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1 # Antibiotic resistance among *Escherichia coli* and *Salmonella*
2 # isolated from dairy cattle feces in Texas
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41 # Abstract

42 This study was conducted to develop and field-test a low cost protocol to estimate the
43 isolate- and sample-level prevalence of resistance to critically important antibiotic drugs
44 among *Escherichia coli* and *Salmonella* isolated from dairy cattle feces. *E. coli* and
45 *Salmonella* were isolated from and screened on selective media, with and without antibiotics
46 respectively. Bacterial isolates were further tested for susceptibility to a suite of antibiotics
47 using disk diffusion. Molecular methods were performed on select bacterial isolates to
48 identify and distinguish genetic determinants associated with the observed phenotypes.
49 Among 85 non-type-specific *E. coli* randomly isolated from MacConkey agar without
50 antibiotics, the isolate-level prevalence of resistance to tetracycline was the highest (8.2%),
51 there was no isolate resistant to third-generation cephalosporin (0.0%) and one isolate was
52 resistant to nalidixic acid (1.2%). Among 37 *E. coli* recovered from MacConkey agar with
53 cefotaxime at 1.0μg/ml, 100% were resistant to ampicillin and 56.8% were resistant to a third-
54 generation cephalosporin (ceftriaxone). Among 22 *E. coli* isolates recovered from
55 MacConkey agar with ciprofloxacin at 0.5μg/ml, 90.9% were resistant to tetracycline whereas
56 77.3% and 54.5% were resistant to nalidixic acid and ciprofloxacin respectively. Sixteen
57 *Salmonella* were isolated and only one demonstrated any resistance (i.e., single resistance to
58 streptomycin). Among *E. coli* isolates that were either resistant or intermediate to ceftriaxone,
59 an AmpC phenotype was more common than an extended spectrum beta-lactamase (ESBL)
60 phenotype (29 versus 10 isolates, respectively). Among 24 *E. coli* isolates that were whole
61 genome sequenced, phenotypic profiles of antibiotic resistance detected were generally
62 substantiated by genotypic profiles. For instance, all isolates with an AmpC phenotype carried
63 a bla_{CMY2} gene. The protocol used in this study is suited to detecting and estimating

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64 prevalence of antimicrobial resistance in bacteria isolated from food animal feces in resource-
65 limited laboratories in the developing world.

66 # Introduction

67 Monitoring of the emergence, spread, and changes in levels of antimicrobial resistant
68 bacteria along the food chain is needed to inform and guide integrated strategies for
69 combating antimicrobial resistance[1]. In most cases, surveillance systems of antibiotic
70 resistant bacteria in food animals target pathogenic bacteria, such as *Salmonella* and
71 *Campylobacter* as well as indicator bacteria, such as *E. coli* and *Enterococcus spp*. After their
72 isolation from samples, genus/species confirmation, and subtyping when necessary, the
73 bacteria of interest are tested for susceptibility to a select number of antibiotics using a
74 standard phenotyping method of choice. Even though the broth microdilution method is
75 preferred in many surveillance systems and in research [2–4],other less technology intensive
76 methods of antibiotic susceptibility testing, such as disk diffusion, may also be used[1,5].
77 After phenotypic antibiotic susceptibility testing, genetic characterization can be done through
78 the detection of targeted genes or through whole genome sequencing (WGS). Additionally,
79 the selective culture and detection of bacteria with rare antimicrobial resistance (AMR)
80 mechanisms is highly recommended[1].

81 In many developed countries, AMR surveillance systems are replacing phenotypic
82 antimicrobial susceptibility testing with WGS[6,7]. In many developing countries, however,
83 surveillance systems are still not well established and the startup costs associated with
84 equipment acquisition and maintenance can be prohibitive [8]. One of the main reasons
85 impeding the establishment of AMR surveillance systems in these countries is the lack of
86 sufficient resources needed for establishment, and then sustainment of the surveillance

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87 systems[8,9]. An inexpensive and reliable protocol that can generate sufficient high quality
88 and reproducible information of the burden of antibiotic resistance among isolated bacteria
89 would be one of the most helpful solutions for establishment of an AMR surveillance system
90 in situation where resources are limited. Studies have demonstrated that the disk diffusion
91 method is a cost effective method that can generate results comparable to other phenotypic
92 methods of antibiotic susceptibility testing, that is, such as the broth microdilution or the agar
93 dilution methods[10]. This method can be used efficiently to establish phenotypic
94 antimicrobial resistance profiles and to detect mechanisms of resistance such as the
95 production of extended spectrum beta-lactamases (ESBLs) among bacterial isolates.

96 We designed a protocol that uses the disk diffusion method to determine the isolate-level
97 prevalence of resistant to various antibiotics and the sample-level prevalence of any bacteria
98 not susceptible to third-generation cephalosporin or quinolone antibiotics. Additionally, the
99 protocol described herein was designed to estimate the proportion of bacteria resistant to
100 third-generation cephalosporins that produce either ESBL or AmpC enzymes. Afterward, the
101 developed protocol was field-tested on fecal samples collected from dairy cattle to estimate
102 isolate-level and sample-level prevalence of AMR among *E. coli* and isolate-level and
103 sample-level prevalence among *Salmonella*.

104 Materials and Methods

105 Sample collection

106 Using a convenience sampling scheme, we collected 85 freshly voided fecal samples from
107 dairy cattle of different age groups at a dairy farm located near Lubbock, Texas. Fecal
108 samples were aseptically collected into sterilized polypropylene specimen containers then
109 kept on wet ice and transported to a microbiology laboratory at Texas Tech University.

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110 As fecal samples were collected from the pen-floor, there was no interaction with
111 vertebrate animals, consequently an approval from an Institutional Animal Care and Use
112 Committee wasn't needed.

113 Isolation of bacteria from fecal samples

114 In the laboratory, 10g of each fecal sample was weighted in a 710mL Whirl Pak® bag
115 (Whirl-Pak, Madison, Wisconsin) and 90mL of buffered peptone water (Becton Dickinson,
116 New Jersey, United States) was added. The mixture was placed in a commercial stomacher
117 for 2 minutes at 230 rpm. Thereafter, the mixture was incubated at 42°C overnight prior to
118 isolation of *E. coli* and *Salmonella.*

119 Isolation and identification of *Escherichia coli*

120 From each overnight non-selective enrichment, a 10μL loopful was streaked onto
121 MacConkey agar (MAC, Hardy Diagnostics, California, United States) to isolate non-type-
122 specific *E. coli*(NTS *E. coli*), meanwhile, another 10 μL loopful was streaked onto MAC
123 supplemented with 1μg/mL of cefotaxime (MAC+CTX) to screen for *E. coli* resistant to third-
124 generation cephalosporins (3GCr *E. coli*). An additional 10 μL loopful was streaked onto
125 MAC supplemented with 0.5μg/mL of ciprofloxacin (MAC+CIP) to screen for *E. coli* not
126 susceptible to quinolones (Qr *E. coli*). All three MacConkey agar plate types were incubated
127 at 37°C overnight. Following the incubation, agar plates were inspected to identify growth of
128 colonies with typical morphology of *E. coli* (i.e., pink, convex, circular and dry colonies with
129 a surrounding pink zone). From each type of MacConkey agar plate, one typical colony was
130 selected and re-streaked onto a similar MacConkey agar plate type for isolation of pure
131 colonies. All well isolated presumptive *E. coli* were tested for indole production using an

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132 indole spot test (Hardy Diagnostics, California, United States) and were confirmed as *E. coli*
133 by detection of the *wecA* gene using a real time polymerase chain reaction (rtPCR).

134 **Isolation and identification of *Salmonella***

135 One mL of each overnight non-selective enrichment was transferred into 9mL of
136 Rappaport-Vassiliadis *Salmonella* broth (Hardy Diagnostics, California, United States) and
137 another 1mL was transferred into 9mL of tetrathionate broth (Hardy Diagnostics, California,
138 United States). Both inoculated broths were incubated at 42°C overnight. After incubation, a
139 10μL loopful of each broth was streaked onto brilliant green sulfa agar (BGS, Becton
140 Dickson, New Jersey, United States) and onto xylose lysine deoxycholate agar (XLD, Hardy
141 Diagnostics, California, United States) to isolate *Salmonella*. In addition, 10 μL loopful of
142 each broth was streaked onto BGS and onto XLD agar plates, each supplemented with
143 1μg/mL of cefotaxime (BGS+CTX and XLD+CTX) to screen for *Salmonella* resistant to
144 third-generation cephalosporins. Another 10μL loopful of each broth was streaked onto BGS
145 and XLD agar plates both supplemented with 0.5μg/mL of ciprofloxacin (BGS+CIP and
146 XLD+CIP) to screen for *Salmonella* not susceptible to quinolones. All agar plates were
147 incubated at 37°C overnight. Following incubation, agar plates were inspected for growth of
148 colonies with morphology typical of *Salmonella* (i.e, pink, circular, dry, convex colonies on
149 BGS; black, circular convex colonies on XLD). From each type of agar plate, a single typical
150 colony was selected to be re-streaked onto the same type of agar plate for isolation of pure
151 colonies. All presumptive *Salmonella* were tested for production of H_2S gas, dextrose
152 fermentation and decarboxylation reaction using lysine iron agar (Hardy Diagnostics,

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153 California, United States) and were confirmed to be *Salmonella* by detection of the *ttrC* gene
154 using rtPCR.

155 Antibiotic susceptibility testing

156 All isolates confirmed as *E. coli* and *Salmonella* were tested for susceptibility to a panel of
157 12 antibiotics using the disk diffusion method in accordance to guidelines of the Clinical
158 Laboratory Standard Institute (CLSI)[11]. The antibiotics and concentration in each disk were
159 amoxicillin-clavulanic acid 20/10µg(AMC), ampicillin 10µg (AMP), azithromycin 15µg
160 (AZI), cefoxitin 30µg (FOX), ceftriaxone 30µg (CRO), chloramphenicol 30µg (CHL),
161 ciprofloxacin 5µg (CIP), colistin 10µg (COL), meropenem 10µg (MER), nalidixic acid 30µg
162 (NAL),streptomycin 10µg (STR), and tetracycline 30µg (TET). Inhibition zone diameters
163 around the antibiotic-impregnated disks were measured in mm and rounded to the closest
164 integer before being compared to the CLSI clinical break points in order to classify each
165 bacterial isolate as resistant, intermediate or susceptible[11]. Because there was no CLSI
166 standard inhibition zone diameters for colistin, these data were interpreted in accordance with
167 a study conducted by Galani and collaborators[12].

168 Based on previous investigation of antimicrobial resistance in the region, all bacterial
169 isolates not susceptible (i.e., intermediate and resistant) to a third-generation cephalosporin
170 (ceftriaxone) were expected to have a phenotype reflecting ESBL- or AmpC-production.
171 Suspected ESBL- or AmpC beta-lactamase-producing bacteria were discriminated by the
172 combination disk test (CDT) according to CLSI guidelines[11] using a second panel of 12
173 antibiotic-impregnated disks. In addition to cefotaxime 30µg (CTX), cefotaxime-clavulanic
174 acid 30/10µg (CTX-CLA), ceftazidime 30µg (CAZ), and ceftazidime-clavulanic acid 30/10µg
175 (CAZ-CLA), the 4 antibiotic disks required by the CDT method, the second panel of

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176 antibiotics included amikacin 30µg (AMK),cefazolin 30µg (CFZ), cefepime 30µg, (FEP),
177 fosfomycin 200µg (FOS), gentamicin 10µg (GEN), imipenem 10µg (IMP), sulfisoxazole
178 300µg (SSS), and trimethoprim/sulfamethoxazole 1.25/23.75µg (SXT).A bacterial isolate was
179 classified to have a phenotype of ESBL-production when the absolute difference between the
180 inhibition zone diameter around ceftazidime (coded as resistant) versus ceftazidime-
181 clavulanic acid (CLA) and/or around cefotaxime (coded as resistant) versus cefotaxime-
182 clavulanic acid was equal to or greater than 5mm. An isolate was classified to have a
183 phenotype of AmpC beta-lactamase production when the difference between the inhibition
184 zone diameter around ceftazidime (coded as resistant) versus ceftazidime-clavulanic acid
185 and/or around cefotaxime (coded as resistant) versus cefotaxime-clavulanic acid was less than
186 5mm[11].

187 *E.coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality
188 control strains.

189 Polymerase chain reaction to detect genes encoding for beta-190 lactamases

191 All isolates confirmed as exhibiting either a phenotype suggestive of ESBL or else
192 AmpC beta-lactamase-production were subjected to DNA extraction using a boiling
193 preparation method. The extracted DNA was used as a template to detected the family of
194 $bla_{CTX\text{-}M}$ genes (genes encoding for ESBL production) or $bla_{\mathrm{CMY\text{-}2}}$ gene (gene encoding for
195 AmpC beta-lactamase production) by conventional PCR (cPCR), using previously published
196 primers[13].

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197 ## Whole genome sequencing (WGS)

198 Following phenotypic AMR characterization, 24 *E. coli* isolates classified as either ESBL
199 or AmpC phenotypes, resistant to NAL or reduced susceptibility to CIP were selected for
200 WGS. The DNA was extracted using a commercial DNA extraction kit (Qiagen,Venlo,
201 Netherland), libraries were prepared using the Nextera XT DBA library preparation kit
202 (Illumina, California, United States) and the sequencing was performed using an Illumina
203 Miseq (Illumina, California, United States). Generated raw reads (fastq files) were assembled
204 using SPAdes 3.9 on the Center for Genomic Epidemiology platform.

205 ## Data analysis

206 The prevalence proportion (expressed as percentage) of bacteria resistant to each tested
207 antibiotic was determined and confidence intervals were calculated as 95% binomial
208 proportions representing Wilson intervals using R.3.0. software. The proportion (expressed as
209 percentages) of samples with bacteria not susceptible (i.e., resistant or intermediate) to a third-
210 generation cephalosporin (ceftriaxone) or else to quinolones (nalidixic acid and/or
211 ciprofloxacin) was calculated by dividing the number of samples with non-susceptible
212 bacteria by the total number of collected samples collected (i.e., only those isolates screened
213 on media with antibiotics were used to calculate the sample-level percentages). Whole
214 genome sequencing data were analyzed using various bioinformatic tools found on the
215 website of the Center for Genomic Epidemiology, including Resfinder3.0 that detect
216 mobilizable genes and chromosomal mutations conferring antibiotic resistance in bacteria.

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217 # Results

218 ## Isolated bacteria

219 In total, NTS *E. coli* were recovered from all 85 fecal samples. The recovery rate of
220 *Salmonella* and of either bacterial species (i.e., *E. coli* or *Salmonella enterica*) presumptively
221 resistant to third-generation cephalosporins or else resistant/reduced susceptibility to
222 quinolones was lower (Table1).

223 **Table 1.Numbers of bacteria isolated from dairy cattle feces at a dairy farm in Texas**

Bacteria	Culture medium of isolation	#of samples	# of isolates	%
E. coli				
NTS *E.coli*	MAC	85	85	100.0%
Pres. 3GCr *E.coli*	MAC + CTX	85	37	43.5%
Pres. Qr *E. coli*	MAC + CIP	85	22	25.9%
Salmonella				
Salmonella	BGS or XLD	85	16	18.8%
Pres. 3GCr *Salmonella*	BGS + CTX or XLD + CTX	85	1	1.2%
Pres. Qr *Salmonella*	BGS + CIP or XLD + CIP	85	0	0.0%

224 #: number; MAC: MacConkey agar; BGS: brilliant green sulfa; XLD: xylose lysine deoxycholate.
225 MAC+CTX, BGS+CTX, XLD+CTX: respective culture medium supplemented with 1 µg/mL of
226 cefotaxime. MAC+CIP, BGS+CIP, XLD+CIP: respective culture medium supplemented with 0.5
227 µg/mL of ciprofloxacin. Pres.3GCr: presumptive third-generation cephalosporin resistant, Pres. Qr:
228 Presumptive quinolone resistant (or reduced susceptibility).

229 ## Antibiotic resistance among isolated bacteria

230 Antibiotic susceptibility testing of isolated bacteria showed that resistance to
231 antibiotics was rare among NTS *E. coli* isolated on MAC when compared to presumptive
232 3GCr *E. coli* screened on MAC+CTX or else presumptive Qr *E. coli* screened on MAC+CIP.
233 Isolate-level prevalence of resistance to tetracycline (8.2%) was the highest among NTS *E.*
234 *coli* isolates while resistance to cefoxitin, colistin, meropenem, ceftriaxone, and ciprofloxacin
235 were completely absent among these bacterial isolates (Table 2).

236 **Table 2. Distribution of inhibition zone diameters of non-type-specific *E. coli* (n=85) isolated on plain MacConkey agar**
237 **(without antibiotic)**

	%	95%	Distribution (number) for inhibition zone diameters in mm																							
ATB	**R**	**CI**	**0**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**>30**
AMC	1.2	0.2 - 6.4							1				1	4	3		8	9	13	11	18	10	3	2	1	1		0
AMP	3.5	1.2 - 9.9	2					1				3	2	2	3	6	11	16	14	10	10	2	2		1			0
AZI	1.2	0.2 - 6.4							1	3	5	10	12	22	7	12	3	3	2	3						1	1	0
FOX	0.0	0.0 - 4.3															2	7	3	3	14	24	14	10	5	3		0
CRO	0.0	0.0 - 4.3																				2		1	1	2	7	72
CHL	4.7	1.8 -11.5	3	1											1		6	1	6	8	11	11	17	10	6	3	1	0
CIP	0.0	0.0 - 4.3												1					1				3	1	1	4	8	66
COL*	0.0	0.0 - 4.3								7	33	25	14	5		1												0
MER	0.0	0.0 - 4.3																		1	2	5	2	1	5	9	20	40
NAL	1.2	0.2 - 6.4	1									1				1		3	5	7	18	14	9	12	11	3		0
STR	7.1	3.3 -14.5	2	1		1	1	1	7	10	17	11	19	11				1		1	1			1				0
TET	8.2	4.0 -16.0	2	5												1	6	10	21	20	16	2	2					0

238 In accordance with the Clinical Laboratory Standards Institute (CLSI) human clinical break points, dark grey, light blue, and white fields represent numbers of isolates with
239 inhibition zone diameters for resistant, intermediate and susceptible Enterobacteriaceae, respectively. COL*: break points for colistin were from the study by Galani et al. 2008.
240 AMC: amoxicillin-clavulanic acid, AMP: ampicillin, AZI: azithromycin, FOX: cefoxitin, CRO: ceftriaxone, CHL: chloramphenicol, CIP: ciprofloxacin, COL: colistin, MER:
241 meropenem, NAL: nalidixic acid, STR: streptomycin, TET: tetracycline. ATB: Antibiotic, R: resistance, CI: Confidence intervals were calculated as 95% binomial proportions and
242 presented as Wilson intervals

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243 Among the 37 presumptive 3GCr *E. coli* screened on MAC+CTX, all isolates were
244 resistant to ampicillin (100%), 21 isolates were resistant to ceftriaxone (55.8%), and none of
245 the isolates was resistant to meropenem (Table 3). In total, 36 out of the 37 presumptive 3GCr
246 *E. coli* were not susceptible (i.e., either resistant or intermediate) to a third-generation
247 cephalosporin (CRO). These isolates were from 36 out of the 85 collected samples. The
248 sample-level prevalence of *E. coli* non-susceptible to third-generation cephalosporin was
249 calculated to be 42.3% (36/85) with a 95% confidence interval of 32.4% - 53.0%.

Table 3. Distribution of inhibition zone diameters of presumptive third-generation cephalosporin resistant *E. coli* (n=37) isolated on MacConkey agar supplemented with 1 µg/mL cefotaxime.

	%	95%	Distribution (number) of inhibition zones diameters in mm																									
ATB	**R**	**CI**	**0**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**>30**
AMC	67.6	51.5 - 80.4	1	6	10	5		1	2		1	1					4	4							1	1		0
AMP	100.0	90.6 - 100	35	1		1																						0
AZI	2.7	0.5 - 13.8				1				4	9	2	2	4	6	2	3	1	1	1								1
FOX	62.2	46.1 - 75.9	1		1		2	1	1	7	10	3			1	1		1					4	2	2			0
CRO	56.8	40.9 - 71.3					6	2			1	1	1	2	3	5	8	5	2	1								0
CHL	27.0	15.4 - 43.0	9	1										1			1	2	2	6	5	1		3	3	2		1
CIP	8.1	2.8 - 21.3	3																	1			1		3	2	4	23
COL*	2.7	0.5 - 13.8						1		3	22	10	1															0
MER	0.0	0.0 - 9.4																						1	1	3	11	21
NAL	10.8	4.3 - 24.7	4															3	4	7	7	6	4	1	1			0
STR	32.4	19.6 - 48.5	11	1					2	4	4	10	2		2	1												0
TET	62.2	46.1 - 75.9	5	15			2	1										1	3	4	5	1						0

In accordance with the Clinical Laboratory Standards Institute (CLSI) human clinical break points, dark grey, light blue, and white fields represent numbers of isolates with inhibition zone diameters for resistant, intermediate and susceptible Enterobacteriaceae, respectively. COL*: break points for colistin were from the study by Galani et al. 2008. AMC: amoxicillin-clavulanic acid, AMP: ampicillin, AZI: azithromycin, FOX: cefoxitin, CRO: ceftriaxone, CHL: chloramphenicol, CIP: ciprofloxacin, COL: colistin, MER: meropenem, NAL: nalidixic acid, STR: streptomycin, TET: tetracycline. ATB: Antibiotic, R: resistance, CI: Confidence intervals were calculated as 95% binomial proportions and presented as Wilson intervals

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257 Most presumptive Qr *E. coli* screened on MAC+CIP were resistant to tetracycline
258 (90.9%), meanwhile 17 isolates were resistant to nalidixic acid (77.3%), 12 isolates were
259 resistant to ciprofloxacin (54.5%), and none of the isolates was resistant to colistin or
260 meropenem (Table4). All of the 22 presumptive Qr *E. coli* were resistant or intermediate to
261 nalidixic acid or else to ciprofloxacin. These isolates were recovered from 22 out of 85
262 collected samples. The sample-level prevalence of *E. coli* non-susceptible to quinolone
263 antibiotics was calculated to be 25.9 % with a 95% confidence intervals of 17.8%- 36.1%. In
264 total 12 out of the 22 Qr *E. coli* isolates were resistant to both nalidixic acid and ciprofloxacin,
265 4 isolates were resistant to nalidixic acid only, 5 isolates were intermediate to nalidixic acid
266 but susceptible to ciprofloxacin and 1 isolate was intermediate to both ciprofloxacin and
267 nalidixic acid.

268 **Table 4. Distribution of inhibition zone diameters of presumptive quinolone resistant *E. coli* (n=22) isolated on MacConkey**
269 **agar supplemented with 0.5 µg/mL of ciprofloxacin**

	%	**95%**	**Distribution (number) of inhibition zone diameters in mm**																								
ATB	**R**	**CI**	**0**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**>30**
AMC	18.2	7.3 - 38.5					1	1	2				1	2	2	3	5	3	1							1		0
AMP	59.1	38.7- 76.7	10	2				1						1	3	3	1	1										0
AZI	9.1	2.5 - 27.8			1				1	7	9	4																0
FOX	9.1	2.5 - 27.8								1	1		1		1		2	1	2	3	5	4	1					0
CRO	4.5	0.8 - 21.8				1											3			1				1	1	3	5	7
CHL	77.3	56.6 - 89.9	16	1												1		1			3							0
CIP	54.5	34.7 - 73.1	8	1		1		2									1	2	1		3	1	1	1				0
***COL**	0.0	0.0 - 14.9								4	15	2	1															0
MER	0.0	0.0 - 14.9																					1	1	2	8	4	6
NAL	77.3	56.6 - 89.9	13	1					1	2		2	1	1	1													0
STR	63.6	43.0 - 80.3	9	1	1			3	3		3	1	1															0
TET	90.9	72.2 - 97.5	14	6														1					1					0

270 In accordance with the Clinical Laboratory Standards Institute (CLSI) human clinical break points, dark grey, light blue, and white fields represent numbers of isolates with
271 inhibition zone diameters for resistant, intermediate and susceptible Enterobacteriaceae, respectively. COL*: break points for colistin were from the study by Galani et al. 2008.
272 AMC: amoxicillin-clavulanic acid, AMP: ampicillin, AZI: azithromycin, FOX: cefoxitin, CRO: ceftriaxone, CHL: chloramphenicol, CIP: ciprofloxacin, COL: colistin, MER:
273 meropenem, NAL: nalidixic acid, STR: streptomycin, TET: tetracycline. ATB: Antibiotic, R: resistance, CI: Confidence intervals were calculated as 95% binomial proportions and
274 presented as Wilson intervals
275

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276 Resistance to antibiotics among *Salmonella* isolated on culture media without
277 antibiotics was very low yielding a single isolate that was resistant to only streptomycin. The
278 sole *Salmonella* isolated on XLD+CTX was confirmed to be resistant to CRO.

279 Phenotypic and genotypic detection of ESBL- and AmpC-
280 producing bacteria

281 The sole *Salmonella* isolated from XLD+CTX plates, exhibited the phenotype of an AmpC
282 beta-lactamase producer; later, this was confirmed by the presence of *bla*$_{\text{CMY-2}}$gene using
283 cPCR.

284 A total of 40 *E. coli* isolates were found to be resistant or intermediate to ceftriaxone.
285 Thirty-six of them were isolated from MAC+CTX and four were isolated from MAC+CIP
286 plates. The second panel of antibiotics showed that these isolates were resistant to at least one
287 of the third-generation cephalosporins tested; furthermore all of them were resistant to
288 cefazolin. Only a very few of these *E. coli* isolates were resistant to sulfisoxazole and
289 trimethoprim/sulfamethoxazole (Table 5).

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299 **Table 5. Distribution of inhibition zone diameters of all *E. coli* isolates (n=40) not susceptible to third-generation**
300 **cephalosporins (i.e., second panel of antibiotics)**

	%	**95%**	**Distribution (number) of inhibition zone diameters in mm**																								
ATB	**R**	**CI**	**0**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**>30**
AMK	0	0.0 - 8.8													2			7	11	9	6	4			1			0
CFZ	100	91.2 - 100	28	2	1	7	1		1																			0
FEP	0	0.0 - 8.8																2	5	2		1		1	1	2	4	22
CTX	82.5	68.0 - 91.2				1	2	7				1		1		3	5	5	8	3		3						1
CTX-CLA										1			1	1	2	4	4	2	8	3	1	1	1		1	4	2	4
CAZ	25	14.2 - 40.2		1							1		3	5	5	12	8	1	3									1
CAZ-CLA										1	1			3	2	2	4	1	6	4	2	3	1	2	5	2		1
FOS	0	0.0 - 8.8																			4	2	2	8	9	5	1	9
GEN	0	0.0 - 8.8												2	1	1	7	7	7	10	4	1						0
IMP	0	0.0 - 8.8																			1	1	1	6	8	9	10	4
SSS	32.5	20.1 - 48.0	13												2	1	2	2	3	1	2		4	5	4	1		0
SXT	40	26.3 - 55.4	16									2	1	1		1				2		1		2		5	2	7
ESBL	47.5	32.9 - 62.5																										
AmpC	50	35.2 - 64.8																										

301 In accordance with the Clinical Laboratory Standards Institute (CLSI) human clinical break points, dark grey, light blue, and white fields represent numbers of isolates with
302 inhibition zone diameters for resistant, intermediate and susceptible Enterobacteriaceae, respectively. CLSI clinical break points for enterobacteriaceae resistant to antibiotic are
303 represented by a vertical solid line in the table. There are no CLSI clinical break points for CTX-CLA and CAZ-CLA. AMK: amikacin, CFZ: cefazolin, FEP: cefepime, CTX:
304 cefotaxime, CAZ: ceftazidime, FOS: fosfomycin, GEN: gentamicin, IMP: imipenem, SSS: sulfisoxazole, SXT: trimethoprim/sulfamethoxazole. ATB: Antibiotic R: resistance. CI:
305 Confidence intervals were calculated as 95% binomial proportions and presented as Wilson intervals. The line with ESBL represents the percentage of *E. coli* isolates producing
306 ESBLs. The line with AmpC represents the percentage *E. coli with a phenotype of AmpC beta-lactamse producers*isolates producing AmpC.

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307
308 The phenotypic combination disk test detected 19 *E. coli* isolates with a phenotype
309 indicating ESBL-production, 20 isolates with a phenotype indicating AmpC beta-lactamase-
310 production and a single isolate that was not confirmed to be resistant to either cefotaxime or
311 ceftazidime. On the other hand, molecular cPCR detected genes encoding for ESBL
312 production ($bla_{CTX\text{-}M\text{-}1}$or $bla_{CTX\text{-}M\text{-}9}$ family genes) in 10 isolates (25.0%), the gene encoding
313 for AmpC beta-lactamase production (i.e., $bla_{\mathrm{CMY\text{-}2}}$) in 29 isolates (72.5%) and none of these
314 genes in one isolate (2.5%).

315 Whole genome sequencing of select *E. coli* isolates

316 Whole genome sequencing of 24 *E. coli* isolates showed that the gene *tet*(A) encoding
317 for a tetracycline efflux pump, was present in 70.8% of sequenced isolates. In general, all the
318 detected genes were in accordance with the phenotypic antibiotic resistance observed in each
319 of the isolate tested. Some exception included the presence of the *aph (3')-Ia* gene that
320 purportedly confers resistance to aminoglycosides, though in our case, in isolates
321 phenotypically susceptible to these antibiotics. The mutation *gyrA[87:D-Y]* that confers
322 resistance to quinolone antibiotics was observed in one isolate exhibiting no phenotypic
323 resistance to either nalidixic acid or ciprofloxacin. The presence of the *mef(B)* gene that
324 encodes for resistance to macrolides was detected in one isolate susceptible to azithromycin.
325 Finally, the mutation *pmrB[161: V-G]* that confers resistance to polymixins was observed in
326 an isolate phenotypically susceptible to colistin (Fig. 1).

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327 Finally, WGS revealed that *E. coli* isolates resistant to nalidixic acid typically had only a
328 single point mutation in the *gyrA* gene or else harbored a plasmid-mediated quinolone-resistance
329 gene (*qnr*). All isolates resistant to both nalidixic acid and ciprofloxacin had a mutation in both
330 *gyrA* and *parE* or *parC* genes of the quinolone resistance determining region (QRDR).

331 # Discussion

332 This study was conducted to field-test a cost-effective and highly valid protocol that
333 could be used to determine the status of antibiotic resistance among *E. coli* and *Salmonella*
334 isolated from food-producing animals, especially where laboratory resources are limited. The
335 protocol used in this study was inspired by different guidelines for antimicrobial resistance
336 detection, including guidelines from the European Food Safety Authority(EFSA)[14], the U.S
337 National Antimicrobial Resistance Monitoring System (NARMS)[2], the European Union
338 Reference Laboratory for Antimicrobial Resistance (EURL-AR)[15], and the Danish Integrated
339 Antimicrobial Resistance Monitoring and Research Program (DANMAP)[16]. Unlike the above
340 mentioned guidelines, this protocol used the disk diffusion method instead of broth or agar
341 dilution methods because disk diffusion is recognized as a simple and low-cost method when
342 compared to other antibiotic susceptibility testing methods[17]. To increase our confidence in
343 recommending the protocol, results of the phenotypic methods used herein were thereafter cross-
344 referenced and validated using by results from relevant molecular methods.

345 In regard to results obtained in this study, all NTS *E. coli* and *Salmonella* isolated on
346 bacterial culture media without supplemented antibiotics were largely susceptible to all
347 antibiotics tested; meanwhile, bacteria isolated on culture media with antibiotics supplemented at
348 sub-breakpoint levels tended to be resistant to more than three antibiotics. This observation
349 provided evidence that when a bacterium acquires resistance to one antibiotic it tends also to be

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350 resistant to other antibiotics. In fact, in most of the cases, different genes encoding antibiotic
351 resistance are known to be co-located on transmissible genetic elements such as plasmids. When
352 a resistance plasmid is transferred to a previously susceptible bacterium, multidrug resistance can
353 be transferred in a single conjugation event[18].

354 Furthermore, percentages of samples that generated presumptive 3GCr *E. coli* (43.5%)
355 and presumptive 3GCr *Salmonella* (1.2%) were lower than the percentages of samples with
356 presumptive 3GCr *E. coli* (89.1%) and presumptive 3GCr *Salmonella* (10.9%) reported in three
357 beef feed lots in Nebraska[19]. In addition, similar to the study in Nebraska[19], we also noted
358 that the number of 3GCr *E. coli* isolates phenotypically or genotypically confirmed to be AmpC-
359 producers was higher than the number of isolates confirmed to be ESBLs-producers. The only
360 identified 3GCr *Salmonella* was confirmed to be an AmpC beta-lactamase producer. In the U.S,
361 resistance to third-generation cephalosporins among *Salmonella* from food animals has
362 historically been largely due to the gene $bla_{\text{CMY-2}}$ encoding for AmpC beta-lactamase
363 production [20].

364 In the present study, nine 3GCr *E. coli* had a phenotype typical of ESBL-production
365 (according to the CDT) while the cPCR showed that these isolates carried $bla_{\text{CMY-2}}$, a gene
366 encoding for AmpC beta-lactamase production, instead of genes encoding for an ESBL. The
367 combination disk test is reported to be an accurate phenotypic method and is widely used to
368 detect ESBL-producing Enterobacteriaceae[21]. Despite its widespread use and solid reputation,
369 the sensitivity and specificity of this method to detect ESBL-producing Enterobacteriaceae is not
370 always 100%[22]. In fact, different authors have reported a number false positive ESBL-
371 producing Enterobacteriaceae by the CDT in their investigations [23–26].

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372 To mitigate misclassification of bacterial isolates as ESBL-producers using phenotypic
373 methods, some of these authors have suggested modifications of standard methods, such as the
374 CDT, in order to increase their efficacy in discriminating ESBL-producing bacteria from AmpC
375 beta-lactamase-producing bacteria[23,24]. Along this line, our study illustrates that when the
376 combination disk test is used to identify ESBs-producing *E. coli*, some precaution should be
377 taken as the test may produce several false positive *E. coli* producing ESBLs, especially when
378 the results are close to 5 mm decision point. A close look at the 9 *E. coli* isolates falsely
379 classified as ESBL-producers showed that the difference between inhibition zone diameters
380 around cefotaxime with clavulanic acid and around cefotaxime was less than 5mm for all the
381 isolates. In contrast, the difference between inhibition zone diameters around ceftazidime with
382 clavulanic acid and around ceftazidime was equal to 5mm or slightly higher than 5mm.The later
383 observation led to the conclusion of classifying these same isolates as ESBL-producers.
384 Importantly, 8 of the 9 isolates were not susceptible to cefoxitin (a second-generation
385 cephalosporin (cephamycin) antibiotic).

386 Based on observations made in this study, we came up with the following rule of thumb:
387 false positive ESBL-producing *E. coli* can be detected by looking at the increase in inhibition
388 zone diameters around CAZ-CLA versus CAZ and around CTX-CLA versus CTX. False
389 positive ESBL-producing *E. coli* can be identified as isolates for which inhibition zone diameters
390 around ceftazidime is increased by 5mm or slightly higher (6mm) due to clavulanic acid while
391 the increase of the inhibition zone diameter around cefotaxime caused by clavulanic acid is less
392 than 5mm. When false positives are identified, we recommend using the information generated
393 by CTX with and without CLA to classify an isolate as ESBL- or AmpC-producer. In addition, a
394 look at bacterial isolates' susceptibility to cefoxitin (a cephamycin) or amoxicillin-clavulanic

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395 acid maybe helpful to conclude that an isolate is not an ESBL-producer but might be an AmpC-
396 producer. It is reported that AmpC-producing Enterobacteriaceae are resistant to cephamycins
397 such as cefoxitin while ESBL-producers are susceptible to cephamycins[27,28].

398 In general, WGS showed that phenotypic antibiotic resistance observed in *E. coli* isolates
399 was a good indicator of the presence of genetic antibiotic resistance determinants, with only few
400 a exceptions. The observed discordance may be explained by the fact that the presence of a
401 resistance gene doesn't always mean its expression. In addition, some genes are cryptic or even
402 misclassified as to their primary purpose in bacterial host function. Results of WGS also showed
403 that all isolates resistant to both NAL and CIP had mutations in both genes of the quinolone
404 resistance determining region (QRDR) while isolates resistant to NAL alone had a single
405 mutation in one of the target genes, else or carried a plasmid mediated quinolone-resistance gene
406 (*qnr* gene). In fact, mutations in the QRDR have been identified as the common genetic
407 determinant conferring a higher level of resistance to quinolone antibiotics while PMQR genes
408 confer moderate resistance[29,30]. Several studies have proven that the detection of genetic
409 determinants of antibiotic resistance by WGS accurately predicts antibiotic resistance phenotypic
410 behavior of bacterial isolates[6,31]. High level of agreement between WGS and phenotypic
411 antibiotic susceptibility testing have been reported [32,33].

412 In conclusion, this study established that bacteria resistant to antibiotics were present in
413 dairy cattle on the study farm but at a low level. The addition of an antibiotic to the culture
414 medium of isolation helped in detecting and later characterizing the few antibiotic resistant
415 bacteria. The developed protocol can help to establish percentages of indicator *E. coli* resistant to
416 various antibiotics, including critically important antibiotics for human medicine, at a relatively
417 low cost and with high reliability, even in the developing world. Furthermore, cPCR and WGS

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418 both supported our phenotypic findings at a high level which increased our confidence in
419 recommending the tested protocol for its use to establish status of AMR in food animals;
420 specifically, when laboratory facilities are limited and financial and other resources are scarce.

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427 # Conflict of interest

428 All authors declare no conflicts of interest.

429

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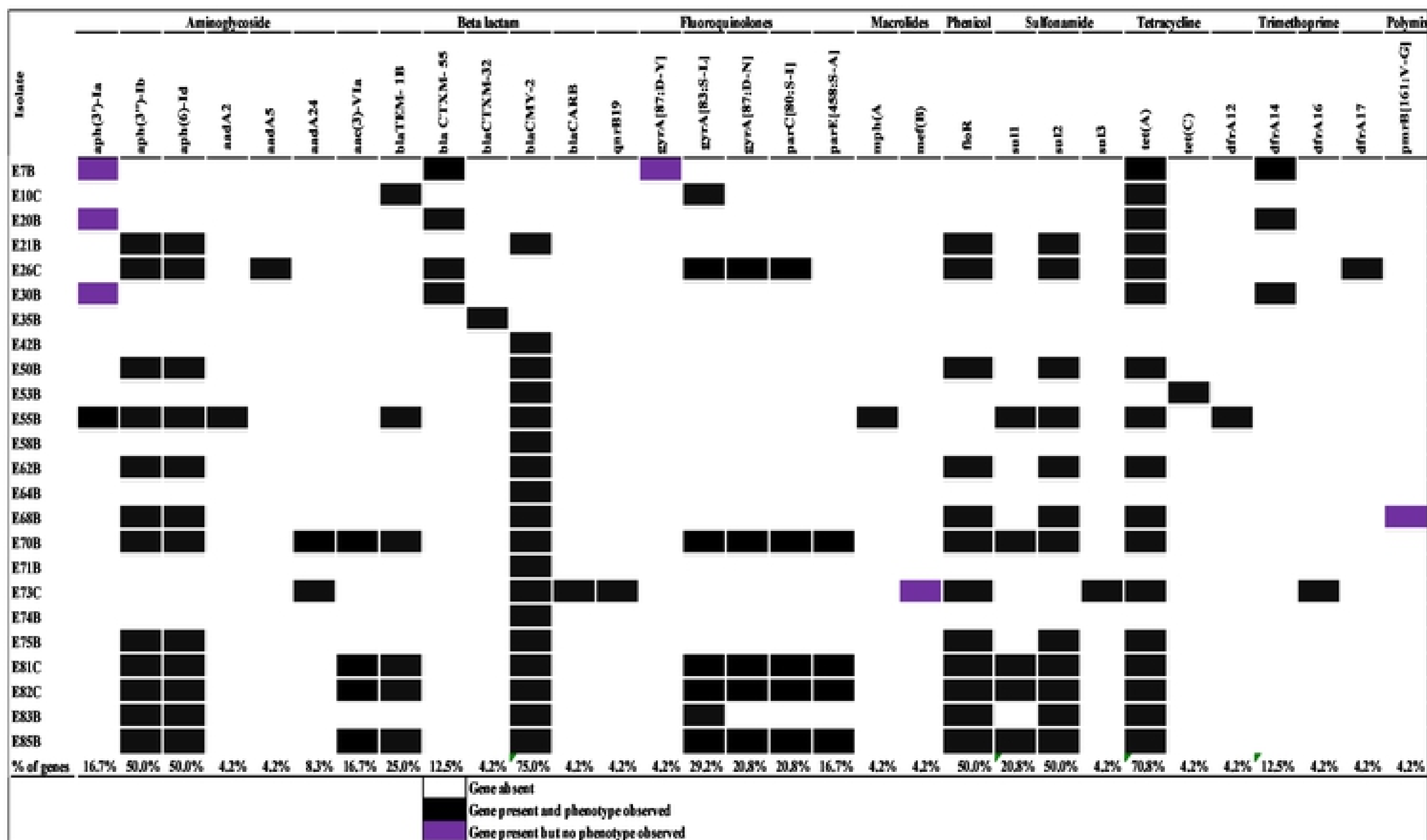

Fig. 1.Genetic determinants of antibiotic resistance among *E. coli* isolated from cattle feces on a dairy farm in Texas. Black cells indicate the presence of a resistance determinant and a phenotype of that determinant in a bacterial isolate. Purple cells indicate the presence of a resistance determinant without a corresponding phenotype in the same isolate. White cells indicate the absence of both resistance determinant and corresponding phenotype.