

1    **A novel method for measuring phenotypic colistin resistance in *Escherichia coli***  
2    **populations from chicken flocks**

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12    **Running title:** Method for measuring colistin resistance in chicken

13    **KEYWORDS**

14    Poultry, colistin resistance, broth microdilution, colistin use.

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23 **ABSTRACT**

24 Colistin is extensively used in animal production in many low- and middle-income countries.

25 There is a need to develop methodologies to benchmark and monitor changes in resistance in

26 commensal bacterial populations in farms. We aimed to evaluate the performance of a broth

27 microdilution method based on culturing a pooled *Escherichia coli* suspension (30-50

28 organisms) from each sample. In order to confirm the biological basis and sensitivity of the

29 method, we prepared 16 standard suspensions containing variable ratios of colistin-susceptible

30 and *mcr-1* encoded colistin-resistant *E. coli* which were grown in 2mg/L colistin. The optical

31 density (OD<sub>600nm</sub>) readings over time were used to generate a growth curve, and were adjusted to

32 the values obtained in the absence of colistin. The median limit of detection of the method was 1

33 colistin-resistant in 10<sup>4</sup> susceptible colonies [1<sup>st</sup> - 3<sup>rd</sup> quartile, 1:10<sup>2</sup> – 1:10<sup>5</sup>]. We applied this

34 method to 108 pooled faecal samples from 36 chicken flocks in the Mekong Delta (Vietnam)

35 over the production cycle. The correlation between this method and the prevalence of colistin

36 resistance in individual colonies harvested from field samples, determined by the Minimum

37 Inhibitory Concentration (MIC), was established. The overall prevalence of colistin resistance at

38 sample and isolate level was 38.9% and 19.4%, respectively. Increased colistin resistance was

39 associated with recent (2 weeks) use of colistin and other, non-colistin antimicrobials (OR=3.67

40 and OR=1.84, respectively). Our method is a sensitive and affordable approach to monitor

41 changes in colistin resistance in pooled *E. coli* populations from faecal samples over time.

## 42     **IMPORTANCE**

43     Colistin (polymyxin E) is an antimicrobial with poor solubility properties, and therefore broth  
44     microdilution is the only appropriate method for testing colistin resistance. However, estimating  
45     colistin resistance in commensal mixed *Escherichia coli* populations is laborious since it requires  
46     individual colony isolation, identification and susceptibility testing. We developed a growth-  
47     based microdilution method suitable for pooled faecal samples. We validated the method by  
48     comparing it with results from individual MIC testing of 909 *E. coli* isolates. We used the  
49     method to investigate phenotypic colistin resistance in 108 pooled faecal samples from 36  
50     healthy chicken flocks, each sampled three times over the production cycle. A higher level of  
51     resistance was seen in flocks recently supplemented with colistin in drinking water, although the  
52     observed generated resistance was short-lived. Our method is affordable, and may potentially be  
53     integrated into surveillance systems aiming at estimating the prevalence of resistance at colony  
54     level in flocks/herds. Furthermore, it may also be adapted to other complex biological systems,  
55     such as farms and abattoirs.

## 56 INTRODUCTION

57 Colistin (polymyxin E) is a last-resort drug used for the treatment of severe multi-drug resistant  
58 (MDR) infections in many countries, and currently is classified by the World Health  
59 Organization (WHO) as a ‘highest priority, critically important’ antimicrobial (1). The  
60 emergence of *mcr-1* plasmid-encoded colistin resistance among Gram-negative bacteria is  
61 considered a serious threat to global health (2). It has been hypothesized that colistin use in  
62 animal production is a major contributing factor to the emergence of colistin resistance  
63 worldwide (3). Colistin is still used in poultry and pig farming in many countries (4). In terms of  
64 frequency, colistin is the most commonly used antimicrobial in chicken production in the  
65 Mekong Delta region of Vietnam (5, 6). Studies in the same region have shown that resistance  
66 against colistin in commensal *Escherichia coli* from chicken flocks is often encoded by the *mcr-*  
67 1 gene (7, 8). At sample level, the prevalence of *mcr-1* in chicken faecal samples in the Mekong  
68 Delta was 59.4%. The prevalence of this gene has also been found to be higher among in-contact  
69 humans (chicken farmers) than in urban individuals (7).

70 *E. coli* is an ubiquitous commensal enteric organism globally used to monitor phenotypic  
71 antimicrobial resistance (AMR) in national surveillance programmes, both in humans and in  
72 animals (9, 10). Given the diversity of this organism within the enteric microbiome, the  
73 characterisation of phenotypic resistance in a mixed population of commensal *E. coli* requires  
74 selecting a representative and sufficiently large number of strains. This is often achieved by  
75 performing differential colony counts on agar media with and without antimicrobials (11).  
76 However, agar-based methods are not appropriate for colistin given the antimicrobials’ poor  
77 solubility (12). Determination of the minimal inhibitory concentration (MIC) by broth  
78 microdilution is regarded as the gold standard for testing of colistin resistance of

79 Enterobacteriaceae (ISO 20776-1) both by the Clinical and Laboratory Standards Institute  
80 (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (12,  
81 13). Establishing accurately the prevalence of resistance at colony level requires the investigation  
82 of a sufficiently large, representative number of isolates from each sample, which is extremely  
83 laborious and costly (8, 11, 14). Therefore, there is a need for developing cost-effective  
84 methodologies for evaluating resistance against colistin in mixed *E. coli* populations from animal  
85 faecal samples. Here, we designed and evaluated a broth microdilution-based method to quantify  
86 colistin resistance in *E. coli* populations from pooled chicken faecal samples. We then related the  
87 observed results to data on antimicrobial use (AMU) from the same flocks.

88 **RESULTS**

89 **Growth of standard suspensions**

90 The AUC<sub>adj</sub> values generated from all susceptible-resistant combinations are presented in Fig.1.  
91 In all cases, AUC<sub>adj</sub> values increased with increasing ratio of resistant to susceptible organisms.  
92 Growth was detected at a ratio of 1 resistant to 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> and 10<sup>1</sup> susceptible strains for  
93 43.7%, 12.5%, 18.5% and 12.5% and 12.5% combinations, respectively. There was no difference  
94 in average AUC<sub>adj</sub> between resistant strains with low (R1 and R2, MIC= 4mg/L) and moderate  
95 (R3 and R4, MIC= 8mg/L) levels of resistance (both AUC<sub>adj</sub>= 0.39). There were significant  
96 difference between average AUC<sub>adj</sub> values using different susceptible strains, with values  
97 ranging from 0.09 to 0.62 (Kruskal Wallis test, p= 0.002). In combinations with resistant strains,  
98 S2 yielded the lowest average AUC<sub>adj</sub> (median 0.09 [1<sup>st</sup> - 3<sup>rd</sup> quartile, 0.07-0.29]) as well as the  
99 lowest limit of detection (average S:R ratio of 10<sup>2</sup>:1). S4 gave the highest median AUC<sub>adj</sub> (0.62  
100 [1<sup>st</sup> - 3<sup>rd</sup> quartile, 0.48-0.69]) as well as the highest limit of detection (average S:R ratio of 10<sup>5</sup>:1).

102 **Study flocks and their AMU**

103 A total of 36 flocks (108 samples) were investigated in this study. The median flock size was 231  
104 [1<sup>st</sup> - 3<sup>rd</sup> quartile, 189-401] chickens. Flocks were raised over a median of 19 [1<sup>st</sup> - 3<sup>rd</sup> quartile,  
105 17-20] weeks. Colistin had been administered to 22/36 (61.1%) flocks. Among flocks given  
106 colistin, the average number of Animal Daily Doses (ADD) per 1,000 chicken-days of this  
107 antimicrobial administered over the production cycle was 149.5 Standard deviation [SD]  $\pm$ 261.6.  
108 Colistin was used more during the early flock cycle period (281.7 SD  $\pm$ 321.2 ADDs per 1,000  
109 chicken-days) compared with the second period (17.4 SD  $\pm$ 18.1 ADDs per 1,000 chicken-days)  
110 (Wilcoxon paired test, p<0.001) (Table 1). This antimicrobial was administrated over a median  
111 of 4 [1<sup>st</sup> - 3<sup>rd</sup> quartile, 2-6] weeks. The data of colistin use among study flocks is displayed in Fig.  
112 S2.

113 In addition to colistin, a total of 28 non-colistin antimicrobials (belonging to 12 classes) were  
114 administered to study flocks.. In decreasing order, oxytetracycline, tylosin, neomycin, ampicillin,  
115 streptomycin and doxycycline were the antimicrobials most used. The average number ADDs  
116 per 1,000 chicken-days of other antimicrobials among flocks using colistin was higher than  
117 flocks did not use colistin (350.9 SD  $\pm$ 383.8 vs. 187.2 SD  $\pm$ 366.2, Wilcoxon test, p= 0.004).  
118 Among both type of flocks, antimicrobials were administrated more commonly during the first  
119 period (average No. ADD per 1,000 chicken-days 629.3 SD  $\pm$ 359.8 and 345.5 SD  $\pm$ 471.5,  
120 respectively) compared to the second period of chicken life (average No. ADDs per 1,000  
121 chicken-days 72.5 SD $\pm$  98.5 and 29.0 SD  $\pm$ 48.6, respectively) (Table 1).

122 **Prevalence of colistin resistance at colony level**

123 A total of 909 *E. coli* strains were isolated from 23 selected samples (~40 *E. coli* isolates/  
124 sample) and were tested for their MIC against colistin. Among those, total of 129 strains (14.2%)

125 were resistant to colistin. Of resistant strains, 75.2% strains had a MIC of 4 mg/L, whereas  
126 24.0% had a MIC of 8mg/L. Only 1 isolate (0.8%) displayed a MIC of 16mg/L (Fig. S1). The  
127 beta-regression model that relates the AUC<sub>adj</sub> to percentage of resistant bacteria in samples is  
128 shown in Fig. 2. The trend over AUC<sub>adj</sub> was highly significant ( $p < 0.001$ ). The equation  
129  $100/(1+e^{4.8-(7.04*AUC_{adj})})$  associated with this model was applied for estimating the prevalence of  
130 colistin resistance at colony level among field samples.

### 131 **Changes of AUC<sub>adj</sub> over production cycle and prevalence of colistin resistance**

132 Overall, there was no significant change colistin resistance (AUC<sub>adj</sub>) over the production cycle  
133 ( $p = 0.569$ , Fig. S3). Among flocks not exposed to colistin, the differences AUC<sub>adj</sub> between  
134 sampling points were small. However, among flocks using colistin, the AUC<sub>adj</sub> values for mid-  
135 production samples (0.54 [1<sup>st</sup> - 3<sup>rd</sup> quartile, 0.07-0.65]) were higher than those of day-olds (0.06  
136 [1<sup>st</sup> - 3<sup>rd</sup> quartile, 0.04-0.52]) (Wilcoxon paired test,  $p = 0.063$ ) and end of production samples  
137 (0.07 [1<sup>st</sup> - 3<sup>rd</sup> quartile, 0.06-0.55]) (Wilcoxon paired test,  $p = 0.046$ ). There was little to no  
138 difference in AUC<sub>adj</sub> values between day-old and end of production samples (Table 1).

139 The prevalence of colistin resistance at sample level was 38.9% (42/108 positive samples). The  
140 prevalence of resistance level of day-old, mid-, and end of production samples was 36.1%,  
141 50.0% and 30.5%, respectively ( $\chi^2$  test,  $p = 0.219$ ). The overall average prevalence of resistance  
142 at colony level was 19.4 SD  $\pm 26.3\%$ . Among flocks using colistin, the highest level of resistance  
143 corresponded to mid-production samples (27.0 SD  $\pm 26.4\%$ ), followed by day-old (15.7 SD  $\pm$   
144 24.7%) and end production (12.8 SD  $\pm 18.1\%$ ) (Kruskal Wallis test,  $p = 0.070$ ). In contrast,  
145 among non-using flocks, day-old samples showed higher prevalence of resistance (28.8 SD  
146  $\pm 36.0\%$ ) compared to mid (17.3 SD  $\pm 28.7\%$ ) and end production (16.2 SD  $\pm 24.8\%$ ) (Kruskal

147 Wallis test,  $p= 0.453$ ). Summary results are presented in Table 1 and individual sample results  
148 are given in Table S1.

149 **Risk factors for colistin resistance**

150 Table 2 shows results for univariable and multivariable analyses. In the multivariable model, use  
151 of colistin during the two weeks prior to sampling (OR= 3.67; 95% [Confidence Interval] CI  
152 0.68-19.7) and use of non-colistin antimicrobials (OR= 1.84; 95% CI 0.88-3.85) were associated  
153 with colistin resistance at sample level.

154 **Estimation of test costs**

155 The reagent and media costs of broth microdilution and Etest for testing one sample based on the  
156 investigation of 10 *E. coli* isolates were ~24.5 and ~63 US dollars (USD), respectively. The cost  
157 for testing one sample by the growth-based method (based on 40 isolates) was ~6.5 USD. In  
158 addition, broth microdilution involved a higher labour cost (average of ~1 person-day per  
159 sample) compared with either the Etest or the growth-based method (~0.5 person-day) (Table  
160 S2).

161 **DISCUSSION**

162 We present here an approach for the phenotypic investigation of colistin resistance in pooled *E.*  
163 *coli* populations from poultry feces using a broth microdilution-based method. Colistin is widely  
164 used in poultry and pig production worldwide (4, 15, 16). In the Mekong Delta of Vietnam,  
165 colistin is typically administered to chicken flocks in drinking water during the brooding period  
166 (1-4 weeks) with a prophylactic purpose (i.e. to prevent disease) (5). Colistin is also included in  
167 some pig and poultry commercial feeds as a growth promoter (AGP) (17). However, from 2020  
168 onwards, AGPs are longer be allowed in Vietnam (Law No. 32/2018/QH14), in line with  
169 legislative restrictions in Thailand (2015) (18), China (2016) (19) and India (2019) (20).

170 In contrast with the study of human patients, where colistin susceptibility testing is required to  
171 inform therapeutic choices (21) our method is aimed at estimating colistin resistance in mixed  
172 commensal *E. coli* populations. Through evaluation of the growth curves of standard *E. coli*  
173 suspensions from faecal samples, our method enables the detection of colistin resistance in a  
174 dichotomous fashion (presence/absence), as well as providing a quantitative assessment of  
175 colistin resistance at colony level (prevalence of resistant *E. coli*). The sensitivity of this  
176 methodology is, however, limited by the number of colonies harvested per sample (30-50), and  
177 may therefore miss colistin resistant strains in situations of very low prevalence. Indeed,  
178 statistically, given a sample of 40 colonies, there is a 5% probability of not detecting colistin  
179 resistance in any of them when the prevalence of resistant falls below 7.5%. Because of this, the  
180 method is more suitable advised for situations of medium to high prevalence of colistin  
181 resistance. The sensitivity could however be potentially increased by collecting several samples  
182 or increasing the number of *E. coli* colonies used in each suspension. For example, detection of a  
183 prevalence of 2% would require the investigation of 150 isolates (~4 samples, each with 30-50  
184 colonies), detection of a prevalence of 1% would require 300 isolates (~8 samples); 0.1% a total  
185 of 3,000 isolates (~75 samples).

186 Although there was a reasonable relationship between prevalence of resistance and  $AUC_{adj}$ , we  
187 observed considerable variation in  $AUC_{adj}$  for similar prevalence values both in our laboratory  
188 validation as well as on flock samples. This suggests variable growth capacity among resistant  
189 strains, which may depend on their relative fitness. In the case of field suspensions containing a  
190 diversity of susceptible and resistant strains, it is also likely that the relative composition of  
191 strains may result in variable growth among the resistant strains due to the liberation of toxins  
192 (i.e. colicins) in the culture media (22). This may also explain the variable limit of detection

193 confirmed in laboratory conditions with different susceptible strains. In general, given identical  
194 prevalence of resistant strains, we observed higher  $AUC_{adj}$  values for individual susceptible-  
195 resistant strain combinations, compared to the mixed of *E. coli* in field samples (Fig 2). It could  
196 be probably explained by less competition exerted in mixes containing a single strain, compared  
197 with heterogenous mixes containing ~40 different strains. Because of these reasons, prevalence  
198 estimates derived from  $AUC_{adj}$  should be always interpreted with caution.

199 We believe that our testing approach is more efficient than isolating and investigating individual  
200 colonies, at a relatively lower cost. However, it requires investment on a microplate reader  
201 costing between 3,000 and 10,000 USD. The technique presented here could potentially be  
202 adapted to the investigation of other types of phenotypic resistance in *E. coli* (i.e. tetracycline,  
203 ampicillin, etc.) but it would necessarily require optimizing working concentrations.

204 At the colony level, we obtained a median prevalence of 19.4% colistin resistance in flocks.  
205 These results are comparable with previous studies on chicken *E. coli* isolates in the area (12-  
206 22%) (7, 8). Furthermore, the observed ~40% resistance at sample level is consistent with a  
207 previous study on chickens in the Mekong Delta of Vietnam, where 5 *E. coli* colonies were  
208 investigated from each of 18 faecal samples (8). In such study, a total of 8/18 (44%) samples  
209 included at least one resistant strain (NT Nhung, personal communication). A PCR-based study  
210 in this region reported that 59.4% chicken samples investigated tested positive for *mcr-1* gene  
211 (7).

212 We demonstrated a short-term increase in phenotypic colistin resistance following administration  
213 of colistin use as well as non-colistin antimicrobials. This contrasts with a study conducted on a  
214 broiler flock in France, where administration of colistin failed to induce colistin resistance in  
215 Enterobacteriaceae (including *E. coli*) (23). However, unlike in Vietnam, colistin use and

216 resistance (including *mcr-1*) is relatively rare in European livestock (10). Overall, we found  
217 relatively high levels of colistin resistance (~40%), even in flocks that had not been given  
218 colistin (33.3%). There was evidence of colistin resistance in mid-production samples from  
219 flocks that had previously tested negative in day-old samples, and had not been administered  
220 colistin (3 of 8 flocks) (data not shown). This suggests that colistin resistance may have been  
221 generated or introduced to study flocks from other sources, such as contaminated water or feed,  
222 or due to contamination with bacteria from other animal species present in these small-scale  
223 farms.

224 Our findings of increased colistin resistance in flocks treated with antimicrobials other than  
225 colistin are intriguing. In a previous study on Mekong Delta pig farms, colistin resistance in *E.*  
226 *coli* strains was associated with use of non-colistin antimicrobials such as quinolones and  
227 cephalosporins (8). The presence of genes conferring for resistance against several different  
228 antimicrobial classes in *mcr*-harboring plasmids may explain these findings, and suggest that the  
229 use of non-colistin drugs may also select for colistin resistance (24).

230 We observed a peak of colistin resistance in mid-production samples among flocks using  
231 colistin, and generally levels of resistance decayed subsequently. This is likely to reflect the  
232 higher frequency of colistin use during the brooding period. A longitudinal study on travelers  
233 colonized by *mcr-1*-carrying bacteria showed that they were able to completely eliminate these  
234 bacteria within one month after returning to their home country (25). The reasons for a reduction  
235 in resistance over time are known and may be due to a combination of factors leading to plasmid  
236 loss and/or fitness costs. However, studies in the laboratory have shown that the presence of  
237 plasmid-mediated colistin resistance has been shown to confer no fitness costs to *E. coli* (26). It  
238 is worthwhile noting that in our study chicken flocks were of local native breed, and they were

239 typically raised over a 4-5 month period, a period much longer than that required by industrial  
240 broilers (typically 1.5 months). This suggests that birds slaughtered earlier may have a higher  
241 prevalence of colistin resistance, and this potentially represents an additional risk to the  
242 consumer.

243 In summary, we developed and validated an affordable method that may be effectively used to  
244 quantify colistin resistance in commensal *E. coli* in chicken flocks. Our method may also be  
245 adapted to benchmark and monitor changes over time in colistin resistance in faecal samples in  
246 other complex biological systems such as abattoirs, slaughter-points and sewage, or even in  
247 human individuals. Our results indicate a high background of colistin resistance even in flocks  
248 not using this antimicrobial. The observed increases after colistin use were short-lived and  
249 suggest that in small-scale farming systems reducing colistin resistance may require increasing  
250 biosecurity as well as restocking colistin-negative day-old chicks.

## 251 MATERIALS AND METHODS

### 252 Study design

253 In order to investigate the biological basis and the limit of detection of the proposed method,  
254 we used four previously characterized *mcr-1* colistin resistant *E. coli* strains, two displaying  
255 moderate-level (MIC= 8mg/L) and two low-level (MIC= 4mg/L) colistin resistance, alongside  
256 four colistin-susceptible strains. We prepared standard bacterial suspensions consisting of a  
257 mix of each of the resistant and the susceptible strains at different ratios; these were incubated  
258 in medium with and without 2mg/L of colistin. A growth curve from each suspension was  
259 obtained by measuring the optical density (OD<sub>600nm</sub>) during incubation. The area under the  
260 curve (AUC<sub>adj</sub>) of each colistin-containing standard suspension was adjusted by the AUC  
261 values obtained from its equivalent colistin-free suspension. We investigated the relationship

262 between the prevalence of resistance at colony level and the observed  $AUC_{adj}$  from the  
263 examination of 30-50 individual *E.coli* isolates from each of 23 samples and obtained a model  
264 equation. We calculated  $AUC_{adj}$  values of suspensions consisting 30-50 *E. coli* colonies  
265 harvested from each of 108 pooled faecal samples from 36 small-scale (single-age) chicken  
266 flocks raised in Dong Thap province (Mekong Delta, Vietnam) (27). We inferred the  
267 prevalence of resistant *E. coli* in flock samples investigated by extrapolation using the model  
268 equation. The contribution of colistin use and other antimicrobials administered to flocks on  
269 the observed phenotypic colistin resistance was investigated by building logistic regression  
270 models with age as primary time variable.

271 **Culture of standard suspensions and calculation of  $AUC_{adj}$**

272 Each of the chosen resistant *E. coli* strains (named R1 to R4, where R1 and R2 had  $MIC=$   
273 4mg/L; R3 and R4 had  $MIC=$  8mg/L) and susceptible (all  $MIC \leq 1$ mg/L) strains (S1 to S4)  
274 were incubated in cation adjusted Mueller Hinton II Broth II (MHB2, Sigma-Aldrich, USA) at  
275 37°C, 200 rpm for 4h (log-phase) and these bacterial inoculum were adjusted to  $10^8$  CFU/mL  
276 ( $OD_{600nm} = 0.1$ ), and then diluted down with MHB2 to  $10^6$  CFU/mL. Each resistant strain was  
277 mixed with a susceptible strain (total 16 combinations) at susceptible: resistant ratios ranging  
278 from 0:1 (susceptible strain only) to 1:0 (resistant strain only). Intermediate ratios were 10:1,  
279  $10^2:1$ ,  $10^3:1$ ,  $10^4:1$ , and  $10^5:1$ . A total of 100 $\mu$ L of each suspension was added into a well of  
280 polystyrene microplate (Corning, USA), containing 100 $\mu$ L of colistin solution (final working  
281 concentration was 2mg/L). In addition, respective colistin-free (control) suspensions were  
282 prepared. Plates were incubated in a microplate reader (SPECTROStar, BMG Labtech,  
283 Germany) at 37°C for 20h, and the turbidity ( $OD_{600nm}$ ) readings were recorded every hour.. All  
284 experiments were conducted in triplicate.

285 The areas under the curves (AUC) generated over the 20-hour observation period were  
286 computed. The AUC value generated from each standard suspension ( $AUC_{[i]}$ ) was related to the  
287 AUC generated by its respective colistin-free control ( $AUC_{adj} = AUC_{[i]} / AUC_{[0]}$ ).

288 **Flock sample and AMU data collection**

289 Fresh pooled faecal samples were collected from each flock at three time-points: (1) day-old  
290 chicks, (2) mid-production (~2-3 months-old) and (3) end of production (~4-6 months-old).  
291 Day-old faecal (i.e. meconium) samples were collected from the crates at the time when  
292 chicks were delivered to the farms. For mid- and end-production sampling, sterile paper liners  
293 were placed near drinkers and feeders in the chicken house/pen to collect deposited droppings.  
294 After a minimum of 10 droppings had been deposited, liners were swabbed using sterile  
295 gauzes. Each of collected gauze was placed in a universal jar and mixed vigorously with  
296 50mL saline buffer. One ml of the resulting eluate was stored at -20°C with glycerol. Data on  
297 AMU had been collected using purposefully designed diaries where farmers were asked to  
298 note down all antimicrobials used. Farmers were instructed to keep all packages of  
299 antimicrobials used on their flocks (5). Sample and data collection were conducted between  
300 October 2016 and October 2018.

301 **Testing of pooled faecal samples**

302 Eluates from pooled faecal samples were plated onto ECC agar (CHROMagar, France) and  
303 incubated at 37°C for 20h. A total of 30-50 *E. coli* (blue) colonies from each agar sample were  
304 picked, pooled and incubated in CAMHB to log-phase. The resulting bacterial suspensions  
305 were investigated as described above.

306

307 **Prevalence of colistin resistance at colony level**

308 We selected a number of positive samples with variable levels of  $AUC_{adj}$ . From each sample, 40  
309 *E. coli* were isolated and tested individually for the MIC by standard broth micro-dilution  
310 method. Pool of 40 isolated *E. coli* for each sample was also subjected for the growth curve  
311 measurement as described previously.

312 **Data analyses and cost estimation**

313 In order to relate the  $AUC_{adj}$  value to the measured prevalence of resistance among selected  
314 samples, we fitted a beta-regression model using the ‘*betareg*’ package in R (28). Both the trend  
315 and the dispersion were allowed to vary over  $AUC_{adj}$  in a linear way.

316 AMU in flocks was quantified for the two periods defined by the sampling schedule: (1) between  
317 restocking and mid-production, and (2) between mid- and end of production. Weekly estimates  
318 of colistin use were expressed as the number of ADDs (number of Animal Daily Doses  
319 administered per 1,000 chicken days) calculated for each of the two periods (5). Risk factors  
320 associated with colistin resistance at mid- and end of production were investigated by logistic  
321 regression. The outcome was colistin resistance (Yes/No) at sample level. The variables  
322 investigated were: (1) Age of chicken flock (weeks); (2) Use of colistin within two weeks prior  
323 to sampling (Yes/No); (3) Number of ADDs per 1,000 chicken-days of colistin in each period;  
324 (4) Colistin resistance of day-old chicks (Yes/No); and (5) Number of ADDs per 1,000 chicken-  
325 days of non- colistin antimicrobials used in each period. The variable Age of chicken flock was  
326 included in all univariable models because it is the principal time variable. Since we had two  
327 measurements per flock (mid and end cycle samples), we used generalized estimation equations  
328 with an exchangeable correlation structure to estimate the parameters using the ‘*geepack*’ R  
329 package (29, 30).

330 The change in AUC<sub>adj</sub> over age of chicken was modeled using a random effects linear regression.  
331 In order to allow for a nonlinear trend, we used a natural spline for the fixed effect term (knots at  
332 0, 8, 12 and 20 weeks). We allowed for a random intercept and linear trend by age.  
333 The overall costs (per sample) of the method described above were calculated based on expenses  
334 on medium, reagents and consumables (excluding staff time, which was estimated separately).  
335 The estimated costs were compared with those incurred in testing one sample by broth  
336 microdilution and Etest in Vietnam as of January 2020. Our calculations were based on the  
337 investigation of 40 *E. coli* isolates per sample using the growth-based method, compared with 10  
338 isolates each by broth microdilution and by Etest.

### 339 **SUPPLEMENTAL MATERIAL**

340 Table S1 Estimated percentage of resistant *E. coli* from 108 samples  
341 Table S2 Estimated costs (in US dollar) of testing 1 sample to determine colistin phenotypic  
342 resistance of *E. coli*  
343 FIG S1 Usage of colistin among study flocks by week.  
344 FIG S2 Changes in AUC<sub>adj</sub> by age (weeks) of chicken at the time of sampling.

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350 We declare that we have no competing interests.

351 N.T.N and JC-M conceived the idea; J.C, G.T and S.B advised on the study design; N.V.C and  
352 B.T.K coordinated field sampling and data collection; N.T.N, N.T.P.Y. and N.V.K.T performed  
353 laboratory experiments. N.T.N, R.B.G and J.C-M conducted data analyses and produced first  
354 draft. All authors commented on subsequent versions.

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448

449 TABLE 1 Description of AMU and estimated prevalence of colistin resistance in 36 small-scale  
 450 chicken flocks stratified by whether farmers administered colistin or not.

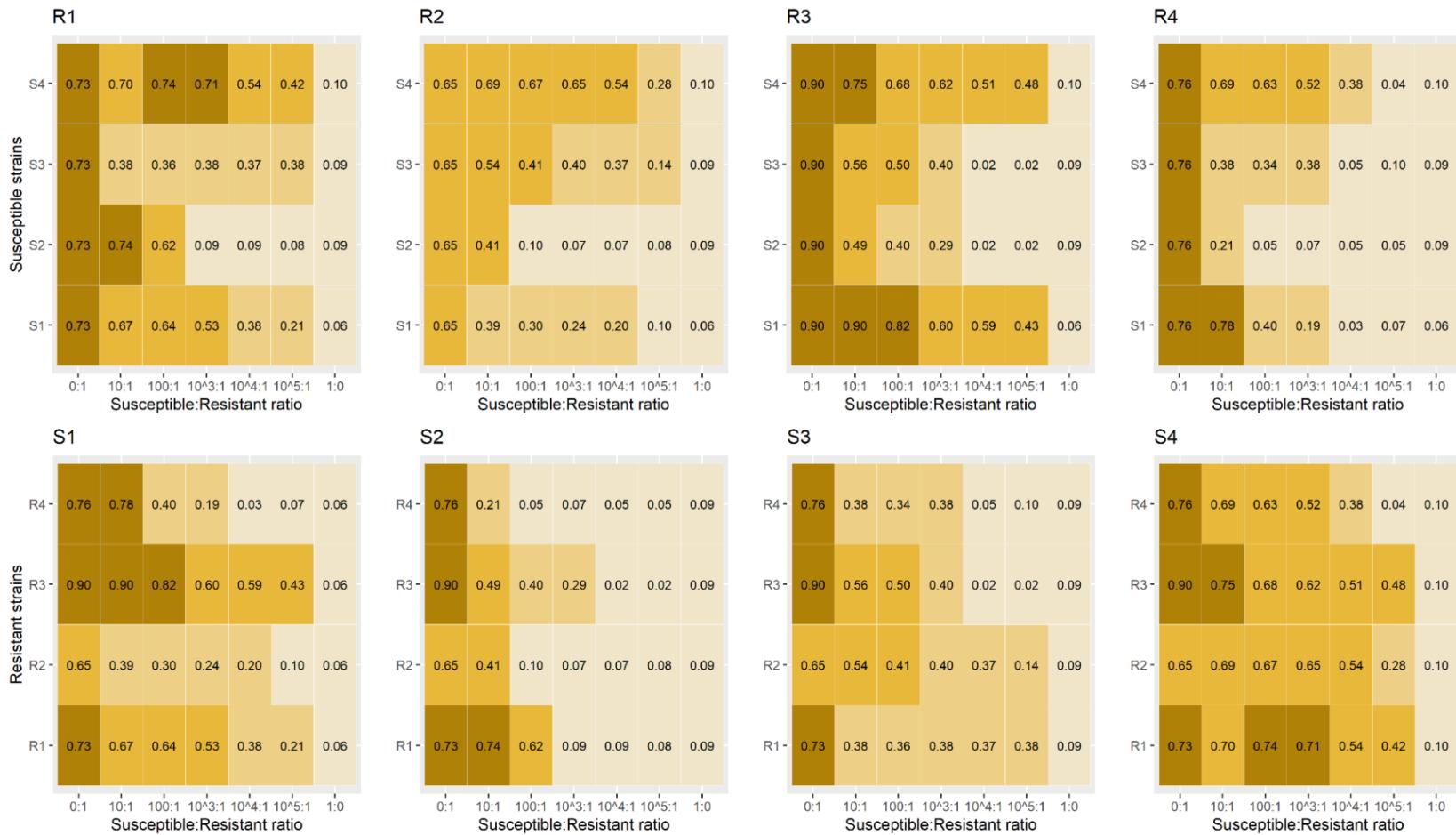
	Flocks not using colistin (n=14)	Flocks using colistin (n=22)	All flocks (n=36)
Cycle duration (weeks) (median [1 <sup>st</sup> - 3 <sup>rd</sup> quartile])	19 [17-20]	20 [17-21]	19 [17-20]
No. chickens (median [1 <sup>st</sup> - 3 <sup>rd</sup> quartile])	249 [194-482]	208 [128-398]	231 [189-401]
No. ADDs of colistin (per 1,000 chicken-days) (mean ± SD)			
First period	0	281.7 ±321.2	172.1 ± 285.1
Second period	0	17.4 ±18.1	10.6 ± 16.4
Whole production cycle	0	149.5 ±261.6	91.4 ± 216.4
No. ADDs of non-colistin antimicrobials (per 1,000 chicken-days) (mean ± SD)			
First period	345.5 ± 471.5	629.3 ±359.8	518.9 ± 424.2
Second period	29.0 ± 48.6	72.5 ± 98.5	55.6 ± 84.7
Whole production cycle	187.2 ±366.2	350.9 ± 383.8	287.3 ± 382.9
No. flocks using colistin two weeks prior to			
Mid-sampling	0	11	11
End of sampling	0	1	1
AUC <sub>adj</sub> (median, [1 <sup>st</sup> - 3 <sup>rd</sup> quartile])			
Day-olds	0.07 [0.04-0.42]	0.06 [0.04-0.52]	0.07 [0.04-0.65]
Mid-production	0.06 [0.03-0.43]	0.54 [0.07-0.65]	0.20 [0.05-0.63]
End of production	0.07 [0.06-0.55]	0.07[0.06-0.55]	0.07 [0.05-0.56]
Prevalence of resistance (%) at sample level (95% CI)			
Day-olds	42.8 (18.8-70.3)	31.8 (14.7-54.9)	36.1 (21.3-53.8)
Mid-production	28.6 (9.5-58.0)	63.6 (40.8-82.0)	50.0 (34.5- 65.5)
End of production	28.6 (9.5-58.0)	31.8 (14.7-54.9)	30.5 (16.9- 48.3)
Estimated prevalence of resistance (%) at colony level (mean ± SD)			
Day-olds	28.8 ±36.0	15.7 ±24.7	20.8 ±29.8
Mid-production	17.3 ±28.7	27.0 ±26.4	23.3 ±27.4
End of production	16.2 ±24.8	12.8 ±18.1	14.1 ±20.7

451 AUC= area under the growth curve; CI= Confidence interval; SD= standard deviation

452 TABLE 2 Logistic regression models investigating risk factors associated with colistin resistance  
453 in chicken flocks at sample level. Models were based on a total of 72 samples (mid and end  
454 production); 29 were positive resistance to colistin.

Variable	Univariable <sup>a</sup>			Multivariable		
	OR	95% CI	p-value	OR	95% CI	p-value
Age of chicken flock (weeks)	0.93	0.84-1.02	0.156	1.04	0.91-1.18	0.605
Use of colistin within last two weeks (Yes/No)	5.30	1.17-24.08	0.030	3.67	0.68-19.70	0.128
No. ADDs per 1,000 chicken-day of colistin <sup>b</sup>	1.66	1.00-2.76	0.049	1.06	0.55-2.06	0.845
Colistin resistance of day-old chicks (Yes/No)	1.45	0.53-3.97	0.461	1.61	0.54-4.84	0.395
No. ADDs per 1,000 chicken-day of non-colistin antimicrobials <sup>b</sup>	2.10	1.18- 3.73	0.012	1.84	0.88-3.85	0.102

455 <sup>a</sup>The variable ‘Age of chicken flock’ was included in all univariable models to calculate estimates for all subsequent  
456 variables. <sup>b</sup>logarithm transformed after adding 1, ADD= animal daily dose; OR= Odds ratio; CI= Confidence interval.

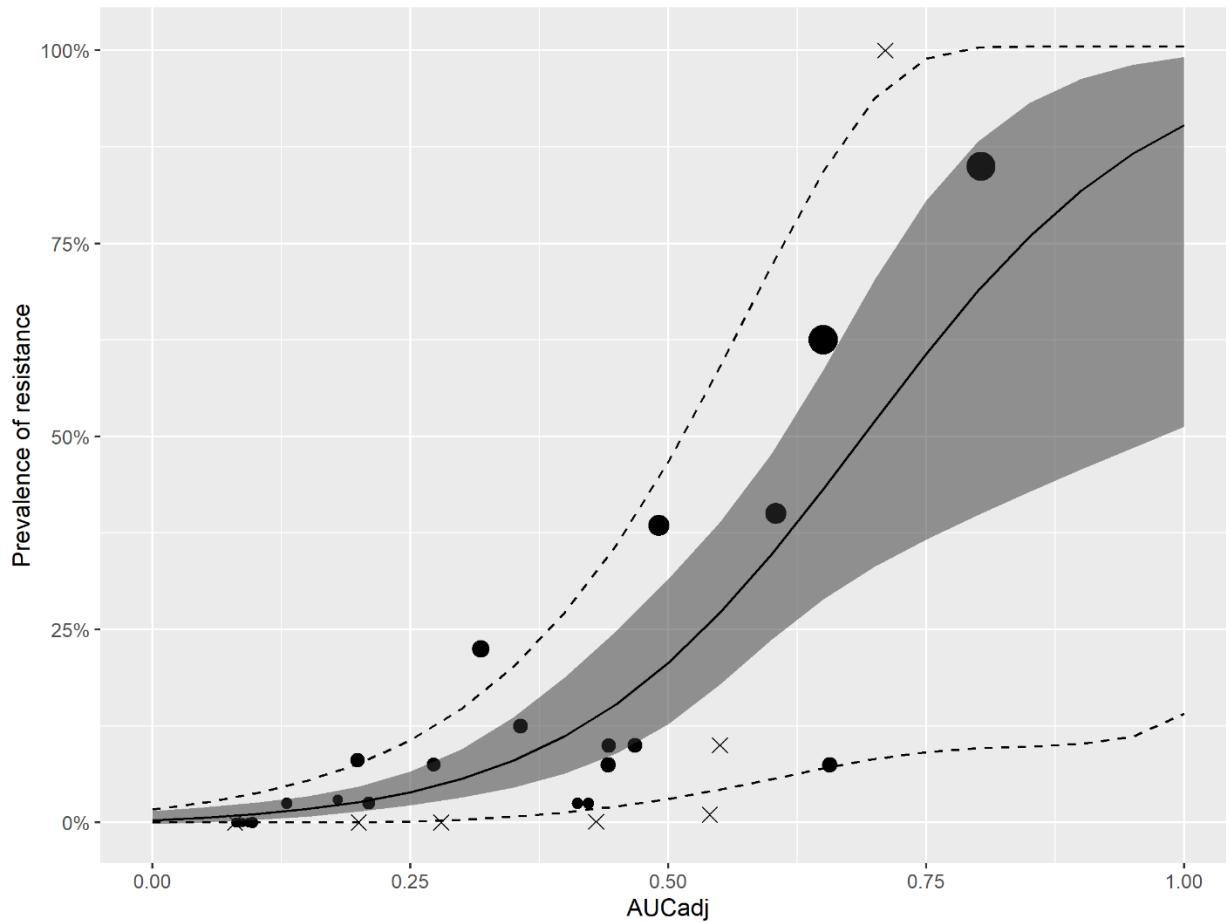


457

458 FIG 1 AUC<sub>adj</sub> of standard suspensions. Positive growth values are represented by increasing strength of color. R= Resistant, S=

459 Susceptible. Average of AUC<sub>adj</sub> of resistant strain 1, 2, 3 and 4 was 0.40, 0.30, 0.41 and 0.26, respectively. Average AUC<sub>adj</sub> of

460 susceptible strain 1, 2, 3 and 4 was 0.41, 0.19, 0.31 and 0.54 respectively.



461

462 FIG 2 Relationship between  $AUC_{adj}$  and prevalence of colistin resistance at colony level. The  
463 figure shows the predicted mean value of resistance with pointwise 95% confidence as shaded  
464 area. The dotted lines give the 5% and 95% prediction intervals. Circle symbols indicated  
465  $AUC_{adj}$  values of mixed *E. coli* in field samples. Size of dot represented the average MIC of each  
466 sample. Cross symbols indicated  $AUC_{adj}$  values of mixed susceptible and resistant strains.