreted using the current EUCAST clinical breakpoints (with MIC cut-off for resistant isolates of > 4mg/l for vancomycin and > 2mg/l for teicoplanin), which compare with ECOFFs that are > 4mg/l for both drugs. Screening for *vanA* and *vanB* resistance genes was carried out using conventional PCR using primers previously described by Woodford et al. (1993).

3.3.5 Multidrug resistance

Multidrug resistance was defined as reduced susceptibility to at least three unrelated antimicrobial classes as specified by the ECDC definition (see Table 5; ECDC, 2016).

Table 5. Antimicrobial groups and the compounds within them

Antimicrobial Group	Antimicrobial(s) included
Aminoglycosides	Gentamicin, streptomycin, amikacin, tobramycin
Penicillin beta-lactam antibiotics	Ampicillin, temocillin – β-lactamase resistant
β-lactam/inhibitor combinations	Piperacillin/tazobactam, cefotaxime/clavulanic acid, ceftazidime/clavulanic acid
Macrolides	Erythromycin, azithromycin
Quinolones	Ciprofloxacin, nalidixic acid
Cephalosporins	Cefoxitin (2nd generation cephamycin); Ceftazidime and cefotaxime (3rd generation cephalosporins); Cefepime (4th generation cephalosporin)
Carbapenems	Meropenem, imipenem, ertapenem
Sulphonamides	Sulfamethoxazole
Chloramphenicol	Chloramphenicol
Tetracyclines	Tetracycline
Polymyxins	Colistin
Glycylcyclines	Tigecycline
Trimethoprim	Trimethoprim
Glycopeptides	Teicoplanin, vancomycin

3.4 Detection of resistance genes using Polymerase Chain Reaction (PCR)

A multiplex PCR technique was used to investigate the presence of *bla*$_{CTX-M}$, *bla*$_{OXA-1}$, *bla*$_{SHV}$, *bla*$_{TEM}$ beta-lactamase genes in *E. coli* isolates that exhibited an AmpC and/or ESBL phenotype, as previously described (Fang *et al.* 2008). PCR was performed using DNA preparations from pure cultures and resulting *bla*$_{CTX-M}$ amplicons were sequenced to determine the CTX-M variant (Randall *et al*. 2011).

3.5 Characterisation of isolates using whole genome sequencing

All isolates of *Salmonella* were further characterised by means of whole genome sequencing (WGS) (Ashton *et al*. 2016; Byrne *et al*. 2014; Dallman *et al*. 2015). Selected isolates of *E. coli* and *Klebsiella* were also characterised by WGS.

3.6 Statistical analysis

Descriptive and statistical analyses of the data were undertaken using Excel 2010 (Microsoft, Redmond, Washington). Relative proportions were compared using the Fisher's Exact Test (GraphPad Software, San Diego, California). A probability value of less than 5% was defined as significant.

3.7 Quality assurance

All the meat testing laboratories that participated in this study are UKAS accredited to ISO 17025, and participate in External Quality Assurance (EQA) schemes. whilst laboratories carrying out MIC determination and further identification of strains also work within quality management systems and participate in EQA schemes.
All analyses were performed by trained and competent staff.

4.0 Results

4.1 Sample numbers submitted for microbiological examination

Overall, 339 samples of chicken and 342 samples of pork mince were examined. These included samples originating from 63 different approved establishments (ie. establishments approved by the FSA, FSS or equivalent competent authority in other EU Member States to undertake certain processes for which hygiene conditions are laid down in Regulation (EC) No 853/2004 e.g. slaughtering of animals). The establishments included 47 with UK approval codes, four in Poland, four in the Netherlands, three in Denmark and one each in Spain, Ireland and Germany. In addition, the approval code was either unclear or not provided for three samples. Based on UK market share data, the majority of samples were purchased from large national retail chains (n = 560), whilst 61 samples were collected from butchers, other retailers or the retailer was described as 'miscellaneous'. At the time of sampling, all of the pork mince samples (342) and 305 of the chicken samples were collected chilled, with the remaining 34 chicken samples collected frozen. The number of chicken and pork samples tested at each laboratory are shown in Table 6.

Table 6. Number of samples tested at each laboratory

Laboratory	Number of Samples Examined		
	Chicken	**Pork**	**Total**
AFBI	49	51	100
APHA	0	291	291
PHE London	134	0	134
PHE Porton	156	0	156
Total	**339**	**342**	**681**

4.2 Detection of target organisms in chicken and pork

The number of samples giving positive results for each target organism is shown in Table 7.

Campylobacter spp. were detected in 85/339 (25.1%) of chicken samples, of which one sample (a whole chicken) gave a result of greater than 1000 cfu/g (Table 8). Overall, *Campylobacter* spp. were detected significantly more frequently in whole chickens (42/120; 35.0%) and leg/wing meat (21/73; 27.4%) compared to breast meat (19/132; 14.4%) (Fishers exact test; p=0.0002 and 0.02 for whole and leg/wing meat, respectively). The difference between whole chickens and leg/wing meat was not statistically significant.

Salmonella was detected in 5/342 (1.5%) of pork mince samples. Using WGS four of these were identified as *S.* Typhimurium (1.2%) and one as *S.* Derby (0.3%). The four samples from which *S.* Typhimurium was isolated all originated from a single UK Approval Number, with three being purchased from two different outlets of the same supermarket chain and one from a separate supermarket chain.

Moreover, two of these strains were indistinguishable by WGS (no SNP differences), and these were both purchased from the same outlet of a supermarket, with use by dates only two days apart (28th and 30th October 2017). The sample with *S.* Derby was purchased from a different supermarket chain and originated from a different UK Approval Number.

Table 7. Number of chicken and pork mince samples giving positive results for each bacteria of interest.

Bacteria	Chicken (n = 339)			Pork (n = 342)		
	No. of positive samples	% positive samples	95% CI	No. of positive samples	% positive samples	95% CI
Campylobacter	85	25.1	20.6-30.0	NE[1]	-	
Salmonella	NE[1]	-	-	5	1.5	0.5-3.4
E. coli	165	48.7	43.4-54.0	35	10.2[2]	7.4-13.9
Enterococci	180	53.1	47.8-58.3	103	30.1[3]	25.5-35.2
Klebsiella	22	6.5	4.3-9.7	127	37.1	32.2-42.4
Presumptive ESBL/AmpC *E. coli*	75	22.1	18.0-26.9	16	4.7	2.9-7.5

[1]NE=not examined

[2] An additional 64 pork samples tested using an enrichment method were positive for *E. coli*

[3] An additional 46 pork samples tested using an enrichment method were positive for enterococci

Table 8. Levels of *Campylobacter* (cfu/g) in retail chicken by sample type, country of origin and storage temperature

	No. (%) of samples with *Campylobacter* counts (cfu/g) in specified range [95 % CI]			
	<10[a]	**10 - 100**	**> 100 – 1000**	**>1000**
Sample type:				
Whole chicken (n=120)	78 (65.0)	27 (22.5)	14 (11.7)	1 (0.8)
Chicken portions[b] (n=219)	176 (80.3)	36 (16.4)	7 (3.2)	0
Country of origin:				
UK (n=305)	223 (73.1)	61 (20.0)	20 (6.6)	1 (0.3)
Non-UK (n=34)[c]	31 (91.2)	2 (5.9)	1 (2.9)	0
Storage temperature:				
Chilled (n=305)	220 (72.1)	63 (20.7)	21 (6.9)	1 (0.3)
Frozen (n=34)	34 (100.0)	0	0	0
Total (n=339)	**254 (74.9)**	**63 (18.6)**	**21 (6.2)**	**1 (0.3)**
95% confidence interval	[70.0-79.5]	[14.8-23.1]	[4.0-9.3]	[0.01-1.6]

[a] Minimum detection limit;

[b] Portions include leg, wing and breast meat samples

[c] Of which 24/34 (70.6%) were frozen

The most frequently detected group of organisms from both chicken and pork samples was enterococci. These were detected at levels of at least 20 cfu/g in 179/339 (52.8%) of chicken samples and 103/342 (30.1%) of pork mince samples. The higher prevalence in chicken compared to pork was statistically significant (Fishers exact test; p = 0.02). Enrichment for enterococci (i.e. testing for presence in a 25 g aliquot) was undertaken in addition to enumeration (if the preceding enumeration test was negative) for an initial 73 samples and this resulted in almost all (72/73; 98.6%) pork mince samples testing positive for enterococci.

E. coli were also detected at levels of at least 20 cfu/g significantly more frequently in chicken (165/339; 48.7%) compared to pork (35/342; 10.2%) (Fishers exact test; p=0.0001). Furthermore, presumptive AmpC and/or ESBL-producing *E. coli* (i.e. those that grew on a selective agar plate containing cefotaxime) were detected more frequently in chicken (75/339; 22.1%) than in pork (16/342; 4.7%) (Fishers exact test; p = 0.0001). *E. coli* were detected by enrichment (i.e. present in a 25 g aliquot) only in 64/73 samples of pork mince tested during a pilot phase of the study, during which both enumeration and enrichment procedures were performed (where only nine of these were positive by enumeration).

In contrast, *Klebsiella* spp. were detected in significantly more pork samples (127/342; 37.1%) compared to chicken (22/339; 6.5%) (Fishers exact test; p<0.0001). The overall detection rate for *Klebsiella* spp. was higher than estimated at the start of the study, and therefore a subset of isolates was subjected to MIC testing. Isolates were selected in such a way as to ensure representation from both chicken and pork samples, as well as from all the different Approval Codes and, as far as possible, the different batch numbers covered within the range of samples tested. Testing of multiple isolates from the same batch/use by date was avoided.

4.3 Determination of minimum inhibitory concentration (MIC) for bacterial isolates

4.3.1 *Salmonella*

All four *S.* Typhimurium isolates from minced pork samples were resistant to ampicillin and tetracycline, with one also showing resistance to chloramphenicol, but were susceptible to all other antibiotics tested with the exception of sulfamethoxazole (i.e. azithromycin, cefotaxime, ceftazidime, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, tigecycline, trimethoprim, cefepime, cefoxitin, ertapenem, imipenem and temocillin). The *S.* Derby isolate was susceptible to all antibiotics except sulfamethoxazole. Susceptibility to sulfamethoxazole was reduced for all five *Salmonella* isolates, but no ECOFF value is available for this drug for *Salmonella*, and therefore it was not possible to interpret these as resistant in relation to an ECOFF values. None of the salmonellae were identified as having an ESBL or AmpC phenotype.

4.3.2 *Campylobacter* species

Determination of MICs was performed for a total of 157 *C. jejuni* and 45 *C. coli* isolates from 79 samples. Isolates from the remaining six positive samples were not recoverable and no MIC determination was undertaken for these. Just under half of the *C. coli* (46.7%) and 38.9% of the *C. jejuni* isolates examined were resistant to ciprofloxacin and 6.7% of the *C. coli* and 7.6% the *C. jejuni* isolates were resistant to erythromycin (Table 9). Over half the *C. coli* (60.0%) and the *C. jejuni* (61.8%) isolates were resistant to tetracycline but all isolates

tested were sensitive to gentamicin and only one *C. coli* isolate was resistant to streptomycin. Two *C. jejuni* isolates derived from the same sample were co-resistant to ciprofloxacin and erythromycin. Multidrug resistance defined as reduced susceptibility to at least three unrelated antimicrobial classes was found in four *C. coli* (8.9% of isolates) and one *C. jejuni* (0.6% of isolates) originating from 5 samples (Table 10). In total 46 isolates were susceptible to all antimicrobials tested including 35 *C. jejuni* and 11 *C. coli* isolates.

Table 9. *Campylobacter* isolates from retail chicken showing resistance to various antimicrobials

Antimicrobials	*C. jejuni* (n = 157)			*C. coli* (n = 45)		
	No. of isolates	%R	95% CI	No. of isolates	%R	95% CI
Erythromycin	12	7.6	4.0-13.0	3	6.7	1.4-18.3
Ciprofloxacin	61	38.9	31.2-47.0	21	46.7	31.7-62.1
Gentamicin	0	0	0-2.3	0	0	0-7.9
Nalidixic acid	64	40.8	33.0-49.9	30	66.7	51.1-80.0
Streptomycin	0	0	0-2.3	1	2.2	0.06-11.3
Tetracycline	97	61.8	53.7-69.4	27	60.0	44.3-74.3

Table 10. Multi-drug resistance profiles in *C. jejuni* and *C. coli* isolates from retail chicken meat.

Antimicrobial resistance (AMR) profile	No. of isolates with the given AMR profile (% of isolates; 95% CI)		
	C. jejuni (n = 157)	*C. coli* (n = 45)	All isolates (n = 202)
Tet, Strep, Nal and/or Cip	0	1	1
Tet, Ery, Nal and/or Cip	1	3	4
Total for all profiles	1 (0.6; 0.02-3.5)	4 (8.9; 2.5-21.2)	5 (2.5; 0.8-5.7)

Species identification and MIC determination was carried out for more than one isolate from 42 samples (Table 11). The same species was detected in the majority (34/42) of these samples but both *C. jejuni* and *C. coli* were recovered from 8 samples. In 33 samples where three or more isolates were tested, the same species was also recovered from the majority (26/33). The proportion of *Campylobacter*-positive samples containing antimicrobial resistant campylobacters was calculated either by counting a sample as 'resistant' even where both resistant and susceptible isolates were present or by counting a sample as 'resistant' only when solely resistant isolates of *C. jejuni* or *C. coli* were detected (Table 12).

Ciprofloxacin resistant *C. jejuni* were detected in 25 (38%), erythromycin resistant in 6 (9%) and tetracycline resistant in 39 (59%) samples of 66 samples from which *C. jejuni* was detected. Ciprofloxacin resistant *C. coli* were detected in 8 (38%), erythromycin resistant in 3 (14%) and tetracycline resistant in 11 (52%) samples of 21 samples from which *C. coli* was detected. The proportion of samples with resistant *C. jejuni* or *C. coli* calculated in this manner (regardless of whether both resistant and susceptible types were found) was similar to the calculated proportion of resistant isolates of total number of isolates (Table 9). Similar results were also obtained by counting a sample as 'resistant' only if all isolates from the sample showed resistance to an antimicrobial, although calculated in this way erythromycin resistance was present in a lower proportion of samples (Table 12).

Table 11. Number of chicken samples in relation to numbers of *C. jejuni* and *C. coli* isolates MIC tested per sample

		Number of *C. coli* isolates in sample subjected to MIC testing					
		0	1	2	3	4	5
Number of *C. jejuni* isolates in sample subjected to MIC testing	0	0	7	1	1	0	3
	1	30	1	0	0	1	0
	2	7	2	0	2	0	0
	3	2	0	2	0	0	0
	4	8	0	0	0	0	0
	5	12	0	0	0	0	0

Table 12. Proportion of campylobacter-positive samples with *Campylobacter jejuni* or *C. coli* isolates resistant to antimicrobials*

Antimicrobial	*C. jejuni* (isolated from 66 samples)		*C. coli* (isolated from 21 samples)	
	Number of samples (%; 95% CI) [only samples where all isolates resistant]	Number of samples (%; 95% CI) [including samples where both resistant and susceptible isolates found]	Number of samples (%; 95% CI) [all isolates in sample resistant]	Number of samples (%; 95% CI) [mixed resistant and susceptible isolates]
Erythromycin	3 (5; 1-13)	6 (9; 3-19)	1 (5; 0-24)	3 (14; 3-36)
Ciprofloxacin	20 (30; 19-43)	25 (38; 26-51)	7 (33; 15-57)	8 (38; 18-62)
Nalidixic acid	22 (33; 22-46)	26 (39; 28-52)	11 (52; 30-74)	14 (67; 43-85)
Tetracycline	33 (50; 37-63)	39 (59; 46-71)	10 (48; 26-70)	11 (52; 30-74)

*One sample contained one streptomycin resistant (and one susceptible) *C. coli*.

4.3.3 *E. coli*

A total of 225 *E. coli* isolates detected by means of the enumeration procedure were subjected to MIC determination (Table 13). Highest percentages of isolates were resistant against ampicillin (72.5% and 54.3% in chicken and pork, respectively), sulfamethoxazole (61.1% and 52.1%), trimethoprim (58.8% and 47.9%) and tetracycline (36.6% and 72.3%), whilst the lowest percentages of isolates were resistant against meropenem and piperacillin/tazobactam (no resistant isolates detected in either case), tigecycline and colistin (both with no resistant isolates from chicken and both 2.1% from pork) and azithromycin (0.8% from chicken and 2.1% from pork).

Table 13. *E. coli* isolates from retail chicken and pork mince showing resistance to various antimicrobials

Antimicrobial	Chicken (n = 131)			Pork (n = 94)		
	No. of isolates	%R	95% CI	No. of isolates	%R	95% CI
Ampicillin	95	72.5	64.0 – 80.0	51	54.3	43.7 – 64.6
Azithromycin	1	0.8	0.02 – 4.2	2	2.1	0.3 – 7.5
Cefotaxime	22	16.8	10.8 – 24.3	33	35.1	25.5 – 45.6
Ceftazidime	19	14.5	9.0 – 21.7	21	22.3	14.4 – 32.1
Chloramphenicol	9	6.9	3.2 – 12.6	22	23.4	15.3 – 33.3
Ciprofloxacin	34	26.0	18.7 – 34.3	12	12.8	6.8 – 21.2
Colistin	0	0	0.0 – 2.8	2	2.1	0.3 – 7.5
Gentamicin	9	6.9	3.2 – 12.6	0	0	0 – 3.9
Meropenem	0	0	0 – 2.8	0	0	0 – 3.9
Nalidixic acid	33	25.2	18.0 – 33.5	3	3.2	0.7 – 9.0
Sulfamethoxazole	80	61.1	52.2 – 69.5	49	52.1	41.6 – 62.5
Tetracycline	48	36.6	28.4 – 45.5	68	72.3	62.2 – 81.1
Tigecycline	0	0	0 – 2.8	2	2.1	0.3 – 7.5
Trimethoprim	77	58.8	50.0 – 67.3	45	47.9	37.5 – 58.4
Piperacillin/tazobactam*	0	0	0 – 2.8	0	0	0 – 3.9
Fully susceptible	18	13.7	8.4 – 20.8	13	13.8	7.6 – 22.5
Resistant to ≥ 3 antimicrobial groups	74	56.5	47.6 – 65.1	52	55.3	44.7 – 65.6

* Piperacillin/tazobactam resistance could not be determined for two isolates

Only 13.7% (18/131) and 13.8% (13/94) of the *E. coli* isolates from chicken and pork samples, respectively, were susceptible to all antibiotics tested. Resistance to three or more groups of antimicrobials (multi-resistance) was seen in 56.5% (74/131) and 55.3% (52/94) of chicken and pork isolates, respectively. One isolate from pork was resistant to 10/15 antimicrobials tested (belonging to 9/13 antimicrobial groups: penicillins, macrolides, cephalosporins, quinolones, polymyxins, sulphonamides, tetracyclines, glycylcyclines and trimethoprim). A further three isolates (two from chicken and one from pork) were resistant to nine antimicrobials (from seven different groups). The percentage of isolates that were resistant was significantly greater for chicken compared with pork for ciprofloxacin (Fishers

exact test; p=0.019), nalidixic acid (p = 0.0001) and gentamicin (p = 0.011). In contrast, the percentages of isolates from pork that were resistant were higher compared to isolates from chicken for cefotaxime (p = 0.003), chloramphenicol (p = 0.0007) and tetracycline (p = 0.0001).

Table 14. Number (and percentage) of presumptive ESBL-producing *E. coli* isolates from retail chicken and pork mince showing resistance to various antibiotics and showing typical resistance phenotypes (ESBL, AmpC or ESBL+AmpC).

Antimicrobial	Chicken (n = 74)			Pork (n = 16)		
	No. of isolates	% R	95% CI	No. of isolates	% R	95% CI
Ampicillin	74	100	94 – 100	16	100	77 – 100
Azithromycin	1	1.4	0.0 – 8.0	0	0.0	0 – 22.7
Cefotaxime	74	100	94 – 100	16	100	77 – 100
Ceftazidime	70	94.6	86.5 – 98.3	15	93.8	69.7 – 99.9
Chloramphenicol	12	16.2	9.4 – 26.4	2	12.5	2.2 – 37.3
Ciprofloxacin	30	40.5	30.1 – 51.9	6	37.5	18.4 – 61.5
Colistin	1	1.4	0.0 – 8.0	0	0.0	0 – 22.7
Gentamicin	19	25.7	17.0 – 36.7	1	6.3	<0.01 – 30.3
Meropenem	0	0.0	0 – 5.9	0	0.0	0 – 22.7
Nalidixic acid	24	32.4	22.8 – 43.8	3	18.8	5.8 – 43.8
Sulfamethoxazole	53	71.6	60.4 – 80.7	14	87.5	62.7 – 97.8
Tetracycline	45	60.8	49.4 – 71.2	10	62.5	38.5 – 81.6
Tigecycline	0	0.0	0 – 5.9	0	0.0	0 – 22.7
Trimethoprim	28	37.8	27.6 – 49.2	13	81.3	56.2 – 94.2
Piperacillin/tazobactam	0	0.0	0 – 5.9	0	0.0	0 – 22.7
Cefepime	64	86.5	76.7 – 92.7	16	100	77 – 100
Cefoxitin	45	60.8	49.4 – 71.2	0	0.0	(0 – 22.7
Ertapenem	0	0.0	0 – 5.9	0	0.0	0 – 22.7
Imipenem	0	0.0	0 – 5.9	0	0.0	0 – 22.7
Temocillin	0	0.0	0 – 5.9	0	0.0	0 – 22.7
Cefotaxime+clavulanic acid synergy	34	45.9	35.1 – 57.2	16	100	77 – 100
Ceftazidime+clavulanic acid synergy	25	33.8	24.0 – 45.2	15	93.8	69.7 – 99.9
ESBL	28*	37.8	26.8 – 49.9	16	100	77 – 100
AmpC	39*	52.7	40.8 – 64.4	0	0.0	0 – 22.7
ESBL+AmpC	6*	8.1	3.0 – 16.0	0	0.0	0 – 22.7

*MIC testing indicated that one presumptive isolate from chicken could not be confirmed as ESBL or AmpC phenotype.

A further 90 isolates of *E. coli* were considered to be presumptive AmpC or ESBL (or both) phenotype following detection in a 25 g portion of meat by selective plating on an agar plate containing cefotaxime. These were selected for an extended MIC assay to confirm AmpC or ESBL phenotype (Table 14). Of these, 48.9% were confirmed as having the ESBL only phenotype, whilst 43.3% demonstrated an AmpC only phenotype and a further 6.7% had the ESBL+AmpC phenotype. All 16 presumptive AmpC/ESBL phenotype isolates from pork were confirmed as having the ESBL only phenotype, whereas of those isolated from chicken, 37.8% were confirmed as ESBL phenotype only whilst 52.7% had the AmpC phenotype and 8.1% had the ESBL+AmpC phenotype.

In relation to the total number of samples tested, *E. coli* organisms demonstrating the ESBL (but not AmpC) phenotype were detected in 44/681 (6.5%) of meat samples tested, including 16/342 (4.7%) of pork and 28/339 (8.3%) of chicken samples. Furthermore, AmpC and ESBL+AmpC *E. coli* phenotypes were only detected in chicken samples (11.5% and 1.8% respectively) with none recovered from pork samples. Overall, the difference in prevalence of the ESBL phenotype between chicken (10.0%) and pork (4.7%) samples (including those that were both ESBL and AmpC) was statistically significant (Fishers exact test; p= 0.008). The AmpC phenotype was also significantly more common in chicken than pork (Fishers exact test; p<0.0001).

4.4.4 Molecular characterisation of resistance genes in *E. coli* isolates

E. coli isolates that had the AmpC and/or ESBL phenotype according to MIC profiles were tested for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$ genes and resulting *bla*$_{\text{CTX-M}}$ amplicons were sequenced to determine the CTX-M sequence type. In total, 40/89 isolates tested were positive for the *bla*$_{\text{CTX-M}}$ gene, of which 13 were also positive for *bla*$_{\text{TEM}}$ (Table 15).

Table 15. Summary of multiplex PCR results for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA-1}}$-like, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ genes

Genes detected	No. of isolates	No. of isolates ESBL/AMPC only	No. of pork/chicken samples	No. of UK origin samples
bla$_{\text{CTX-M}}$	27	27/0	10/17	24
bla$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$	13	13/0	6/7	12
bla$_{\text{TEM}}$	7	2/5	0/7	4
bla$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$	7	6/1	0/7	6
bla$_{\text{SHV}}$	2	2/0	2/0	0
bla$_{\text{OXA-1}}$-like	0	NA	NA	NA
Negative for all genes tested	33	0/33	0/33	32

Of the 40 isolates positive for *bla*$_{\text{CTX-M}}$, 39 had their CTX-M sequence type determined (Table 16). The most common CTX-M type detected was CTX-M-1. One isolate was positive for *bla*$_{\text{CTX-M}}$ but the precise CTX-M variant could not be determined, possibly due to the

presence of a variant and further work would be needed to confirm this. One CTXM-15 positive *E. coli* isolate was derived from a chicken breast sample of German origin while the other CTXM-15 positive *E coli* was isolated from pork mince of UK origin. It is likely that the 33 isolates that were negative for bla_{CTX-M}, bla_{OXA-1}, bla_{SHV}, bla_{TEM} genes might harbour an AmpC gene such as $bla_{CIT.}$

Table 16. Summary of different CTX-M types detected

CTX-M sequence type	No. of isolates
CTX-M-1	32
CTX-M-14	2
CTX-M-15	2
CTX-M-32	3

4.3.5 *Klebsiella* spp.

A total of 85 *Klebsiella* isolates were selected for MIC determination comprising 54 *Klebsiella oxytoca* and 31 *K. pneumoniae* isolates. While all of the isolates were resistant to ampicillin (which is intrinsic for *K. oxytoca* and *K. pneumoniae*), the rates of resistance to the other antibiotics tested were low, except for tetracycline resistance in isolates from chicken (11.8 %; Table 18).

Although 100% of isolates were found to be ampicillin-resistant, this is an expected result since klebsiellas are intrinsically resistant to this drug. When ampicillin resistance was discounted from the calculations for multi-drug resistant (MDR) isolates (i.e. three or more excepting ampicillin resistance) two isolates were classed as MDR. One of the MDR isolates was resistant to trimethoprim, chloramphenicol, tetracycline and amoxicillin-clavulanic acid, whilst the remaining MDR isolate (identified as *K. pneumoniae*) showed a pattern of resistance that was consistent with an ESBL phenotype. This was isolated from a chicken breast fillet purchased from a supermarket. This isolate harboured a bla_{SHV-2} gene (ascertained by WGS) explaining the ESBL phenotype.

Table 18. Number (and percentage) of *Klebsiella* isolates from retail chicken and pork mince showing resistance to various antibiotics

Antimicrobial	Chicken (n = 17)			Pork (n = 68)		
	No. of isolates	%	95% CI	No. of isolates	%	95% CI
Ampicillin	17	100	78.4 – 100	68	100	94.7 – 100
Augmentin	1	5.9	0.2 – 28.7	1	1.5	0.4 – 7.9
Azithromycin	0	0	0 – 19.5	1	1.5	0.4 – 7.9
Cefotaxime	1	5.9	0.2 – 28.7	0	0	0 – 5.3
Ceftazidime	1	5.9	0.2 – 28.7	0	0	0 – 5.3
Chloramphenicol	1	5.9	0.2 – 28.7	3	4.4	0.9 – 12.4
Ciprofloxacin	1	5.9	0.2 – 28.7	0	0	0 – 5.3
Colistin	0	0	0 – 19.5	0	0	0 – 5.3
Gentamicin	1	5.9	0.2 – 28.7	0	0	0 – 5.3
Meropenem	0	0	0 – 19.5	0	0	0 – 5.3
Nalidixic acid	1	0	0 – 19.5	0	0	0 – 5.3
Tetracycline	2	11.8	1.5 – 36.4	3	4.4	0.9 – 12.4
Tigecycline	0	0	0 – 19.5	2	2.9	0.4 – 10.2
Trimethoprim	1	5.9	0.2 – 28.7	1	1.5	0.4 – 7.9
Piperacillin/tazobactam	0	0	0 – 19.5	0	0	0 – 5.3
Amikacin	0	0	0 – 19.5	0	0	0 – 5.3
Cefepime	0	0	0 – 19.5	0	0	0 – 5.3
Ceftazidine/avibactam	0	0	0 – 19.5	0	0	0 – 5.3
Cefoxitin	0	0	0 – 19.5	0	0	0 – 5.3
Ertapenem	0	0	0 – 19.5	0	0	0 – 5.3
Tobramycin	1	5.9	0.2 – 28.7	0	0	0 – 5.3
ESBL	1	5.9	0.2 – 28.7	0	0	0 – 5.3
Fully susceptible	0	0	0 – 19.5	0	0	0 – 5.3
Resistant to ≥ 3 antimicrobial groups	1	5.9	0.2 – 28.7	1	1.5	0.4 – 7.9

4.3.6 Enterococci

Enterococci were isolated from 298 samples. Three isolates (1.0%) showed resistance to vancomycin and one (0.3%) to teicoplanin. Two isolates were resistant to vancomycin alone, reflecting a resistance that was not conferred by *van*A. Of these one was *Enterococcus gallinarum* displaying endogenous *vanC* activity, the other was PCR negative for *vanB*. These were from a sample of chicken leg meat and a portion of pork mince. The third isolate, an *Enterococcus faecalis* from a whole chicken, was resistant to both vancomycin and teicoplanin, indicating a *van*A type of resistance which was confirmed by PCR. The samples in which these resistant isolates were detected were from three separate retailers.

5. Discussion

The Food Standards Agency identified antimicrobial resistance (AMR) in *Campylobacter* from chickens and *Salmonella* in pork as a surveillance priority following the AMR systematic review published in 2016 (Food Standards Agency, 2016). The study reported here provides data on current rates of resistance for *Salmonella* and *Campylobacter* but also hygiene indicator bacteria isolated from retail chicken and pork mince samples collected from all four countries of the UK. However, it should be noted that the study took place over a two-month period in September and October 2017, and therefore did not aim to detect any seasonal variations in bacterial prevalence and antimicrobial resistance. Testing of samples and subsequent AMR analyses were largely in line with the requirements set out in Commission Implementing Decision 2013/652/EU (European Commission, 2013), but with the additional isolation of *Klebsiella* spp. for AMR determination.

Salmonella Of 342 pork mince samples tested, *Salmonella* was detected in only five (1.5%). According to EC Regulation 2073/2005 (European Commission, 2005), *Salmonella* must not be detected in a 10 g portion of minced meat whilst it is on the market during its shelf-life. Although the quantity of sample tested in this study (25 g) was greater than the statutory requirement, to increase the sensitivity for the purposes of monitoring trends in AMR, the presence of *Salmonella* was taken as an indicator of unsatisfactory quality, and was reported to the FSA for follow-up with the retailer. Four of the positive samples originated from the same processing plant, with all four isolates being identified as *S.* Typhimurium, two of which were indistinguishable by WGS. All four were resistant to ampicillin and tetracycline, and one to chloramphenicol, with all also showing reduced susceptibility to sulfamethoxazole. The fifth isolate, identified as a strain of *S*. Derby with reduced susceptibility to sulfamethoxazole only, originated from a separate processing plant. Subsequent comparison of WGS results with recent human isolates identified cluster matches (< 5 SNPs difference) between two *S*. Typhimurium isolates from pork and three patient isolates, indicating that these patients may have become infected through exposure to meat from the same farm or processing plant. Given the small number of positive *Salmonella* samples in pork, with only two processing plants represented by these positive samples, it is not possible to determine whether the AMR profiles observed in these isolates are representative of the overall trends for foodborne salmonellae in the UK.

An EU study in 2016 (European Food Safety Authority, 2018) found high levels of resistance to ciprofloxacin (64.7%) and nalidixic acid (61.5%) in salmonellae isolated from broiler meat, whilst resistance to third-generation cephalosporins (cefotaxime and ceftazidime) was low or not detected, with the exception of Portugal which reported high levels of resistance at 39.4%. Resistance to ampicillin, tetracycline and sulfamethoxazole ranged from moderate to extremely high, depending on the species of *Salmonella* and reporting country.

Campylobacter *Campylobacter* species were detected in 25% of chicken samples, with the highest prevalence being observed in whole chickens, followed by leg and wing portions. Chicken breast meat had a significantly lower rate of contamination with *Campylobacter* than other portions or whole chickens, possibly due to the higher level of processing, often involving removal of the skin, in this type of sample. It was not possible to draw conclusions regarding significant differences between *Campylobacter* contamination rates in UK compared to non-UK chicken, or chilled versus frozen, since the numbers of non-UK and frozen samples were very small. Overall, under half of *Campylobacter* isolates were resistant to ciprofloxacin (40.6%) and nalidixic acid (46.5%), whilst 61.4% showed resistance to tetracycline. The rate of resistance to nalidixic acid amongst *C. coli* isolates was greater than for *C. jejuni*. These results are broadly similar to a study of *Campylobacter* prevalence in raw whole UK-produced chicken on retail sale in the UK (PHE 2017), which also showed that approximately half of both *C. jejuni* and *C. coli* isolates were resistant to ciprofloxacin. In that

study only 1.9% of *C. coli* and none of the *C. jejuni* isolates were resistant to erythromycin. In comparison, rates of erythromycin resistance observed in this current study were higher (6.7 and 7.6%, respectively). However, this observation must be treated with caution as the current study included fewer isolates and also represented other types of chicken meat products. For 2016, data on broiler isolates from 6 EU member states reported 2.2 % of *C. jejuni* and 13.1 % of *C. coli* erythromycin resistant (EFSA & ECDC 2018). In the UK, 3.3 % of *C. jejuni* and 11.1 % *C. coli* isolates from humans were reported as erythromycin resistant in 2016 (EFSA & ECDC 2018).
The proportion of isolates resistant to erythromycin in the current study were not significantly different compared with the percentages of resistant isolates from a 2004-2006 UK study (reporting 2.9% for *C. jejuni* and 14.5% for *C. coli*), whilst in the earlier study the proportions of isolates resistant to ciprofloxacin were lower, especially for *C. jejuni* (CLASSP 2010; study data from 2004-2006).
EU studies from 2014 and 2016 (EFSA & ECDC 2016; EFSA & ECDC 2018) found the highest percentages of isolates resistant to ciprofloxacin, nalidixic acid and tetracyclines in campylobacters from broilers, turkeys and meat derived from these animals, with fewer isolates resistant to erythromycin and gentamicin and with generally higher percentages of *C. coli* resistant than *C. jejuni*.

E. coli Higher percentages of *E. coli* were detected with resistance to fluoroquinolones (ciprofloxacin and nalidixic acid) from chicken than from pork, but higher percentages of *E. coli* were detected with resistance to chloramphenicol and tetracycline in pork compared to chicken. These differences may reflect the use of different antibiotic groups during the rearing of chickens and pigs. The UK Veterinary Antibiotic Resistance and Sales Surveillance (VARSS) Report for 2016 indicates that tetracyclines accounted for 45% of antibiotic usage in the pig industry followed by 16% for macrolides, with only approximately 0.05 mg/kg of fluoroquinolones being used in 2016, representing approximately 0.01% of total antibiotic usage (Veterinary Medicines Directorate, 2017). Fluoroquinolones accounted for roughly 0.003% of antibiotic usage in the chicken industry, with tetracyclines and amoxicillin accounting for approximately 13.95% and 37% respectively.

ESBL-producing *E. coli* are of particular public health concern, due to their resistance to third-generation cephalosporin antibiotics, which are used to treat severe infections in human medicine. A gradual increase in the use of third-generation cephalosporins in food animal production may be linked to the increasing prevalence of ESBL-producing bacteria in cattle, poultry and pigs (Joint Working Group of DARC and ARHAI, 2012). Detection of ESBL-producing *E. coli* was more frequent in retail chicken (10.0%) compared to pork (4.7%). However, in this study, significantly fewer ($p<0.0001$; Fishers exact test) chicken samples tested positive for ESBL-producing *E. coli* compared to a 2016 UK survey where 29.7% of samples were positive using similar methodology (APHA 2017). An earlier smaller survey from 2013/14 detected ESBL-producing *E. coli* in 65.4% of 159 chicken samples (Randall et al, 2017). Whilst there were minor differences in the sampling strategies and methodologies (for example, the proportion of whole chickens versus portions sampled) between the two latter surveys, the drop between 2013/14 and 2016 from 65.4% to 29.7% of chicken meat samples being positive for ESBL-producing *E. coli* was significant ($p < 0.001$). In 2012, the British Poultry Council, which represents more than 90% of the UK poultry meat production, banned the use of all cephalosporins in flocks used for poultry meat production. Between 2012 and 2016, overall sales of 3rd/4th generation cephalosporins for use in livestock and fish farmed for food reduced from 0.20 to 0.15 mg/kg (i.e. a 25% reduction), with no recorded usage of this group of antimicrobials in the poultry industry in 2015 or 2016 (Veterinary Medicines Directorate, 2017). It is possible that this reduction in cephalosporin use is associated with the decrease in isolation of ESBL-producing *E. coli* from chicken meat seen across these three studies.

Methodological differences, variations in sample type (e.g. whole chickens versus chicken portions) and seasonality, however, may also account for some of the difference in ESBL prevalence. Across the EU, presumptive ESBL-producing *E. coli* were detected in 35.9% of chicken retail meat samples in 2016 (EFSA 2018).

In this study, ESBL-producing *E. coli* were detected in a higher proportion (4.7%) of minced pork samples compared to a survey of 312 pork samples (retail pork fillets, chops and diced/sliced pork) tested as part of the harmonised EU survey in 2015 where 1.6% were positive (APHA 2016). In another smaller survey conducted in 2013/14, 2.5% of 79 pork samples tested positive for ESBL-producing *E. coli* (Randall et al. 2017). The differences in the percentages of pork samples positive for ESBL-producing *E. coli* between this study and previous studies may reflect different sample types, seasonality and/or differences in methodologies. Across 22 EU member states 7.0% of pig meat samples yielded presumptive ESBL-producing *E. coli* and 0.4% yielded *E. coli* with an ESBL + AmpC phenotype in 2014 (EFSA 2017).

Of 89 ESBL *E. coli* isolates tested for specific β-lactamase resistance genes, 40 (46%) were positive for the $bla_{\text{CTX-M}}$ gene, of which 13 were also positive for bla_{TEM}. The most common CTX-M type detected was CTX-M-1 (detected in 32 isolates). CTX-M-15, which is recognized as one of the most widely distributed CTX-M enzymes, was detected in two *E. coli* isolates (one from chicken and one from pork). Isolates that were negative for $bla_{\text{CTX-M}}$, $bla_{\text{OXA-1}}$, bla_{SHV}, bla_{TEM} genes may have had other resistance genes e.g. an AmpC gene such as bla_{CIT}.

Klebsiella The percentages of *Klebsiella* isolates with resistance were generally lower than for *E. coli* isolates. Since a specific test was carried out using an agar plate that contained cefotaxime to select presumptive ESBL-producing *E. coli*, it is unsurprising that a larger proportion of samples yielded ESBL-producing *E. coli* than ESBL-producing Klebsiellae. However, even when considering the indicator *E. coli* strains that were isolated using a method that did not make use of selective antibiotics in the agar, the percentages of resistant *E. coli* were considerably higher than for *Klebsiella* spp., especially for cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, nalidixic acid, tetracycline and trimethoprim. This difference may indicate that the *Klebsiella* and *E. coli* strains do not naturally co-exist in the same animals for sufficiently long periods of time for exchange of mobile (horizontally transferable) resistance genes to occur. This is backed up by the fact that *E. coli* were detected more frequently in chicken whereas *Klebsiella* spp. were detected in significantly more pork samples. Only 21 samples of meat (13 pork and 8 chicken) gave positive results for both *E. coli* and *Klebsiella* spp. (data not shown).

Enterococci Enterococci were isolated more frequently than the other target organisms from both chicken and pork. However, the percentages of isolates with resistance to teicoplanin and vancomycin were low, with only 1% of isolates showing vancomycin resistance. A study of 21 whole, raw chickens in the UK in 2000 (Jørgensen et al, 2002) showed the presence of vancomycin-resistant enterococci (VRE) in 19/21 chicken carcasses. However, this marked difference in prevalence may reflect differences in methodology since the Jørgensen study involved detection by both enrichment and enumeration methods, whereas the study reported here used only an enumeration procedure on the majority of samples. Enrichment is a more sensitive method, in that it allows the detection of very low numbers of bacterial cells (usually less than 10 cells) in a 25 g portion of sample, whilst the enumeration procedure has a detection limit of 20 cfu per gram (approximately 50-fold less sensitive). Therefore, it is difficult to make a direct comparison of results from the two studies. An EU ban on the use of the glycopeptide antibiotic, avoparcin, in animal production in 1997 resulted in reports of decreased prevalence of VRE in Italy (Pantosti et al, 1999), the Netherlands (van den Bogaard et al,

2000), Denmark (Bager et al, 1999) and Germany (Klare et al, 1999). It is possible that the low rates of VRE prevalence in the study reported here compared to the earlier Jørgensen study may reflect the reduction in the use of glycopeptide antibiotics in animal production in the UK. There are at least nine recognised phenotypes of acquired glycopeptide resistance (Ahmed and Baptiste, 2017), of which VanA and VanB are the most prevalent and globally widespread. Of the three VRE isolates detected in this study, one was confirmed as *vanA* positive, one was identified as an *E. gallinarum* for which vancomycin resistance is intrinsic and one was negative for *vanA* and *vanB*, suggesting vancomycin resistance was due to other mechanisms.

Livestock-associated MRSA has caused concern within the EU, and an EFSA opinion in 2009 recommended that periodic monitoring of intensively reared animals should be carried out (European Food Safety Authority, 2009). However, to date, this strain has rarely been detected in the UK, and an FSA risk assessment published in 2017 concluded that the risk to human health from the preparation, handling and/or consumption of LA-MRSA / MRSA contaminated foodstuffs in the UK was very low (Food Standards Agency, 2017). Whilst MRSA was not included in this study, Fox et al (2017) reported the detection of MRSA in 7.3% of 124 raw meat samples on retail sale in England. Therefore, this may be an area requiring further surveillance in the future.

It has previously been suggested (Zhao et al, 2003; Warren et al, 2008; Dhanji et al, 2010) that imported foods may be responsible for introducing resistant bacteria into the UK, due to the less tightly regulated use of antibiotics in animal production in some other areas of the world. Whilst a small number of samples (approximately 40) in this study had a non-UK approval code, only five chicken samples (and no pork mince) were from outside the EU. Samples were collected according to market share, and therefore it appears that imported meat does not currently constitute a significant proportion of the retail chicken and pork mince market in the UK. Indicator *E. coli* isolates with resistance to greater than three antimicrobial groups were detected in one pork and three chicken samples from non-UK countries of origin (all within the EU) and *E. coli* isolates demonstrating an ESBL and/or AmpC profile were detected in ten chicken samples (both EU and non-EU origin). However, it was not possible to demonstrate any statistically significant differences compared to UK products, due to the small number of samples in this category. The contribution to AMR in the food chain of imported foods, including other food groups that are more commonly imported from outside the EU, may require consideration in future studies.

6. Conclusion

This study has provided data on AMR prevalence in chicken and pork mince on retail sale in the UK. It has provided some reassurance that AMR prevalence is currently low in enterococci and *Klebsiella* species, but has highlighted the potential need for continued monitoring relating to ESBL-producing *E. coli* and erythromycin resistance in *Campylobacter* species. Results from this study will provide a valuable baseline against which further surveillance data can be compared in future to identify trends and changes in resistance rates. Whilst there is an ongoing need to monitor the emergence of antimicrobial resistance, the risk to consumers can be reduced by following the ‘4C’s when transporting, storing and preparing food.
The 4Cs are:

- cleaning well
- cooking thoroughly
- chilling correctly
- avoiding cross-contamination

Thorough cooking is crucial as it can destroy bacteria that may be present in foods including those that are AMR. Good hygiene practices at all stages of the food chain are also important as this will help to reduce the risk of spreading AMR bacteria to other foods and surfaces.

7. Recommendations for further studies

It would be beneficial to continue this surveillance of AMR in retail chicken and pork over a longer time period, in order to identify any seasonal differences in AMR prevalence as well as increasing the number of imported samples included in the dataset.

The inclusion of livestock associated MRSA in the testing parameters in future would also be of interest, to increase the understanding of the prevalence of this organism in the UK food chain and to compare data with that generated through the EU surveillance projects.

A broader assessment of AMR prevalence in imported foods (of both animal and non-animal origin) will be important in future, since it is recognised that antibiotic usage in humans and animals is less well-regulated in other parts of the world than it is within the EU.

The number of *Salmonella* isolates in this study was small. However, *Salmonella* isolates from a wide range of food samples are isolated by food microbiology laboratories during routine examinations and submitted to the PHE GBRU laboratory for further typing using whole genome sequencing. A more detailed review of the WGS data would allow information on AMR profiles to be collated for these isolates, thus providing a larger dataset on AMR in *Salmonella* isolates in the UK food chain.

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APPENDIX I

NATIONAL INFECTION SERVICE

FOOD, WATER AND ENVIRONMENTAL MICROBIOLOGY

EXAMINATION OF CHICKEN AND PORK FOR ANTIMICROBIAL-RESISTANT BACTERIA

AMENDMENT HISTORY

Controlled document reference	
Controlled document title	**Examination of chicken and pork for antimicrobial-resistant bacteria**

The amendment history is shown below. On issue of revised or new documents each controlled document should be updated by the copyholder in the laboratory.

Page	Section(s) involved	Amendment
All	All	New method issued

EXAMINATION OF CHICKEN AND PORK FOR ANTIMICROBIAL-RESISTANT BACTERIA

INTRODUCTION

Scope

This method describes the test procedures required for the examination of raw chicken and pork products for an FSA-funded study of antimicrobial resistance.

Background

The Food Standards Agency requires data on the prevalence of antimicrobial resistance (AMR) in retail chicken and pork to inform AMR risk assessment in the food chain, to monitor trends in emerging AMR issues, to track progress with interventions aimed at tackling AMR and to contribute to the wider international effort on AMR surveillance.

PRINCIPLE

Chicken and pork samples will be collected from retail and/or wholesale in England, Wales, Northern Ireland and Scotland and tested for pathogens (*Salmonella* in pork and *Campylobacter* in chicken) using standard methods. Enumeration of indicator bacteria (*Klebsiella, E. coli* and enterococci) will also be carried out, using standard methods where available, and in-house methods where no standard exists. Any target organisms isolated will be forwarded to specialist laboratories for AMR testing.

DEFINITIONS

Antimicrobial resistance
Growth of bacteria in Minimum Inhibitory Concentration (MIC) tests in the presence of agreed antibiotics at levels above the cut-off points shown on the EUCAST website: https://mic.eucast.org/Eucast2/.

Salmonella species
Micro-organisms that form typical or less typical colonies on solid selective agar media and which display the biochemical and serological characteristics described in ISO 6579:2017.

Campylobacter species
Micro-organisms which form typical or less typical colonies on solid selective agar media incubated at 41.5°C and which display the morphological, biochemical and growth properties described in ISO 10272-2:2006.

E. coli
Micro-organisms which, under the test conditions specified, grow in the presence of bile salts at 44°C and show a positive β-glucuronidase reaction.

Enterococci
Micro-organisms which form typical colonies on the surface of the selective agar medium described and which demonstrate aesculin hydrolysis in the confirmatory test specified.

Klebsiella species
Micro-organisms which grow with typical morphology on the selective agar medium described, and which are confirmed by biochemical array and/or MALDI-TOF as belonging to the *Klebsiella* genus.

Extended Spectrum Beta-Lactamase Producing E. coli (ESBLs)
Micro-organisms which grow with typical morphology on the selective agar medium described, and which are confirmed by biochemical array and/or MALDI-TOF as being *E. coli*.

SAFETY CONSIDERATIONS

General Safety Considerations

Normal microbiology laboratory precautions apply.

All laboratory activities associated with this SOP must be risk assessed to identify hazards. Appropriate controls must be in place to reduce the risk to staff or other groups. Staff must be trained to perform the activities described and must be provided with any personal protective equipment (PPE) specified in this method. Review of this method must also include a review of the associated risk assessment to ensure that controls are still appropriate and effective. Risk assessments are site specific and are managed within safety organiser.

Specific Safety Considerations

Not applicable

Laboratory Containment

All procedures can be carried out at Containment Level 2.

EQUIPMENT

Usual laboratory equipment and in addition:

- Top pan balance capable of weighing to 0.1g
- Gravimetric diluter (optional)
- Stomacher

- Vortex mixer
- Automatic cycling incubator: 30±1°C and 44±1°C
- Colony Counter (optional)
- Stomacher bags (sterile)
- Automatic pipettors and associated sterile pipette tips capable of delivering up to10 mL and 1 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)
- Sterile spreaders

CULTURE MEDIA and reagents

Equivalent commercial dehydrated media may be used; follow the manufacturer's instructions.

Media for the detection of *Salmonella* are as described in ISO 6579:2017.

Media for the enumeration of *Campylobacter* are as described in ISO 10272-2:2006.

Buffered peptone water

Enzymatic digest of casein	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate dodecahydrate	9.0 g
or anhydrous disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Water	1L
pH 7.0 ± 0.2 at 25°C	

Peptone saline diluent (Maximum recovery diluent)

Peptone	1.0 g
Sodium chloride	8.5 g
Water	1 L
pH 7.0 ± 0.2 at 25 °C	

Tryptone Bile Glucuronide agar (TBX)

Enzymatic digest of casein	20.0 g
Bile salts No.3	1.5 g
5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (BCIG) cyclohexylammonium salt	75 mg
Dimethyl sulfoxide (DMSO)	3 ml
Agar	15.0 g
Water	1L
pH 7.0 ± 0.2 at 25°C	

Slanetz and Bartley Glucose Azide Agar (SB)

Tryptose	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Di – Potassium hydrogen phosphate	4.0 g
Sodium azide	0.4 mg
Tetrazolium chloride	0.1mg
Agar	10.0 g
Water	1 L
pH 7.2 ± 0.2 at 25°C	

Bile Aesculin Agar (BAA)

Peptone	8.0 g
Bile salts	20.0 g
Ferric citrate	0.5 g
Aesculin	1.0 g
Agar	15.0 g
Water	1 L
pH 7.1 ± 0.2 at 25°C	

MacConkey with cefotaxime (1.0mg/l) (McCon+CTX)

Pancreatic digest of gelatin	17.0 g
Peptones (meat and casein)	3.0 g
Lactose	10.0 g
Bile salts No. 3	1.5 g
Sodium chloride	5.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Water	1 L
pH 7.1 ± 0.2 at 25°C	

Selective Supplement: Cefotaxime sodium salt stock solution prepared in bi-distilled water.

Aliquots of aqueous cefotaxime stock solution (concentration 1 mg/mL) can be stored at -20° C.

Note: It is important to take the activity of the drug into account to ensure that 1 mg/mL active compound is used. E.g. if the manufacturer has given an activity of 50%, 2 mg/mL should be prepared to give an active concentration of 1 mg/mL.

Klebsiella Selective HiCrome™ Agar (KA)

Peptone, special	12.0 g
Yeast extract	7.0 g
Sodium chloride	5.0 g
Bile salts mixture	1.5 g

Chromogenic mixture	0.2 g
Sodium lauryl sulphate	0.1 g
Agar	15.0 g
Water	1 L
pH 7.1 ± 0.2 at 25°C	

Nutrient Agar

'Lab-Lemco' powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1 L
pH 7.4 ± 0.2 at 25°C	

Dorset Egg Slopes

Charcoal swabs

SAMPLE PROCESSING

Sample receipt

Samples will be delivered either in person or by courier from the sampling contractor, Hallmark Veterinary Compliance Services. On receipt, the following information must be recorded:

1) Date and time of receipt
2) Temperature of samples (if above 8°C, determine whether the deviation is sufficiently significant to affect results).
3) Unique ID number (if possible, use a barcode reader to avoid transcription errors).
4) Retailer from which sample was purchased
5) Brief sample description (e.g. chicken breast / whole chicken / pork mince in tray).

Full sample details will be recorded by Hallmark on an electronic spreadsheet that will be available for the laboratory to check and refer to. However, in order to allow the lab's own records to be accurately cross-checked against Hallmark's records, the retailer and sample description will be recorded on the LIMS system along with the unique ID allocated by Hallmark.

Note: If possible, return the insulated sample container to Hallmark for re-use.

Sample preparation and dilutions

Weigh out at least 27 g of sample and prepare a 10^{-1} homogenate in buffered peptone water (BPW).

Homogenise for between 30 seconds and 3 minutes in a stomacher. The homogenisation time required will depend on the manufacturer instructions and the type of sample being examined.

Decant 20 ml of homogenate into a sterile container for inoculation of direct plates. (Prepare further decimal dilutions as required in Peptone Saline Diluent.)

Retain the remaining 250 ml to be incubated as an enrichment broth.

Inoculation and incubation

Inoculate 0.5 ml of 10^{-1} homogenate onto the surface of a TBX plate and incubate at 30 ± 1°C for 4 ± 1 h and then 44 ± 1°C for 21 ± 3 h.

Inoculate 0.5 ml of 10^{-1} homogenate onto the surface of an SB plate and incubate at 37 ± 1°C for 48 ± 2 h.

If enumeration of *Klebsiella* is required (subject to pilot study), inoculate 0.5 ml of 10^{-1} homogenate onto the surface of a KA plate and incubate at 37 ± 1°C for 24 ± 3 h.

For chicken samples only, plate 1 ml of the homogenate over 3 CCDA plates and also 100 µl onto one CCDA plate. Incubate CCDA plates in a microaerophilic atmosphere at 41.5 ± 1°C for 44 ± 4 h.

Incubate the remaining 250 ml BPW homogenate at 37 ± 1°C for 18 ± 2 h.

Sub-culture to selective media

Sub-culture the bpw pre-enrichment broth (using a 10 µl loop) to:

- MacConkey+CTX – incubate at 44 ± 1°C for 20 ± 2 h.
- KA – incubate at 37 ± 1°C for 24 ± 3 h.
- **For pork samples only:** selective broths (and subsequent sub-culture to selective agar plates) as described in ISO 6579:2017

Identification and counting of colonies

Remove plates from the incubator and, where possible, count the colonies on plates with between 10 and 150 colonies.

E. coli colonies are blue or blue-green on TBX agar. No confirmation is required.

Suspect *Enterococcus* species colonies are red, maroon or pink in colour on SB agar. Confirm as described below.

Klebsiella species form purple-magenta coloured colonies on KA agar.

ESBL-producing *E. coli* form red, non-mucoid colonies on MacConkey+CTX agar.

Confirmation

For *Salmonella* and *Campylobacter* pick 5 (or less if less present) colonies and confirm as described in ISO 6579:2017 and ISO 10272-2:2006, respectively.

Enterococci:

Pick five isolated suspect colonies and sub-culture onto a section of a BAA plate. Incubate at 44 ± 1°C for 24 ± 2 h. Positive identification is the production of a brown to black colouration, indicating aesculin hydrolysis.

Klebsiella:

Pick five isolated suspect colonies and sub-culture onto a section of a nutrient agar plate. Incubate at 37 ± 1 °C for 24 ± 2 h. Confirm identity by inoculating a 20E API strip or by using MALDI-TOF.

ESBL-producing *E. coli*:

Sub-culture five suspect purple/red colonies by re-streaking onto MacConkey agar containing 1mg/l CTX. Incubate at 37 °C for 18-22 h.
Confirm *E. coli* identity using a validated method (e.g. 20E API, MALDI-TOF or oxidase & indole tests).

QUALITY CONTROL

Porton FW&E Laboratory will provide quality control material prior to the start of the study.

Agar plates for *Klebsiella* procedure will be prepared by PHE, and QC checks performed, prior to distribution to other participating laboratories.

Agar plates for *ESBL* procedure will be prepared by APHA, and QC checks performed, prior to distribution to other participating laboratories.

REPORTING OF RESULTS

The test report specifies the method used, all details necessary for complete identification of the sample and details of any incidents that may have influenced the result

Lower detection limit

If plates prepared from the 10^{-1} dilution of the product contain no colonies report the result as

Less than 20 CFU per g

Or **Less than 10 CFU per g** for Campylobacter

For enrichment methods, report as

Detected / not detected in 25 g.

Upper detection limit

If there are only plates containing more than 150 colonies but confirmation is possible, report as greater than the highest count for the dilution used (*e.g.* if 10^{-1} dilution used upper limit will be greater than $3.0x10^3$ CFU per g).

Results of public health significance

The presence of *Salmonella* in pork mince is unsatisfactory according to EC 2073/2005 (as amended). This must therefore be reported to the Project Lead, caroline.willis@phe.gov.uk, as soon as possible after the result has been confirmed (and no more than two weeks after the commencement of testing the sample).

ANOMALOUS RESULTS

Where anomalous results are obtained (for example, an unusual number of samples being positive for a particular organism on the same day of testing), appropriate checks must be made on results before they are reported. These checks should include:

1) Review of sample type and other test results for the same sample, to assess general plausibility of results
2) Review of sterility controls for the day of testing
3) Review of results for other samples tested at the same time, to ensure that there is not an unusual proportion of failures overall
4) Review of result entry on the LIMS system, to ensure that there are no transcription errors, that confirmatory tests have been performed as appropriate and that any calculations have been performed correctly.
5) Review of confirmatory controls for the day that confirmatory tests were set up
6) Consideration of any relevant IQC, EQA or media QC tests that may have been performed on the same days as the samples in question (i.e. to rule out cross-contamination with control strains).
7) Review of recent environmental monitoring results for the relevant laboratory areas.
8) Review of sample details entered onto the LIMS system, to ensure any results are reported in relation to the correct sample and location.

Once appropriate checks have been made, report such results to the Project Lead, caroline.willis@phe.gov.uk, as soon as possible.

REFERRAL OF CULTURES

For each organism, send five isolates (or all isolates if less than five) to the appropriate laboratory for MIC testing, as shown below. Campylobacter strains should be sent on charcoal swabs, as soon as they have been isolated. All other organisms should be sent on Dorset Egg agar slopes, and may be collected together for weekly submission to the appropriate lab.

Organism	**Laboratory**	**Address**	**Contact Name**

Salmonella	APHA	Field Epidemiology & Surveillance, Department of Bacteriology, Woodham Lane, Addlestone, Surrey, KT15 3NB	Luke Randall (020 8026 9853)
E. coli including ESBL E. coli	APHA		
Klebsiella	PHE AMRHAI	61 Colindale Avenue, London, NW9 5EQ	Matthew Ellington (0208 327 7306)
Enterococci	PHE AMRHAI		
Campylobacter	PHE GBRU	61 Colindale Avenue, London, NW9 5EQ	Craig Swift (0208 200 4400)

APPENDIX: FLOWCHART SHOWING THE PROCESS TO EXAMINE CHICKEN AND PORK