

Scotland's Rural College

Detection of extended spectrum lactam, AmpC and carbapenem resistance in Enterobacteriaceae in beef cattle in Great Britain in 2015

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1 **Detection of Extended Spectrum Beta-Lactam (ESBL), AmpC and carbapenem resistance in**
2 **Enterobacteriaceae in beef cattle in Great Britain in 2015**

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15 **Running title:** ESBL-producing *E. coli* in beef cattle

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18 * **Corresponding author:** Luke Randall. Email: Luke.Randall@apha.gsi.gov.uk.

23 **Aims:** This study investigated the occurrence and genetic diversity of Enterobacteriaceae with
24 Extended-Spectrum Beta-Lactamase (ESBL), AmpC and carbapenemase mediated resistance in
25 British beef cattle, and related risk factors.

26 **Methods and Results:** Faecal samples (n=776) were obtained from farms in England & Wales
27 (n=20) and Scotland (n=20) in 2015. Isolates from selective agars were identified by MALDI ToF
28 mass spectrometry. Selected isolates were characterised by multiplex PCR (*bla*_{CTX-M}, *bla*_{OXA}, *bla*_{SHV}
29 and *bla*_{TEM} genes), whole genome sequencing (WGS), MICs and PFGE. None of the faecal samples
30 yielded carbapenem resistant *E. coli*. Ten (25%) of the farms tested positive for ESBL-producing
31 CTX-M Enterobacteriaceae, fifteen (37.5%) of the farms were positive for AmpC phenotype *E. coli*
32 and none were positive for carbapenem resistant *E. coli*. WGS showed a total of 30 different
33 resistance genes associated with *E. coli*, *Citrobacter* and *Serratia* from ESBL agars, and co-location
34 of resistance genes with *bla*_{CTX-M1}. Buying bulls and bringing in fattening cattle from another farm
35 were identified as significant risk factors for positive samples harbouring CTX-M
36 Enterobacteriaceae or AmpC phenotype *E. coli* respectively.

37 **Conclusions:** Beef cattle on a proportion of farms in GB carry ESBL-producing
38 Enterobacteriaceae. Factors, such as operating as a closed herd, may have an important role in
39 reducing introduction and transmission of resistant Enterobacteriaceae. The results indicate
40 management factors may play an important role in impacting ESBL prevalence. In particular,
41 further study would be valuable to understand the impact of maintaining a closed herd on reducing
42 the introduction of resistant Enterobacteriaceae.

43
44 **Significance and Impact of the study:** This is the first study showing the presence of ESBL-
45 producing Enterobacteriaceae in British beef cattle.

46
47 **Keywords:** Antibiotics, Enterobacteria, Epidemiology, Resistance, Veterinary

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49

50 **Introduction**

51 Antimicrobial resistance (AMR) is of major concern for both animal and human health. In recent
52 years Enterobacteriaceae with extended-spectrum β -lactamases (ESBLs) have been associated with
53 increased resistance to critically important cephalosporin antibiotics (Canton and Coque, 2006,
54 Schmid et al., 2013), and have been increasingly isolated from humans (Lau et al., 2008), including
55 healthy people (Kirchner et al., 2013, Card et al., 2014, Card et al., 2015, Kirchner et al., 2017),
56 companion animals (Carattoli et al., 2005), food animals (Horton et al., 2011, Randall et al., 2011,
57 Randall et al., 2014a, Kirchner et al., 2017), including some that also harbour *mcr* plasmid mediated
58 colistin resistance (Figueiredo et al., 2016, Duggett et al., 2018), and some foods (Warren et al.,
59 2008, Egea et al., 2012, Kola et al., 2012, Peternel et al., 2014, van Hoek et al., 2015, Tekiner and
60 Özpınar, 2016). The resistance is mainly conferred by ESBL enzymes, which are normally plasmid
61 encoded, and of which the CTX-M group has become a dominant type, particularly in *Escherichia*
62 *coli* (Snow et al., 2012, Schmid et al., 2013). Whilst in a human clinical setting, increased mortality
63 for bloodstream infections due to ESBL-producing *E. coli* versus non-ESBL *E. coli* has previously
64 been reported (Gastmeier et al., 2012), previous studies suggest that clonal spread of isolates from
65 food and farm animals to humans is unlikely, although dissemination of genes encoding plasmid
66 mediated AmpC (pAmpC)/ESBL may occur (de Been et al., 2014, Bonten and Mevius, 2015,
67 Borjesson et al., 2016).

68 Prevalence of ESBL-producing *E. coli* in cattle has been demonstrated to vary greatly between
69 countries; ranging from 35.4% to 86.7% of positive farms and from 1% to 32.8% of positive
70 animals (Snow et al., 2012, Schmid et al., 2013, Dorado-Garcia et al., 2016, Gonggrijp et al., 2016,
71 Hille et al., 2017). In the United Kingdom, the first case of CTX-M ESBL-producing *E. coli* was
72 reported in calves in a dairy herd in 2004 (Teale et al., 2005). A longitudinal study of one
73 commercial dairy farm found 82.8% of calves were positive for ESBL-producing *E. coli* (Watson et
74 al., 2012). Several other studies have focused on the presence of ESBL-producing *E. coli* in dairy
75 cattle in the UK (Liebana et al., 2006, Horton et al., 2011, Snow et al., 2011, Watson et al., 2012)

76 but according to a recent study there is a lack of information on the occurrence of ESBL-producing
77 *E. coli* in the gut of British beef cattle (Horigan et al., 2016). In 2011 to 2012, a study in Germany
78 investigated the presence of ESBL-producing *E. coli* in ~ 600 samples from 30 mixed dairy and
79 beef cattle farms, 15 beef cattle farms and 10 control farms that had not used antibiotics for at least
80 6 months (Schmid et al., 2013). Samples from mixed dairy and beef farms were significantly more
81 likely to be positive for ESBL-producing *E. coli* than samples from beef cattle farms (Schmid et al.,
82 2013). Additionally, isolates from mixed dairy and beef farms were significantly more resistant to a
83 number of antibiotics than isolates from beef cattle farms (Schmid et al., 2013).

84 The aim of this study therefore was to investigate the presence of ESBL-producing, AmpC-
85 producing and carbapenem resistant Enterobacteriaceae in beef cattle from farms in England, Wales
86 and Scotland, using six different selective agars to isolate Enterobacteriaceae from faeces following
87 non-selective enrichment. Isolates identified by MALDI ToF were further characterised by PCRs,
88 whole genome sequencing (WGS), Minimum Inhibitory Concentrations (MICs) and Pulsed Field
89 Gel Electrophoresis (PFGE). The secondary objective was to investigate potential risk factors
90 associated with the presence of ESBL-producing Enterobacteriaceae with an emphasis on *E. coli*.

91

92 **Materials and methods**

93

94 **Study population and farm selection**

95 Commercial British cattle farms were selected from those participating in an existing larger study
96 on *E. coli* O157 in cattle of slaughter age as previously described (Henry et al., 2017). For the
97 present study, 40 farms (Scotland n=20 and England & Wales n=20) were systematically selected
98 from the source population from which samples were collected on or after 13th April 2015. When
99 the farms to be sampled in a week was determined for the larger study, one Scottish farm and one
100 from England & Wales were randomly selected from these. On receipt of the samples, whether
101 those selected farms provided agreement for their samples to be used for other research purposes
102 was checked, and if a selected farm could not be used, the next available alternative was substituted.

103 **Sample size calculation**

104 The population of 40 farms was selected to provide a crude estimate of herds positive for ESBL-
105 producing Enterobacteriaceae, i.e. herd-level prevalence, such that a 5% prevalence would be
106 detected with an accuracy of 6.8% , so a prevalence between 0 - 11.8% could be detected with 95%
107 confidence. Having 20 English/Welsh and 20 Scottish farms, only allowed for the detection of large
108 differences in prevalence (>10%) between the two sets of countries.

109

110 **Farm visits and data collection**

111 Each study farm was visited once between April and October 2015. From each farm, individual
112 faecal pat samples were collected from the group of finishing cattle closest to slaughter on the day
113 of the visit. The number of animals sampled on each farm was determined by the size of the
114 relevant group of cattle according to a protocol designed for the larger study, which assumed that if
115 8% of animals were positive for *E. coli* O157, there would be a 0.9 probability of identifying groups
116 containing at least one *E. coli* O157 positive animal (Chase-Topping et al., 2007, Gunn et al., 2007,
117 Henry et al., 2017). As ESBL prevalence in beef cattle was unknown it was assumed that it would
118 be of a similar prevalence to *E. coli* O157. Information on general farm characteristics, management
119 practice and health status was collected during an interview with the farmer using a standardised
120 questionnaire administered by the survey staff.

121

122 **Testing of faecal samples**

123 Faecal samples were enriched in buffered peptone water (BPW) by adding 1 gram (\pm 0.1 gram) of
124 faecal sample to 9 mls of BPW, before incubating at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18 to 22 hours. Enriched
125 samples (10 μ l) were plated to MacConkey – “MCA” agar for non-selective isolation of *E. coli*, to
126 agars selective for ESBL (CHROMagar™ ESBL – “CA-ESBL” and Oxoid Brilliance™ ESBL –
127 “BRILL”) and AmpC phenotypes (CHROMagar™ ECC + 16 mg/L cefoxitin – “CA-AMP”), and to
128 agars selective for carbapenem resistant organisms (chromID® carba – “CARBA” and chromID®

129 OXA-48 – “OXA-48”). Plates were incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18 to 22 hours, with the exception
130 of the MCA agar which was incubated at $44^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18 to 22 hours.

131 All isolates of interest (one *E. coli* and/or Enterobacteriaceae type colony per sample per agar type)
132 on agars were purified by streaking a single colony to a second plate of the original isolation media,
133 prior to further tests.

134

135 **Identification of isolates**

136 Purified isolates were identified by MALDI ToF mass spectrometry (Autoflex II, Bruker Daltonics
137 Ltd, UK) using the manufacturer’s Biotyper database identification software, as previously
138 described (Mellmann et al., 2008, Randall et al., 2015).

139

140 **Testing for *bla*_{CTX-M}, *bla*_{OXA}, *bla*_{SHV} and *bla*_{TEM} genes by PCR**

141 Isolates that grew on the two ESBL selective agars (CA-ESBL and BRILL) were tested for the
142 presence of ESBLs genes *bla*_{CTX-M}, *bla*_{OXA}, *bla*_{SHV} and *bla*_{TEM} using a multiplex PCR as previously
143 described (Fang et al., 2008). Farms that were positive for *bla*_{CTX-M} isolates by PCR were
144 categorised as “CTX-M positive farms.”

145

146 **Minimum inhibitory concentrations (MICs)**

147 MICs were performed against a selected sub set of *E. coli* (n=152) from the different agars. The
148 subset of *E. coli* isolates was selected to include isolates with an AmpC phenotype (n=13, from CA-
149 AMP) or ESBL phenotype from ESBL selective agars (n=21), as well as isolates from non-selective
150 agar (MCA) from different farms (n=118). The *E. coli* isolates from non-selective agar were
151 selected to provide information on antibiotic resistance in background *E. coli* from the different
152 farms.

153 MICs were performed using an agar dilution method based on that of the British Society for
154 Antimicrobial Chemotherapy – BSAC (Andrews, 2001, BSAC, 2015). MICs were determined for

155 apramycin; amoxicillin : clavulanate (fixed concentration of 2 mg/L clavulanate); azithromycin;
156 cefotaxime; ceftazidime; ciprofloxacin; colistin; neomycin; sulphamethoxazole : trimethoprim (19 :
157 1) and tetracycline. MIC range, MIC₅₀ and MIC₉₀ values were calculated for each antibiotic for
158 subsets of *E. coli* from different media.

159

160 **Pulsed-field gel electrophoresis (PFGE)**

161 Selected isolates (n=152, same isolates as selected for MICs) were analysed by pulsed field gel
162 electrophoresis (PFGE) after genomic DNA digestion by *Xba*I, according to methods described
163 previously (Ribot et al., 2006, Horton et al., 2016). Different strains were defined by using a cut-off
164 of 80% similarity as per published guidance (Tenover et al., 1995, Ribot et al., 2006).

165

166 **Whole genome sequencing (WGS)**

167 DNA for WGS was prepared as previously described (Anjum et al., 2016). A total of 100 isolates of
168 interest (Table 1) were selected for WGS based on criteria such as ESBL or AmpC phenotypes on
169 agars, presence of *bla*_{CTX-M} genes by PCR, farm, or based on MIC or PFGE results.

170 DNA was sequenced using Illumina MiSeq and the resulting sequences were checked for quality
171 and AMR genes as previously described (Anjum et al., 2016). The WGS “FASTQ” files before and
172 after (for comparison) assembling using “SPAdes” (Bankevich et al., 2012) were analysed in batch
173 analysis mode using DTU pipelines “MLST” (Larsen et al., 2012), “SerotypeFinder” (Joensen et al.,
174 2015) and “SpeciesFinder” (Larsen et al., 2014). Plasmids’ contigs were analysed using the
175 assembled genomes as described previously (Duggett et al., 2017).

176 AMR genes for six *E. coli* isolates (all ST3476) that originated from the same farm and exhibited
177 identical AMR genotypes were fully or partially attributed to a single contig with an *IncII rep* gene
178 using “ABRICATE” (Anonymous, 2018). For one of the isolates, “UNICYCLER” produced a
179 circular contig of ~107 kb (p185-*IncII*), and this was used as the reference (Wick et al., 2017) for a
180 plasmid comparison across strains using “BRIG” (Alikhan et al., 2011).

181

182 **Statistical analysis – epidemiology**

183 There were five outcome measures for the statistical analyses. These were the number of samples
184 positive for: CTX-M ESBL-producing *E. coli* (CTX-M *E. coli*), as determined by PCR; CTX-M
185 ESBL-producing non-*E. coli* (CTX-M non-*E. coli*), as determined by PCR; CTX-M ESBL-
186 producing Enterobacteriaceae (CTX-M Enterobacteriaceae), as determined by PCR; AmpC
187 phenotype *E. coli* (AmpC *E. coli*) on CA-AMP selective agar, and finally, carbapenem resistant *E.*
188 *coli* on OXA-48 and CARBA selective agars. CTX-M Enterobacteriaceae were classed as those
189 samples that were positive by PCR for *bla*CTX-M.

190 Crude prevalence and 95% confidence interval of each of the outcomes was estimated at herd and
191 individual sample (animal) level. A herd was considered positive for each outcome if at least one
192 sample tested positive in a given test.

193 Descriptive statistics on general farm characteristics and management practices were generated.
194 Continuous variables, if appropriate, were categorised based on their distribution. Binary and
195 categorical variables with few observations (less than 5%) recorded were excluded from analyses
196 due to a low variance or re-categorised to increase the number of observations, if appropriate.
197 Univariable and multivariable mixed effect logistic regression models were used to identify
198 potential risk factors at sample level for the following two outcomes considered of most
199 importance: CTX-M *E. coli* and AmpC *E. coli*. Farm ID was added as a random effect to account
200 for the non-independence of samples from the same farm. Only variables significant at $P < 0.25$ in
201 the univariable analysis were considered for multicollinearity checks using the Fisher-exact test
202 (due to structure of the data). Two variables were considered to be collinear if a $P < 0.01$ was
203 obtained and the variable with the stronger association with the outcome or better biological
204 plausibility was kept for further analysis. Multivariable mixed effect logistic regression models
205 were constructed using a forwards stepwise process starting with exposure variables with the
206 strongest association (based on the results from univariable analysis) with the outcome. Each time

207 an exposure variable was added, a change in the estimates of odds ratio was visually examined and
208 a likelihood ratio (LR) test was done to assess whether the variable should remain in the model. The
209 statistical significance level, α , was set at a value of 0.05. Production type (beef specialist, suckler
210 beef, dairy with beef cattle and “other” – combination of beef specialist and suckler beef) was
211 considered to be *a priori* confounder (Schmid et al., 2013) and was therefore forced into the
212 multivariable models. All statistical analysis were carried out in Stata 14 .1 (StataCorp, Texas,
213 USA).

214

215 **Results**

216

217 **Farm characteristics**

218 The median number of cattle on the 40 study farms was 73 (min=2, max=550), with a higher
219 number of cattle on Scottish farms than on English and Welsh farms (Table 2). The most common
220 production type amongst the study farms was suckler beef (22 farms). Two of the study farms were
221 certified as organic production systems. The majority of the farms (28 farms) were open herds that
222 had purchased cattle within the last 12 months, 16 farms in Scotland and 12 farms in
223 England/Wales. The group of cattle sampled were housed at the time of sampling, as opposed to
224 kept outdoor (grazing), on twenty-one farms (12 Scottish and nine English/Welsh). More
225 information on farm characteristics and management practices is summarised in Supporting
226 Information Table S1.

227

228 **Herd- and sample- apparent prevalence of ESBL bacteria**

229 In total, 776 faecal samples were collected from the 40 farms, ranging between 1 and 60 (mean
230 19.4) samples per farm (Table 2). On two farms, less than five samples were collected.

231 A total of 1,117 isolates from these samples were identified by MALDI ToF (Table 1), including
232 425 isolates from the two ESBL agars (BRILL and CA-ESBL), 518 isolates from the CA-AMP
233 agar, 161 isolates from the two agars to select carbapenem resistant bacteria (CARBA and OXA-

234 48) and 13 isolates from MCA agar (most isolates from MCA were identified as presumptive *E. coli*
235 only based on being lactose fermenters). Isolates from the agars that screened for carbapenem
236 resistance were identified as *Aeromonas* (n=24 samples positive), *Ochrobactrum* (n=17 samples
237 positive) and *Pseudomonas* (n=207 samples positive) species, and carbapenem resistant *E. coli* were
238 not isolated.

239 A total of 110 isolates from ESBL selective agars were tested for the presence of ESBLs genes
240 *bla*_{CTX-M}, *bla*_{OXA}, *bla*_{SHV} and *bla*_{TEM}. All of these isolates were identified by MALDI-ToF as
241 *Citrobacter species* (n=20), *Escherichia coli* (n=54), *Morganella morganii* (n=25), *Proteus species*
242 (n=9) and *Serratia fonticola* (n=2). None of these isolates were positive for *bla*_{OXA} or *bla*_{SHV} genes.
243 The *bla*_{CTX-M} was detected in all *Escherichia coli* and *Serratia fonticola* isolates tested, and in 18 of
244 the *Citrobacter species* isolate tested, but not in the other bacterial species tested. The *bla*_{TEM} was
245 detected in 5 CTX-M positive *E. coli* only.

246 Ten (25%) farms were positive for CTX-M Enterobacteriaceae (*Citrobacter*, *Escherichia* or
247 *Serratia*), nine (22.5%) farms were positive for CTX-M *E. coli* by PCR (all of which were also
248 positive for CTX-M Enterobacteriaceae), with similar herd prevalence in both study regions (Table
249 3). Three (7.5%) farms tested positive for CTX-M non-*E. coli* (*Citrobacter* or *Serratia*, Table 1) by
250 PCR, all of which were Scottish farms. Two of the CTX-M non-*E. coli* positive farms were also
251 positive for *E. coli* carrying CTX-M genes. In total 15 (37.5%) farms were positive for AmpC
252 phenotype *E. coli*, with a slightly higher proportion of positive farms found in Scotland compared to
253 England and Wales (Table 3). The difference was not statistically significant ($P = 0.327$).

254 Crude estimates (not adjusted for the study design) of sample level prevalence are summarised in
255 Table 3. In total, 52 (6.7%) samples originating from ten farms were positive by PCR for CTX-M
256 Enterobacteriaceae, with slightly higher proportion of samples positive from England and Wales
257 compared to Scotland (Table 3), but the difference was not statistically significant ($P = 0.389$).
258 CTX-M *E. coli* (by PCR) were detected in 33 (4.2%) samples (from nine farms) and a significantly
259 higher number of samples tested positive in England and Wales, compared to Scotland ($P < 0.001$).

260 CTX-M positive non-*E. coli* (by PCR) were isolated from 20 (2.6%) samples originated from the
261 three positive Scottish herds. A total of 81 (10.4%) samples were positive for AmpC phenotype *E.*
262 *coli* with significantly higher proportion of AmpC *E. coli* positive samples amongst Scottish herds
263 ($P<0.001$).

264

265 **PFGE**

266 PFGE showed that the *E. coli* isolates from the different farms were diverse based on differences in
267 PFGE banding patterns (Figure 1). However, where isolates were tested from different samples
268 from the same farm, there were some examples of the same PFGE pattern from different samples
269 suggesting distribution of the same clone within the farm.

270

271 **MICs**

272 A summary of MIC results for the 10 antibiotics performed against *E. coli* isolates can be seen in
273 Table 4, with MIC₅₀ and MIC₉₀ values. For apramycin, azithromycin, colistin and neomycin, the
274 isolates showed similar MIC₅₀ and MIC₉₀ values regardless of the agar of origin. As would be
275 expected, isolates from ESBL agars (CA-ESBL or BRILL) or CA-AMP agar tended to show
276 reduced susceptibility to the β -lactam antibiotics cefotaxime and ceftazidime, and to the potentiated
277 β -lactam antibiotic amoxicillin/clavulanate, compared to isolates from MCA. Additionally, isolates
278 from ESBL and CA-AMP agars tended to have increased MIC₅₀ and / or MIC₉₀ values for
279 sulphamethoxazole/ trimethoprim (SXT) and / or tetracycline compared to isolates from MCA.
280 Also, isolates from ESBL agars, but not from CA-AMP agar, tended to have increased MIC₉₀ values
281 for ciprofloxacin, compared to isolates from MCA.

282

283 **Risk factors at the sample level**

284 Risk factors analyses were carried out for the following two outcomes: CTX-M *E. coli* and AmpC
285 *E. coli*. Of the sixty-four potential risk factors, 14 were excluded due to a low variance or due to

286 holding information collected only from a group of farms (i.e. additional information on housing
287 collected from farms where cattle were housed as opposed to being kept outdoor – grazing)
288 (Supporting Information Table S1). Based on the results of the univariable analysis ($P \leq 0.25$),
289 variables considered for the multivariable analyses are summarised in Table 5. In the multivariable
290 analysis, accounting for the effect of production type, buying breeding bulls and changes in feeding
291 in the last two weeks before the sample collection, significantly increased odds of a sample being
292 positive for CTX-M *E. coli* (Table 6). Presence of cats significantly decreased odds of a sample
293 being positive for CTX-M *E. coli* (OR=0.03, 95% CI: 0.0-0.3) when added to the model, however it
294 was excluded due to collinearity with the production type.

295 Bringing in cattle for fattening from other farms and the spreading of a farm's own manure were
296 significantly associated with an animal being positive in the univariable analysis for AmpC *E. coli*
297 (Table 5). In the multivariable analysis, bringing in cattle for fattening from other farms, animals on
298 farms located in Scotland and being a dairy farm with beef cattle present, had significantly
299 increased odds of being positive for AmpC *E. coli* (Table 6). There was no collinearity ($P < 0.01$)
300 detected between the three exposure variables included in the model, although a significant
301 association was detected between production type and bringing in cattle for fattening from other
302 farms ($P = 0.016$).

303

304 **Whole genome sequencing (WGS), multidrug resistance and plasmid analysis**

305 WGS was performed on selected isolates (n=100), of which 96 isolates gave results suitable for
306 analysis using DTU pipelines as summarised in Table 1. There was a reasonable correlation
307 between the AMR genotype and MIC resistance phenotype for isolates tested (Table 4), bearing in
308 mind that not all isolates that were MIC tested were also sequenced.

309 The presence of antibiotic resistance genes detected by WGS in *Citrobacter*, *E. coli* and *Serratia*
310 (only *Citrobacter*, *E. coli* and *Serratia* were found to be positive for *bla*_{CTX-M} genes) from the
311 different agars is shown in Table 7.

312 As would be expected, the 30 isolates from MCA were positive for the least number of resistance
313 genes, with only 5 resistance genes encoding resistance to a total of three different classes of
314 antibiotic (Table 7). Resistance genes in these isolates were also found to older antibiotics, with no
315 ESBL or quinolone resistance genes detected. From the 16 isolates from CA-AMP there were a
316 total of 16 different resistance genes detected, representing resistance to 8 different classes of
317 antibiotic (Table 7). Isolates from CA-AMP, as would be expected, included three different AmpC
318 genes, but also genes encoding resistance to phenicol, quinolones and other classes of antibiotics,
319 but not ESBL genes (Table 7). The 24 isolates from ESBL agars had the most resistance genes
320 (n=30), encoding resistance to 8 classes of antibiotics, including five different *bla*_{CTX-M} genes
321 detected (Table 7).

322 There were six *E. coli* isolates (all ST3476) that originated from the same farm and exhibited
323 identical AMR genotypes (*bla*_{CTX-M-1}, *sul2*, *tetA*). A plasmid comparison across strains was
324 performed using BRIG (Figure 2). The BRIG image indicated that there was high similarity
325 amongst the isolates, and that the *bla*_{CTX-M-1}, *sul2* and *tetA* were co-located on the plasmid.
326 However, whilst the IncII *bla*_{CTX-M-1} plasmid present in these isolates essentially had the same
327 backbone, it harboured some differences in its gene content. When compared to plasmids on NCBI,
328 p185-IncII showed 99% identity to an isolate (GenBank Accession: KT779550.1) from a one-day-
329 old chick treated in an experimental study with a 3rd generation cephalosporin in France in 2016
330 (Baron et al., 2016). Interestingly, a seventh *E. coli* from the same farm, also ST3476, contained no
331 AMR genes or IncII suggesting that the plasmid was either lost or had not transferred to this host.

332

333 **Discussion**

334

335 This is the first study aiming to investigate the occurrence of ESBL-producing Enterobacteriaceae
336 (mainly *E. coli*) in the gut of beef cattle in the UK, and to perform characterisation of isolates.

337 The source population for this survey was the 270 farms that participated in a larger study on *E. coli*
338 O157 in cattle in Great Britain (Henry et al., 2017). The 40 herds included in this current study were

339 not selected at random, participation in additional research was voluntary and the subset selected
340 was seasonal compared to the original study (April - October 2015 compared with September 2014
341 - November 2015). Therefore, there is potential for bias in the estimates of occurrence, if these
342 factors are systematically associated with any of the outcomes of interest. No formal validation of
343 these data was carried out. The estimated average herd size (based on number of cows) of the study
344 farms was smaller than that reported for dairy and beef herds in 2014 (AHDB, 2016), which was
345 based on animals of two years of age or over with offspring, except for Wales, where all animals
346 over two years of age were included. This may indicate that a number of the farms were not
347 representative of the British cattle population. It is however unknown whether herd size would have
348 influenced the status of the farms, and whether this bias would have been important to our study's
349 ability to be representative of the cattle population. In our study, herd size was not significantly
350 associated with the presence of *E.coli* CTX-M and AmpC phenotype *E. coli* positive samples.

351 The results showed no sample/herd being positive for carbapenemase-producing *E. coli*. Isolates
352 from the carbapenem agars were identified mainly as *Aeromonas*, *Ochrobactrum* and *Pseudomonas*
353 species. These bacteria occur in habitats such as soil, sewage and the guts of mammals, are
354 generally considered as opportunist pathogens, and may be intrinsically resistant to carbapenem
355 antibiotics, although this was not investigated.

356 CTX-M Enterobacteriaceae and CTX-M *E. coli* positive herds were found in both study regions, but
357 no significant differences in herd-level prevalence were observed. However, the results at regional
358 level should be interpreted with caution as the sample size was not sufficient to estimate the herd
359 prevalence at this level with a high degree of accuracy, and only large differences (>10%) would be
360 detected with confidence.

361 At sample level, significantly higher proportion of samples positive for CTX-M *E. coli* were
362 detected in herds from England and Wales compared to the herds in Scotland ($P < 0.001$). On
363 average the same number of samples was collected from the majority of study farms in both regions
364 which made comparison good. However, on one farm, only one sample was collected, which was

365 less than expected, resulting in potential misclassification of the herd status with respect to the study
366 outcomes. Furthermore, the presented estimates of positive samples are crude estimates, as they
367 don't account for the complex study design (i.e. non-random selection of farms from the
368 subpopulation of farms participating in the existing larger study), so should be considered as an
369 indication of the level of occurrence of ESBL producing Enterobacteriaceae in beef cattle prior to
370 slaughter. The estimated prevalence for the three ESBL outcomes were below the 8% expected
371 prevalence used for the sample size calculation which will have decreased the confidence around
372 these estimates.

373 Compared to two similar studies carried out in dairy and beef cattle in Germany (Schmid et al.,
374 2013, Hille et al., 2017), the study results suggest that herd- and sample-level estimates of CTX-M
375 *E. coli* in beef cattle in GB were much lower. Whilst the methodology of isolation of ESBL-
376 producing and AmpC phenotype *E. coli* in these two studies were not identical to those used in this
377 study, they were similar in that they did involve a pre-enrichment stage and then plating onto
378 selective agar (MacConkey agar plates containing 1 mg/l cefotaxime).

379 Other European studies, although not directly comparable, estimated cattle prevalence (at the
380 animal level) of ESBL producing Enterobacteriaceae as 8.4% in Switzerland (Reist et al., 2013),
381 and of CTX-M *E. coli* as 4.8% in France (Hartmann et al., 2012).

382 Knowledge of the risk factors is important to inform identification of suitable control measures and
383 surveillance programmes. No associations were detected between the cattle treatment in the last
384 three months before sampling or health problems in the last two weeks before sampling. This
385 suggests that there were also other factors that may have played an important role in introduction
386 and/or transmission of resistant organisms, and/or lack of power to detect a difference given the
387 small sample size. The identified risk factors related mainly to herd management and are in
388 agreement with previous studies. Bringing new animals in significantly increased the risk of the
389 sample being positive for CTX-M *E. coli* and for AmpC phenotype *E. coli* as observed by Reist et
390 al. (2013) and Snow et al. (2012).

391 Changes in feeding two weeks before sampling significantly increased the risk of the sample testing
392 positive for CTX-M *E. coli* (Table 5). This could be indicative of an increased stress as a result of
393 the change and increased disease susceptibility of the animals as also reported by Hille et al. (2017).
394 Dairy herds with beef cattle had significantly increased odds of the sample being positive for AmpC
395 phenotype *E. coli* as previously reported (Reist et al., 2013, Schmid et al., 2013, Hille et al., 2017).
396 In dairy herds, cows are often treated with antibiotics at time points throughout their life, whereas
397 cattle in beef herds are more likely to be treated during the earlier stages of their life rather than
398 close to slaughter (Hille et al., 2017). Further explanations can be related to certain management
399 practices on dairy farms, such as feeding waste milk to calves. A survey showed 90% of UK farms
400 fed waste milk to calves (Brunton et al., 2012), whilst another study showed that 21.4% of waste
401 milk samples contained residues of the cephalosporin cefquinome, which was significantly
402 associated with CTX-M bacteria in the waste milk (Randall et al., 2014b).

403 Whole genome sequencing provided an insight into the resistance genes detected in *E. coli* isolated
404 from the different agars (e.g. isolates from MCA, CA-AMP and ESBL agars) which correlated with
405 the major phenotypes present. Although isolates from ESBL and CA-AMP agar both had resistance
406 genes to 8 different classes of antimicrobials, the isolates from ESBL agar has almost twice the
407 number of resistance genes compared to isolates from CA-AMP with many isolates from the former
408 harbouring multiple allelic variants of genes encoding resistance to the same antimicrobial. Plasmid
409 analysis of six *E. coli* isolates that originated from the same farm and exhibited identical AMR
410 genotypes (*bla**CTX-M-1*, *sul2*, *tetA*) showed that the *bla**CTX-M-1*, *sul2* and *tetA* genes were co-
411 located on the same plasmid. Therefore, the use of cephalosporin, sulphonamide and tetracycline
412 antibiotics could all co-select for this plasmid; a similar observation has also been made by others
413 for MDR isolates (Canton and Ruiz-Garbajosa, 2011). Presence of multiple isolates of the same ST
414 harbouring a similar CTX-M-1 IncII plasmid isolated from different samples provided evidence
415 that this *E. coli* clone can easily disseminate between animals on the same farm. Some differences
416 in the IncII plasmid gene content confirmed the elasticity of plasmid genomes as we have shown

417 previously with *mcr-1* harbouring plasmids which have been detected in the UK and worldwide
418 (Duggett et al, 2017).

419 In conclusion, this study provided new evidence of the level of occurrence of ESBL-producing
420 Enterobacteriaceae in GB beef cattle and the presence of antibiotic resistance genes in such isolates.
421 None of the farms were positive for carbapenem resistant *E. coli*. There were no regional
422 differences between farms in England, Wales and Scotland, thus a proportion of beef cattle in GB
423 can be considered to harbour ESBL producing Enterobacteriaceae. Analysis of risk factors was
424 limited by the scale of this study, but a number of significant factors were identified at animal level.
425 These related mainly to farm management that indicate potential routes for introduction and
426 transmission of resistant organisms / genes, with the possible addition of stress as a result of
427 changes in feeding.

428

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436

437 **Conflict of interest**

438 No conflict of interest declared.

439

440

441 **Supporting Information – See table S1**

442

443 **Table 1.** Summary of general farm characteristics and management practices. Data collected from
444 40 farms (England/Wales n=20, Scotland n=20) in 2015.

445

446

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- 651

652 **Table 1.** Background details of isolates that were whole genome sequenced (n=96)

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Isolation agar(s)	No. of isolates identified by MALDI ToF	No. of isolates selected for WGS	No. of farms with isolates on which WGS was performed	No. of isolates identified as <i>Citrobacter</i> , <i>E. coli</i> or <i>Serratia</i> * from WGS	No. of WGS isolates that tested positive for <i>bla</i> _{CTX-M} by PCR	No. of farms with WGS isolates positive by PCR for <i>bla</i> _{CTX-M}
MCA	13 (834†)	34	27	30	0	0
ESBL agars	425	37	16	24	23	10
CA-AMP	518	24	18	16	0	0
CARBA and OXA-48	161	1	1	0	0	0

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655 * - *Citrobacter*, *E. coli* and *Serratia* were the only three bacterial species that were positive by PCR for *bla*_{CTX-M}. In absence of MALDI ToF
 656 results for most isolates from MCA, bacterial identity was taken from WGS results.

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658 † – Most (834) isolates from MCA were identified as presumptive *E. coli* (lactose fermenters), and were not identified by MALDI ToF

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662 **Table 2.** Summary of frequency distributions of continuous variables describing the number
663 of cattle (by different age groups) and number of samples collected for all farms (n=40).
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Variable		Mean (SD*)	Percentiles				
			10 th	25 th	50 th	75 th	90 th
Number of samples collected	England & Wales	19.4 (11.2)	9	12	19	23	24
	Scotland	19.4 (9.8)	10	12	19	23	31
	Combined	19.4 (10.4)	10	12	19	23	29
Number of cattle in the sample group	England & Wales	16.6 (17.3)	6	8	14	19	22
	Scotland	16.1 (10.8)	7	9	15	19	29
	Combined	16.3 (14.2)	6.5	9	14	19	26
Youngest animal sampled in the group (months)	England & Wales	13.7 (4.9)	8	11	12	16	21
	Scotland	15.8 (4.9)	10	11	17	22	24
	Combined	14.8 (5.3)	10	11	13	18	24
Oldest animal sampled in the group (months)	England & Wales	21.6 (13.7)	12	14	17	26	33
	Scotland	21.3 (10.5)	12	15	21	25	26
	Combined	21.4 (12.1)	12	14	19	25	29
Number of heifers per farm	England & Wales	9.8 (15.2)	0	0	3	8	36
	Scotland	12.2 (21.2)	0	0	4.5	12.5	41
	Combined	11.0 (18.2)	0	0	3	11	36
Number of cows per farm	England & Wales	51.4 (75.9)	0.5	14	26	49	160
	Scotland	39.8 (41.5)	0	0	35	59	110
	Combined	45.6 (60.7)	0	6	29	50	125
Number of calves (≤ 1 year old)	England & Wales	28.0 (35.3)	0	10	20	29	88
	Scotland	51.0 (79.4)	0	3	29	62	113
	Combined	39.3 (61.7)	0	7	21	41	108
Cattle 12-30 months	England & Wales	25.9 (30.8)	0	6.5	14	37	73
	Scotland	50.4 (70.4)	1	8	26	60	122
	Combined	38.1 (57.3)	0	8	18	53	86
Number of bulls over 1 year of age	England & Wales	2.2 (4.4)	0	0	1	2	5
	Scotland	1.1 (1.2)	0	0	1	2	3
	Combined	1.6 (3.2)	0	0	1	2	4
Total number of cattle per farm	England & Wales	117.0 (129.6)	23	42	64	137	351
	Scotland	153.9 (145.9)	29	40	99	222	370
	Combined	135.5 (137.5)	28	41	73	177	367

665 * SD = standard deviation

666 **Table 3.** Herd- and sample- level crude prevalence (and 95% confidence intervals). Data collected as part of the study of 40 farms conducted
 667 between April and October 2015.

% positive (95% Confidence interval)			
Herd-level	England & Wales (N=20)	Scotland (N=20)	Combined (N=40)
<i>E. coli</i> CTX-M (PCR)	25 (8.6-49.1)	20 (5.7-43.7)	22.5 (10.8-38.4)
Non- <i>E. coli</i> CTX-M (PCR)	0	15 (3.2-37.9)	7.5 (1.6-20.4)
Enterobacteriaceae CTX-M (PCR)	25 (8.6-49.1)	25 (8.6-49.1)	25 (12.7-41.2)
<i>E. coli</i> AmpC phenotype	30 (11.9-54.3)	45 (23.1-68.5)	37.5 (22.7-54.2)
Sample-level	England & Wales (N=388)	Scotland (N=388)	Combined (N=776)
<i>E. coli</i> CTX-M (PCR)	7.5 (5.1-10.6)	1.0 (0.0-2.6)	4.2 (2.9-5.9)
Non- <i>E. coli</i> CTX-M (PCR)	0	5.1 (3.1-7.8)	2.6 (1.5-3.9)
Enterobacteriaceae CTX-M (PCR)	7.5 (5.1-10.6)	5.9 (3.8-8.8)	6.7 (5.0-8.7)
<i>E. coli</i> AmpC phenotype	3.8 (2.1-6.3)	17.0 (13.4-21.1)	10.4 (8.3-12.8)

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Table 4. Minimum inhibitory concentration (MIC) (mg/L) parameters of 10 antibiotics against selected *E. coli*

Species	Agar	No. tested	MIC range (mg/L) and (MIC ₅₀ / MIC ₉₀)									
			AMC	APR	AZM	CAZ	CTX	CIP	CST	NEO	SXT	TET
<i>E. coli</i> *	MCA	119	0.25-128 (2 / 4)	1-64 (4 / 4)	0.5-8 (4 / 4)	0.06->128 (0.25 / 1)	0.015-8 (0.06 / 0.13)	0.008-2 (0.015 / 0.03)	0.13->128 (0.5 / 1)	1-64 (1 / 2)	0.25->128 (1 / 2)	0.13->128 (1 / 2)
<i>E. coli</i>	ESBL agars †	22	2->128 (16 / >128)	2-4 (4 / 4)	2-4 (4 / 4)	0.25-64 (2 / 16)	4-128 (32 / 64)	0.008-8 (0.015 / 0.5)	0.25->128 (0.25 / 0.5)	1-4 (1 / 2)	0.5->128 (2 / >128)	1->128 (64 / 64)
<i>E. coli</i>	FOX	11	2->128 (128 / >128)	1-8 (4 / 4)	2-8 (4 / 8)	2-32 (8 / 32)	1-8 (2 / 8)	0.008-0.25 (0.015 / 0.03)	0.25-64 (0.5 / 0.5)	1-64 (2 / 4)	0.25->128 (2 / >128)	1->128 (1 / >128)
All <i>E. coli</i>	All above	152	0.25->128 (4 / 16)	1-64 (4 / 4)	0.5-8 (4 / 4)	0.06->128 (0.5 / 8)	0.015-128 (0.06 / 32)	0.008-8 (0.015 / 0.06)	0.13->128 (0.5 / 1)	1-64 (1 / 2)	0.25->128 (1 / 4)	0.13->128 (1 / 64)
All CTX +	ESBL agars †	22	2->128 (16 / >128)	2-4 (4 / 4)	2-4 (4 / 4)	0.25-64 (2 / 16)	4-128 (32 / 64)	0.008-8 (0.015 / 0.5)	0.25->128 (0.25 / 0.5)	1-4 (1 / 2)	0.5->128 (2 / >128)	1->128 (64 / 64)
AMR genes detected by WGS in isolates that can confer resistance to associated antibiotic. Not all of the above isolates were whole genome sequenced.			<i>bla</i> _{ACT} <i>bla</i> _{CMY-2} <i>bla</i> _{CMY-77} <i>bla</i> _{DHA-1} <i>bla</i> _{DHA-2}	<i>mphA</i>	<i>bla</i> _{CTX-M-1} <i>bla</i> _{CTX-M-8} <i>bla</i> _{CTX-M-15} <i>bla</i> _{CTX-M-24} <i>bla</i> _{CTX-M-32}	<i>bla</i> _{CTX-M-1} <i>bla</i> _{CTX-M-8} <i>bla</i> _{CTX-M-15} <i>bla</i> _{CTX-M-24} <i>bla</i> _{CTX-M-32}	<i>qnrB5</i> <i>qnrB19</i> <i>qnrS1</i> <i>gyrA</i> † <i>parC</i> ‡	<i>pmrA</i> ‡ <i>pmrB</i> ‡ <i>phoP</i> ‡ <i>phoQ</i> ‡	<i>aph(3')-Ic</i>	<i>dfr+</i> <i>sul2, 3</i>	<i>tet(A)</i> <i>tet(B)</i> <i>tet(D)</i> <i>tet(41)</i>	

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AMC - amoxicillin : clavulanate (fixed concentration of 2 mg/L clavulanate), APR – apramycin, AZM – azithromycin, CAZ – ceftazidime, CTX – cefotaxime, CIP – ciprofloxacin, CST – colistin, NEO – neomycin, SXT - sulphamethoxazole : trimethoprim (19 : 1), TET – tetracycline. ND – MIC₅₀ values were not determined for less than 10 isolates.

* – Isolates from McC agar were presumptive *E. coli* on the basis of growing as lactose fermenters at 44°C. Other *E. coli* were confirmed as such by MALDI-ToF.

† – ESBL agars - CA-ESBL or BRILL.

‡ - Genes with non-synonymous SNPs.

682 **Table 5.** Results of exposure variables associated with the presence of CTX-M *E.coli* and
683 AmpC phenotype *E. coli* positive samples in the univariable mixed effect logistic regression
684 models ($P \leq 0.25$) and the production type as a *priori* variable.

Variable	Level	OR	95% CI	P-value
CTX-M <i>E. coli</i>				
Production type	Suckler beef	1		0.824
	Specialist finisher	0.32	0.00-15.93	
	Dairy with beef	0.13	0.00-13.68	
	Other*	0.00	-	
Region	England & Wales	1		0.233
	Scotland	0.17	0.00-3.11	
Cats	No	1		0.023
	Yes	0.02	0.00-0.59	
Breeding bulls other farms/buying bulls	No	1		0.004
	Yes	57.78	3.53-944.92	
Isolation of other farm animals	No	1		0.077
	Yes	14.29	0.74-274.05	
Changed feeding last 2 weeks	No	1		0.033
	Yes	22.06	1.27-383.04	
Changed location last 2 weeks	No	1		0.064
	Yes	14.06	0.85-231.28	
	Level	OR	95% CI	P-value
AmpC <i>E. coli</i>				
Production type	Suckler beef	1		0.293
	Specialist finisher	6.76	0.42-109.81	
	Dairy with beef	12.46	0.73-213.16	
	Other ¹	2.70	0.14-51.87	
Region	England & Wales	1		0.059
	Scotland	6.85	0.92-50.79	
Ducks on farm	No	1		0.172
	Yes	10.22	0.36-288.45	
Geese on farm	No	1		0.150
	Yes	19.44	0.33-1114.07	
Horses	No	1		0.168
	Yes	0.15	0.01-2.22	
Fattening other farm	No	1		0.013
	Yes	27.93	1.98-393.28	
Bulls sharing	No	1		0.213
	Yes	7.43	0.31-175.78	
Cattle to show events	No	1		0.242
	Yes	11.27	0.19-654.80	
Cattle housing	Grazing (outdoor)	1		0.138

	Housed	4.43	0.62-31.77	
Isolation of other farm animals	No	1		0.101
	Yes	5.46	0.71-41.81	
Spread manure own	No	1		0.040
	Yes	0.12	0.01-0.90	

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686 OR = crude (unadjusted odds ratio), CI = confidence interval, P-value (Wald chi-squared).

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688 * Other = combination of beef specialist and suckler beef

689 **Table 6.** Risk factors associated with the presence of CTX-M *E.coli* and AmpC phenotype *E. coli* positive samples in the final multivariable
 690 mixed effect logistic regression model in beef cattle in Great Britain.

<i>E. coli</i> type	Management practice	Level	OR	95% CI	P-value
* CTX-M by PCR	Buying bulls	No	1		
		Yes	57.7	2.4-1379.2	0.012
	Changes in feeding last 2 weeks	No	1		
		Yes	14.3	1.4-147.6	0.026
† AmpC phenotype	Bringing in fattening cattle from other farm	No	1		
		Yes	34.3	1.8-660.2	0.019
	Region	England & Wales	1		
		Scotland	6.3	1.0-38.4	0.047
	Management type	Suckler beef	1		
		Beef specialist	0.58	0.0-9.3	0.701
		Dairy with beef	13.4	1.3-134.6	0.027
	Other ‡	0.46	0.0-7.1	0.580	

691
 692 OR = odds ratio, CI = confidence interval

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 694 * Wald chi-squared = 12.63, $P=0.027$

695 † Wald chi-squared = 13.25, $P=0.021$

696 ‡ Other = combination of beef specialist and suckler beef

697

698 **Table 7.** Resistance genes detected by WGS in *Citrobacter*, *E. coli* and *Serratia** isolates from different agars.
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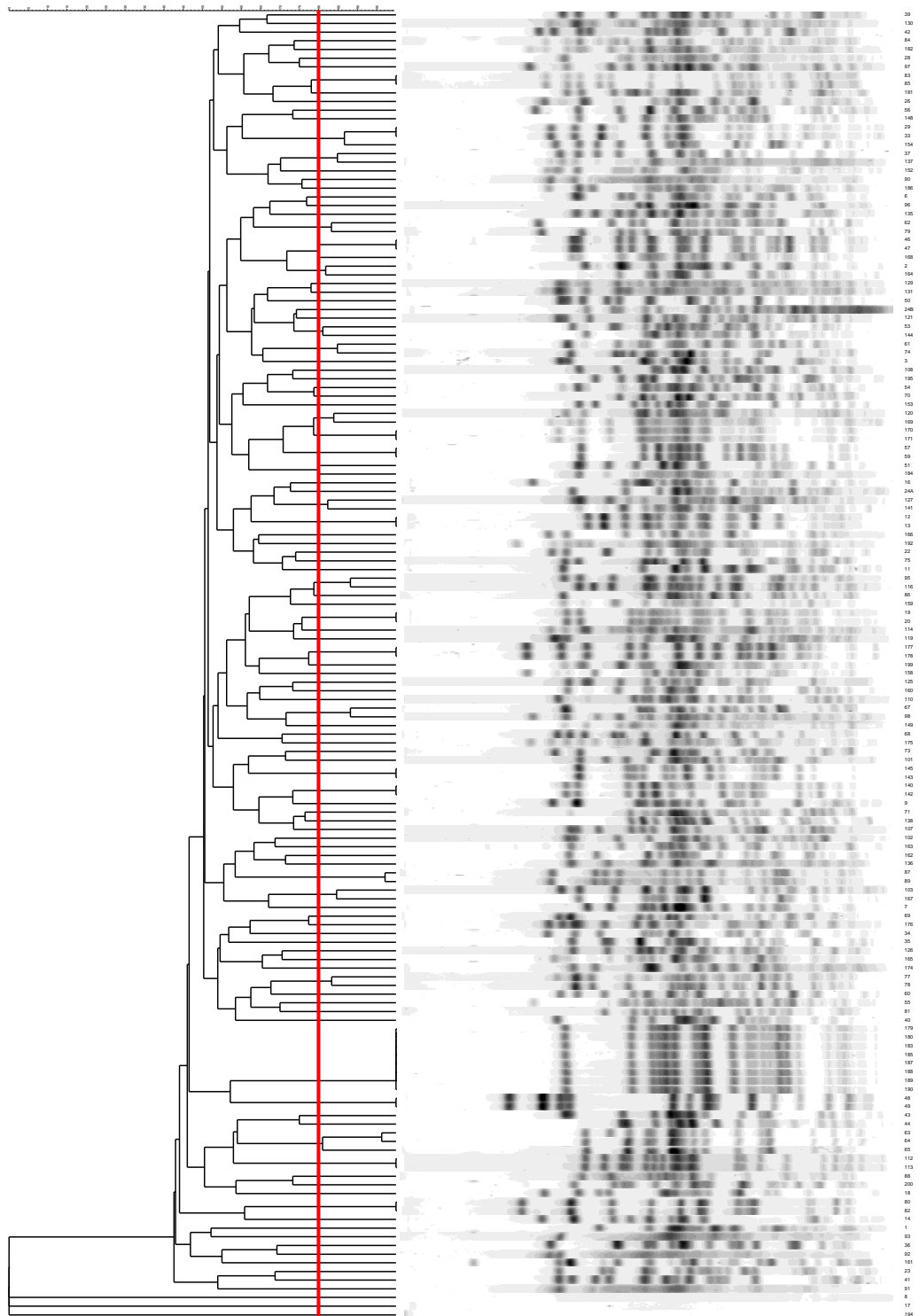
Resistance to antibiotic class	Agar origin for isolation (number of isolates)		
	MCA (n=30)	CA-AMP (n=16)	ESBL - BRILL & CA-ESBL (n=24)
Aminoglycosides	<i>strA, B</i>	<i>aac(6')-Ic; aph(3')-Ic; strA, B</i>	<i>aac(3)-Iid, aadA1, A2; strA, B</i>
Beta-lactams	<i>blaTEM-1C</i>	<i>blaACT-7; blaCMY-70; blaCMY-77; blaTEM-1B.</i>	<i>blaCTX-M-1, 8, 15, 24, 32; blaDHA-2; blaFONA-2, 3, 5; blaTEM-1B</i>
Fosfomycin	-	<i>fosA</i>	-
Macrolides	-	-	<i>mph(A)</i>
Phenicol (e.g. chloramphenicol)	-	<i>catA1; floR</i>	<i>catA2; cmlA1; floR</i>
Quinolones	-	<i>qnrB6</i>	<i>qnrB19; qnrS1</i>
Sulphonamides	<i>sul2</i>	<i>sul2</i>	<i>sul 2, 3</i>
Tetracyclines	<i>tetA</i>	<i>tet(41, B)</i>	<i>tet(A, B, D)</i>
Trimethoprim	-	<i>dfrA5</i>	<i>dfrA1, 5, 12, 17</i>
Total number of resistance gene	5	16	30

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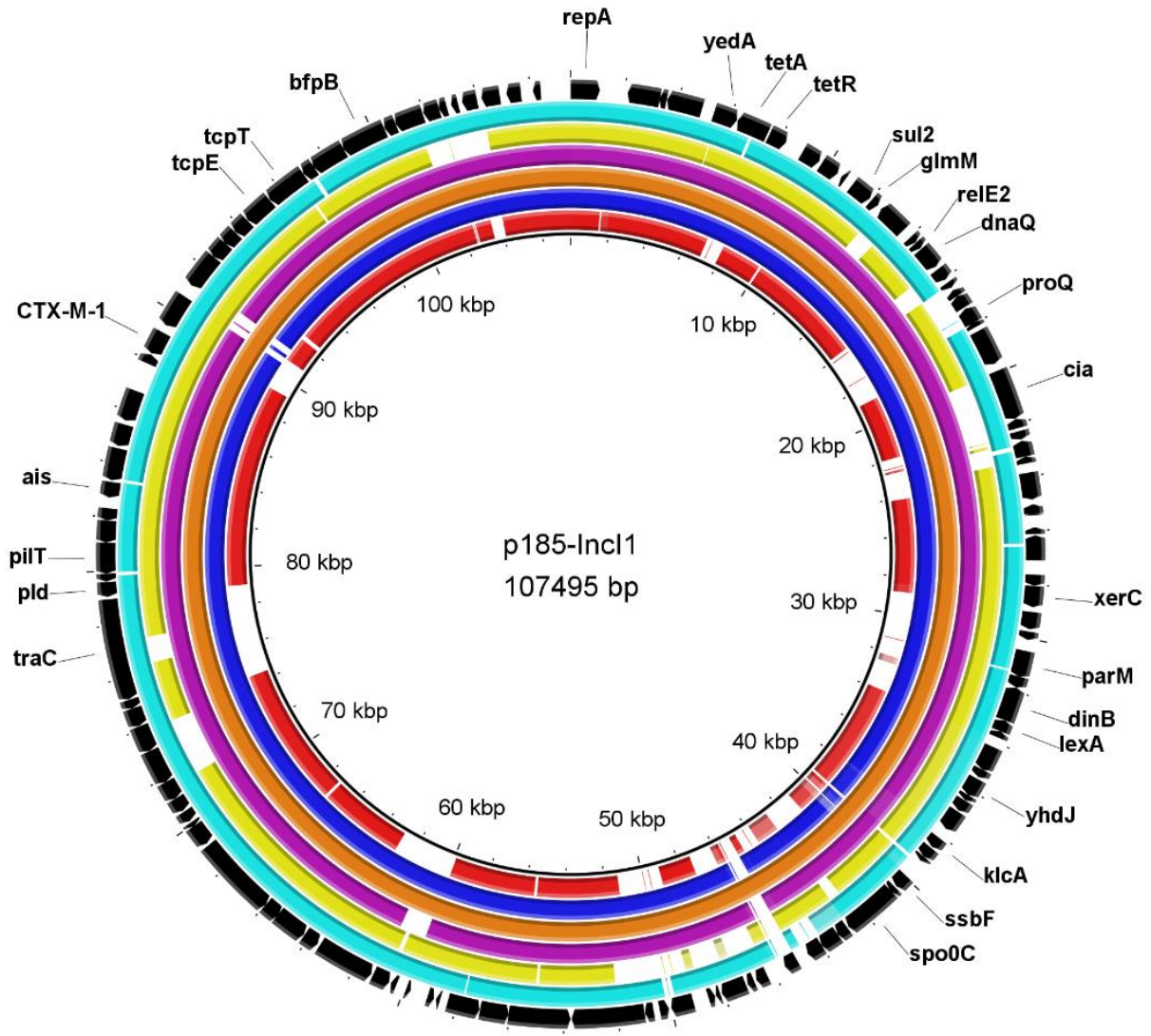
701 * – The presence of antibiotic resistance genes were compared in this table for *Citrobacter*, *E. coli* and *Serratia* isolates only, so that the same
 702 bacterial species were compared across different agars.

703 **Figure 1.** PFGE profiles of *E. coli* (n=152) isolates from cattle after *Xba*I digestion.

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705 **Figure 2.** Common circulating plasmid from different samples from the same farm.
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-  100% identity
-  70% identity
-  50% identity




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-  100% identity
-  70% identity
-  50% identity




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-  100% identity
-  70% identity
-  50% identity


187


-  100% identity
-  70% identity
-  50% identity

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-  100% identity
-  70% identity
-  50% identity

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-  100% identity
-  70% identity
-  50% identity

 Genes

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Legend for figure 2.