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The use of colistin and other critical antimicrobials on pig and chicken farms in southern Vietnam and their association with resistance in commensal *Escherichia coli*

Running title: Antimicrobial resistance in Vietnamese farms

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Abstract

Antimicrobial resistance (AMR) is a global health problem; emerging semi-intensive farming systems in Southeast Asia are major contributors to the AMR burden. We accessed 12 pig and chicken farms at key stages of production in the Tien Giang province (Vietnam) to measure antimicrobial usage and to investigate the prevalence of AMR against five critical antimicrobials (β-lactams, 3th cephalosporins, quinolones, aminoglycosides and polymyxins) and their corresponding molecular mechanisms among 180 *Escherichia coli* isolates. Overall, 94.7mg (interquartile range (IQR) 65.3-151.1) and 563.6mg (IQR 398.9-943.6) of antimicrobials were used to produce 1kg of live weight of chicken and pig, respectively. A median of 3 (out of 8) critical antimicrobials was used on pig farms. *E. coli* isolates exhibited a high prevalence of resistance against ampicillin (97.8% and 94.4% for chickens and pigs, respectively), ciprofloxacin (73.3% and 21.1%), gentamicin (42.2% and 35.6%) and colistin (22.2% and 24.4%). The prevalence of recently discovered colistin resistance gene, *mcr-1*, was 19-22% and had strong agreement with phenotypic colistin resistance. We conducted plasmid conjugation experiments with 37 *mcr-1* gene positive *E. coli* isolates and successfully observed transfer of this gene in 54.0% isolates through a plasmid of approximately 63Kb, consistent with that identified earlier in China. We found no significant correlation between total usage of antimicrobials at farm level and AMR. These data provide additional insight into the role of *mcr-1* in colistin resistance on farms in Asia and begin to outline the dynamics of phenotypic and genotypic AMR in semi-intensive farming systems in Vietnam.

Importance statement

Our study provides accurate baseline information on levels of antimicrobial use, as well as on the dynamics of phenotypic and genotypic resistance for antimicrobials of critical importance among *E. coli* over the different stages of production in emerging pig and poultry production systems in Vietnam. *E. coli* isolates showed a high prevalence of resistance (>20%) against critically important antimicrobials such as colistin, ciprofloxacin and gentamicin. The underlying genetic mechanisms identified for of colistin (*mcr-1* gene) and quinolone (*gyrA* gene mutations), are likely to play a major role in AMR to those compounds. Conjugation experiments led to the identification of a 63 Kb plasmid, similar to that recently identified in China, as the potential carrier of the *mcr-1* gene. These results should encourage greater restrictions of such antimicrobials in southeast Asian farming systems.

Introduction

Antimicrobials are widely used in animal production to prevent and treat disease, and to improve growth performance (1). Nearly all antimicrobial classes important for human medicine are also used in animal production (2). The association between antimicrobial use (AMU) and resistance (AMR) in commensal bacteria has been established in poultry and pig farms (3, 4). However, most studies to date have been conducted in intensive production systems prevalent in developed countries. In contrast, little data are available from semi-intensive systems prevalent in developing countries, where farmers typically have little access to veterinary support.

Of particular concern is the prevalent use of antimicrobials considered by the World Health Organization (WHO) to be of critical importance for human medicine (5) in Vietnamese pig and poultry farms. These antimicrobials include penicillins, third generation cephalosporins, quinolones, aminoglycosides, polymyxins and macrolides (6-8).

E. coli is a commensal organism of the gastrointestinal tract and a widely used marker to monitor AMR in livestock and meat (9, 10). Although a high prevalence of AMR among commensal organisms has been reported on animal farms in Vietnam (7, 11), little is known about the molecular mechanisms of resistance in this organism in the country. Within *E. coli*, reduced susceptibility and resistance against quinolones is known to be associated with mutations in the DNA gyrase gene (*gyrA*) (12), and/or the presence of plasmid mediated quinolone resistance (PMQR) determinants, including the Qnr proteins, the acetylating AAC(6')-Ib-cr enzyme and QepA efflux pumps (13). Common aminoglycoside resistance mechanisms within the Enterobacteriaceae include expression of adenylyltransferases and phosphoryltransferases encoded by *aadA* and *strA*/*B*, respectively which are located within multi-drug resistance (MDR) integrons (14). Very recently, plasmid-mediated colistin resistance,

encoded by the *mcr-1* gene was discovered in *E. coli* isolated from pigs, chickens and humans in China. In view of the higher prevalence of positive samples in animal isolates, it is likely to that the *mcr-1* resistance mechanism originated in animals and subsequently spread to humans (15).

In this study we aimed to (1) measure levels of antimicrobial usage and AMR and assess the prevalence of a selection of nine AMR associated genes encoding resistance against antimicrobials that are commonly used in human healthcare among commensal *E. coli* at different stages of production; and (2) to investigate the relationship between phenotypic and genotypic markers of resistance, and between AMU and AMR. To address these aims we conducted a longitudinal study along the production cycle in 12 pig and meat chicken farms typical of emerging semi-intensive systems in the Mekong Delta region of Vietnam.

Materials and methods

Selection of study farms

A total of six chicken (C1 to C6) and six pig units (P1 to P6) representative of emerging semi-industrial production farming systems in the Mekong Delta region of Vietnam were studied. The selected chicken farms met the following criteria: (1) they raised chickens for meat production; (2) they had between 500 and 10,000 chickens; and (3) they performed all in/all out management. The selected pig farms met the following criteria: (1) they raised pigs from farrowing to slaughter at any one time on the farm; and (2) they had at least 50 pigs at any time on farm.

Farm sampling

From each farm rectal/cloacal swabs were collected from chickens and pigs on three consecutive visits: (1) day of arrival of chicks from the hatchery (chickens) or 1-2 days after

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96 farrowing (pigs); (2) mid-production (chickens: 25-30 days-old; pigs: 60-65 days-old); and (3)
97 immediately before sale (chickens: 45-48 days-old; pigs: 125-130 days-old). On the first visit to
98 each farm, 10 randomly-selected animals that were tagged using leg rings (chickens) or ear
99 notches (pigs). These same animals were sampled on subsequent visits.

100 ***Data collection***

101 Data on administration of antimicrobials by the farmer and in feed were collected using
102 questionnaires during the farm visits. Data on AMU was defined for two distinct periods: (1)
103 between restocking/birth to second sampling; and (2) between second and third sampling. Visits
104 were conducted between November 2013 and June 2014 by trained veterinarians affiliated with
105 the Tien Giang Sub- Department of Animal Health (SDAH).

106 ***Isolation of E. coli***

107 Rectal/cloacal swabs collected on each visit from the target animals were pooled and tested
108 as one analytical sample. In order to isolate *E. coli*, samples were streaked directly onto
109 MacConkey agar (Oxoid, UK) and were subsequently incubated at 37°C overnight. Up to five
110 colonies showing typical *E. coli* morphology were confirmed using standard biochemical tests
111 (motility, indole, lactose/glucose fermentation, methyl red, citrate, urease, hydrogen sulfide, and
112 gas production).

113 ***Phenotypic testing of AMR***

114 Colonies confirmed as *E. coli* were phenotypically tested for their susceptibility against
115 ampicillin (10µg), ceftazidime (30µg) (Oxoid, UK) by the Kirby-Bauer disk diffusion test.
116 Ciprofloxacin, gentamicin and colistin resistance was investigated by determining the minimum
117 inhibitory concentration (MIC) using Etest (Biomerieux, France). The reference strain *E. coli*

ATCC 25922 was used for quality control purposes. In order to establish the susceptibility status of test strains, the guidelines on breakpoints provided by the Clinical and Laboratory Standards Institute (16) were followed. The strains were considered to have colistin resistance if their MIC was >2 µg/mL as described by European Committee on Antimicrobial Susceptibility Testing (17). Colonies that were intermediate in resistance based on the inhibition zone were also regarded as resistant. An MDR organism was defined as a strain is resistant to at least three different antimicrobial classes.

PCR amplification of AMR genes and DNA sequencing

Rapid DNA preparation was performed by a boiling technique that includes a heating step at 95^{o}C in 15 minutes of colonies in a total volume of 200µl of distilled water followed by a centrifugation step of the cell suspension. PCR amplification of the quinolone (*qnrA/B/S*, *qepA*, *aac(6')-Ib-cr* and *gyrA)*, aminoglycoside (*aadA* and *strA/B*) and colistin resistance genes (*mcr-1*) was performed using previously published primer sets (15, 18-20). PCR amplification was performed using a Tprofessional Thermocycler (Biometra, Germany) and BioTaq polymerase (Bioline, UK). PCR amplicons were examined by electrophoresis and UV visualization on 1.5% agarose gels containing Nancy (Sigma, Germany). Amplicons produced from all strains specific for *gyrA* were sequenced with the same primers used for amplification. The forward and reverse strands of all PCR products were sequenced using BigDye terminators on ABI sequencer (Life Technologies, USA).

Plasmid conjugation experiment

The potential transmissibility of the *mcr-1* gene was investigated by performing a *mcr-1* containing plasmid conjugation experiment. We independently mated *mcr-1* plasmid containing strains (donor) with pan-susceptible sodium azide resistant *E. coli* J53 (recipient). Bacteria were

conjugated for 12 hours in Luria-Bertani (LB) broth at 37^{o}C and transconjugant strains were selected on LB agar plates supplemented with colistin (2mg/L) and sodium azide (100mg/L). From each plate colonies were counted and this number was used to estimate transfer frequency per donor in successful transfer experiments. Plasmids were extracted from the donors and transconjugants using the method described by Kado and Liu (21). DNA was separated on 0.7% agarose gels and visualized under UV light after staining with ethidium bromide. The approximate size of plasmid was determined after comparison with *E. coli* 39R861, containing four plasmids of 147, 63, 36 and 7 Kb.

Data analyses

The amount of in-feed antimicrobials consumed (mg) to produce one kilogram of live chicken and pig was estimated from the concentration of antimicrobials in each feed product (as indicated in the label) and the feed conversion rate (FCR) (the amount of feed required to produce one kilogram of animal live weight). The FCR values used were 2.85 for chickens and 3.90 for pigs (22, 23). Since some feed products had ambiguous labeling (i.e. the manufacturer indicated that they contained one of two or more different antimicrobials) separate calculations were performed on the assumption that either of the listed antimicrobials was present.

The amount of antimicrobials administered by the farmer (i.e. excluding commercial medicated feeds) to produce one kilogram of live animal was estimated by dividing total amount of each antimicrobial used (mg) by the estimated bodyweight (kg) of animals at the end of their production cycle. Since weight data on chickens and pigs at slaughter were not available, we assumed that the weight of chicken and pig raised after two months and five months were 1.5 kg and 92.5kg, respectively (22, 23).

In order to explore the association between antimicrobial usage and AMR, we calculated the Pearson's correlation coefficient between quantities of antimicrobials used in each farm and the average number of antimicrobials against which *E. coli* from those farms was resistant for chicken and pig farms separately. The potential association between the use (vs. not use) of specific antimicrobials during periods one and two and the observed phenotypic and genotypic AMR (outcome) of isolates recovered on sampling visits two and three was investigated using univariable risk ratios (RR) for chicken and pig farms. In all cases, the baseline group referred to the prevalence of AMR among isolates from farms that did not use antimicrobials on that period.

The agreement between phenotypic and genotypic resistance for ciprofloxacin, gentamicin, colistin was determined using the kappa statistic (κ). Statistical analyses were performed using 'epicalc' and 'epiR' packages in R version 3.0.2 (The R Foundation for Statistical Computing).

Results

Farms and isolates

The six chicken farms ranged in size from 500 to 6,000 chickens per farm (mean= 2,572; standard deviation (SD) ± 2,318); the six pig farms ranged in size from 73 to 250 pigs per farm (mean= 108; SD± 61). We isolated a total of 180 *E. coli* (5 isolates/visit farm) during the routine farm visits.

Antimicrobials in commercial feed

All farms used commercial feed. A total of four and 13 different feed products were found in chicken and pig farms, respectively. The identity of the feed product (and its antimicrobial composition) could be established for three of the chicken and 10 of the pig feed products. A total of two chicken and nine pig products contained at least one antimicrobial (Table 1).

185 However, 10/11 feed products the label indicated that the product contained one (only one) of
186 several antimicrobials listed (up to five).

187 A total of two and 13 different antimicrobials were listed in chicken and pig feed products,
188 respectively. Enramycin and chlortetracycline were the most common antimicrobials present in
189 pig feeds (in five and four products, respectively). Colistin, amoxicillin and neomycin (all
190 considered to be of critical importance by the WHO) were present in three, two and one of the
191 pig feed products investigated, respectively. No antimicrobials considered to be of critical
192 importance by the WHO were present in any of the three chicken feeds. Antimicrobials were
193 supplemented into pig feed at significantly higher concentrations (median 45 mg/kg) than
194 chicken feeds (median 10 mg/kg) (p<0.001). The median estimated amounts of in-feed
195 antimicrobials used to produce one kilogram live weight of chicken and pig were 57.0 (IQR, 28.5
196 to 57.0) mg and 507.0 (IQR, 312.0 to 877.5) mg, respectively.

197 ***Antimicrobials administered for prophylactic and therapeutic purposes***

198 In addition to antimicrobials present in feed, all chickens and pigs were administered
199 antimicrobials by the farmer at least once in their life cycle. A total of 10 and 15 different
200 antimicrobial products were used in chicken and pig farms, respectively. Fifty percent of the
201 chicken products and 66.7% of pig products contained two or more antimicrobial compounds in
202 their ingredients. A total of 17 different antimicrobials belonging to eight classes were identified
203 (Table 2). Three chicken products and nine pig products contained antimicrobials regarded of
204 critical importance for human medicine (neomycin, kanamycin, gentamicin, spiramycin, colistin
205 and norfloxacin).

206 On chicken farms antimicrobials were administered for prevention of disease on 11 (73.3%)
207 instances and for treatment on four occasions (26.7%) (due to respiratory disease). In pig farms

antimicrobials were administered for treatment of respiratory and enteric disease on 18 (66.7%) instances, on four (14.8%) instances for disease prevention, and on five (18.5%) instances for both purposes. In chicken farms antimicrobials were administered using water (100% cases), whereas antimicrobials were administered by injection (93.3% cases) in pig farms.

The farm estimated amount of antimicrobial administered per kg of live weight were 52.0 (IQR, 29.7 to 101.2) mg and 46.1 (IQR, 34.0 to 75.9) mg for chickens and pigs, respectively.

Phenotypic testing of AMR among E. coli isolates

The highest prevalence of resistance to specific antimicrobials among *E. coli* isolates was against ampicillin (97.8% and 94.4% for chickens and pigs, respectively), followed by ciprofloxacin (73.3% and 21.1%), gentamicin (42.2% and 35.6%), colistin (22.2% and 24.4%) and ceftazidime (1.1% and 7.8%) (Figure 1). The overall prevalence of MDR (defined here as resistance to at least three classes of antimicrobials) among chicken isolates was significantly higher than the organisms isolated from pigs (43.3% vs. 24.4%, respectively) (p=0.01, Fisher's exact test).

Organisms isolated from chickens exhibited a significantly higher prevalence of resistance against gentamicin at mid- and end of production combined (51.7%) compared to the day of arrival (23.3%) (p=0.02, Fisher's exact test). Organisms isolated from pigs exhibited a higher prevalence of colistin resistance at mid-production compared to farrowing (36.6% vs. 3.3%) (p=0.002, Fisher's exact test). Compared with mid-production, pig isolates from end of production had significantly decreased prevalence of resistance against ciprofloxacin (33.3% vs. 6.7%), gentamicin (50.0% vs. 20.0%) and a lower rate of MDR isolates (40.0% vs. 10.0%) (p<0.03 in all cases, Fisher's exact test).

PCR screening for AMR associated genes

Quinolone resistance in *E. coli* is determined predominantly by mutations at codons 83 and 87 in the *gyrA* gene. A total of 33.3% of chicken isolates and 18.3% of pig isolates had a single mutation in the *gyrA* gene. Double mutations were found in 43.3% of chicken isolates and 13.3% of pig isolates. A total of 24.4% chicken and 11.1% pig *E. coli* isolates produced amplicons for the PMQR gene *qnrA*. 23.3% chicken isolates and 43.3% pig isolates were positive for *qnrS*. We detected the *aac(6')-Ib-cr* gene in two pig isolates. None of the isolates from chicken or pig harboured either the *qnrB* or the *qepA* genes (Figure 2). Isolates from chickens had higher prevalence of *gyrA* double mutations ($p<0.001$, Fisher's exact test) and *qnrA* ($p=0.03$, Fisher's exact test) compared to isolates from pigs. In contrast, isolates from pigs had higher prevalence of *qnrS* gene compared to isolates from chickens ($p=0.007$, Fisher's exact test). Among the organisms isolated from pigs, the prevalence of double mutation in *gyrA* gene was higher among isolates from mid-production (26.6%) compared with the isolates from end of production (3.3%) ($p=0.03$, Fisher's exact test). There was no difference in prevalence of PMQR determinants between different stages of production.

The prevalence of *aadA* gene (encoding for aminoglycoside resistance) was higher among pig isolates (80.0%) compared with chicken isolates (51.1%) ($p<0.001$, Fisher's exact test). In contrast, the prevalence of the *strA/B* gene was lower in pig isolates (20.0%) than chicken isolates (47.8%) ($p=0.001$, Fisher's exact test). The prevalence of *strA/B* was higher among chicken isolates from the mid-production (60.0%) compared with the day-old chicks (26.7%) ($p=0.02$, Fisher's exact test). In contrast, no changes in prevalence of *strA/B* was observed between stages of pig production.

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252 The prevalence of the recently discovered plasmid-mediated colistin resistance gene, *mcr-1*,
253 was 18.9% and 22.2% in pig and chicken *E. coli* isolates, respectively (Figure 2). The prevalence
254 of *mcr-1* did not change significantly along production cycle. Among 20 and 17 *mcr-1* positive
255 *E. coli* from chickens and pigs, respectively, colistin resistance could be successfully transferred
256 by conjugation in 70.0% and 35.3% of isolates. The estimated transfer frequency in successful
257 experiments was of 10^{-1} to 10^{-3} cells per donor cell. All transconjugants acquired one plasmid of
258 approximately 63 Kb in size. Furthermore, seven of the 20 transconjugants contained additional
259 plasmids that were <63 Kb or >100 Kb in size.

260 ***Relationship between phenotypic and genotypic resistance***

261 Double mutations in *gyrA* gene exhibited the highest agreement with ciprofloxacin
262 resistance for both chicken and pig isolates (κ= 0.43 and 0.73, respectively) (Table 3). A single
263 *gyrA* mutation was associated with low levels of resistance to ciprofloxacin (median MIC of 3
264 (IQR, 1.0 to 32) μg/ml in chicken and 0.75 (IQR, 0.19 to 3) μg/ml in pig isolates) and double
265 mutations were associated with high levels of resistance (median MIC of 32 μg/ml (IQR, 24 to
266 32 μg/ml and 4 to 16 μg/ml for chicken and pig isolates, respectively)). Amplification of PMQR
267 had a lower agreement with phenotypic ciprofloxacin resistance (κ< 0.2) in all isolates from
268 chickens and pigs.

269 Among the *E. coli* organisms isolated from chickens, the presence of *aadA* and *strA/B*
270 exhibited a reasonable agreement with gentamicin resistance (κ=0.33 and 0.26, respectively)
271 (Table 3). Among pig isolates, the presence of *strA/B* had the strongest agreement with
272 gentamicin resistance (κ= 0.19).

273 The presence of *mcr-1* gene had very strong agreement with colistin resistance in both
274 chicken (κ= 1.0) and pig isolates (κ= 0.84). Among the *mcr-1* positive strains, the median MIC

against colistin was 4 µg/ml (IQR, 3 to 4 µg/ml and 4 to 6 µg/ml for chicken and pig isolates, respectively).

Association between antimicrobial use and antimicrobial resistance

We found no correlation between total usage of antimicrobials at farm level and AMR (calculated as average number of resistances against *E. coli* were resistant per farm) ($p>0.83$). However, the use of quinolones and cephalosporins in the period between birth to mid-production was statistically associated with ciprofloxacin resistance (RR= 9.0 and RR= 5.0, respectively) and colistin resistance (RR= 11.0 and RR= 4.17, respectively) in mid-production of pig farms (Table 4). Cephalosporins use in the first period of production was strongly associated with the detection of the *strA/B* gene in isolates from the second sampling (RR= 5.0). Mutations in *gyrA* among *E. coli* isolates were strongly associated with the use of phenicols (RR= 20.0), tetracyclines (RR= 10.0) and beta-lactams (RR= 5.0) throughout the whole production cycle and the use of quinolones (RR= 4.5) and cephalosporins (RR= 4.17) in the first period. No association between antimicrobial use and resistance was observed in chicken farms.

Discussion

There are very few published longitudinal studies quantifying AMU and describing AMR in poultry and pigs in developing countries (3, 4). Our work represents an important contribution towards understanding the interplay between AMU and AMR on farms in Southeast Asia.

Our results indicate comparable quantities of antimicrobial administered by farmers to produce chicken meat, compared with pig meat (52.0 mg per kg of live weight vs. 46.1 mg, respectively). However, consumption of antimicrobials in feed was considerably higher in pigs compared to chickens, resulting in overall greater amounts of antimicrobial consumed by pigs

(563.6 mg per kg live weight), compared with chickens (94.7 mg per kg live weight). Critically important medicines for human such as colistin, neomycin, gentamicin, kanamycin and norfloxacin were also used on studied farms. In pig farms, a median of 3 (out of 8) critical antimicrobials was used to raise animals. A previous study in Vietnam showed much higher amounts of antimicrobial administered by farmer in meat chicken farms (470.4 mg per chicken produced, equivalent to 276.7 mg per kg assuming an average weight of 1.7 kg) (6). These differences may reflect management practices related to intensification of farming systems. In this study, farms were much larger (mean of 2,572 chickens per farm), whereas in our previous study all farms were <2,000 chickens. The same study showed that larger production units used up to five times less antimicrobials per time unit compared with smaller units (6). Antimicrobials administered to chickens were mostly used to prevent disease (73.3%); in contrast most antimicrobials administered to pigs were used for disease treatment (66.7%). Chickens were often administered antimicrobials through water or feed whereas pigs were administered mostly through injection. This may result in higher levels of AMR in the gastrointestinal microbiota in the chicken species.

The prevalence of AMR among *E. coli* in this study was comparable to previous studies in the area (7, 11). However, we found a higher prevalence of resistance against ciprofloxacin and gentamicin (73.3% and 42.2%, compared with 21.0 to 24.2% and 10.8 to 15.0%, respectively). Our results show that mutations in the *gyrA* sequence play a key role for development of resistance against quinolone antimicrobials, whereas PMQR associated mechanisms investigated appeared to less common (12, 24). The presence of both *aadA* and *strA/B* were most commonly associated with gentamicin resistance in chickens and pigs, respectively. Published studies have

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shown an association between these genes and streptomycin and other aminoglycosides among *E. coli* isolates from poultry and pigs (25, 26).

A particular concern is the high prevalence (22%-25%) of colistin (a polymyxin antimicrobial) resistance among pig and chicken *E. coli* isolates. Colistin is regarded as a last-line antimicrobial for the treatment of severe human infection cause by MDR gram-negative bacteria. Historically most colistin resistance mechanisms have involved chromosomal mutations (27). More recently studies from Europe and Asia have reported the emergence of *mcr-1* gene in Enterobacteriacea of chicken and pig origin (15, 28, 29). Our results indicated a similar prevalence of plasmid-mediated *mcr-1* gene in *E. coli* isolates to those reported the Chinese study. We additionally observed very strong agreement between colistin phenotypic resistance and the presence of the *mcr-1* gene both in chicken and pig isolates. The plasmids containing the *mcr-1* gene was of similar size (~63 Kb) to that reported in China (15) and showed a very high *in vitro* transfer rate between *E. coli* strains. These results suggest that this *mcr-1* containing plasmid is probably widespread globally by now. Our findings indicated that colistin is commonly used in chicken and pig farms (in 4/12 farms as prophylactic/therapeutic drug) as well as in potentially in feed in three pig farms, as has been shown in previous studies in Vietnam (6, 8). We did not, however, observe an increased prevalence in colistin resistance among the four farms where the farmers had administered colistin. In addition, about ~20% isolates from day-old chicks tested positive, suggesting vertical transmission from breeder flocks and the capacity of colistin resistant strains to survive the hatchery process. Given the ambiguous labeling of the feed products with regards to their antimicrobial content, it is not possible to determine whether colistin was also used via the feed on three further farms. Our findings suggest that the use of

quinolones and cephalosporins may select for colistin resistance in pigs, although the reasons for this are currently unknown.

Our results reflect the complexity of AMR and the difficulties in accurately establishing the specific genetic mechanisms underlying specific types of AMR due to the multitude of potential genetic mechanisms. Especially in the case of colistin (*mcr-1* gene) and quinolones (*gyrA* gene mutations), we believe that the genetic mechanisms identified that are likely to have major contribution to AMR to those compounds. In contrast, the genes assayed (*aadA* and *strA/B* genes) seemed to had a lower contribution to gentamicin resistance.

We did not find an overall relationship between total antimicrobial usage and AMR on farms. In contrast we found some unexplained univariable relationships between the use of certain antimicrobials and certain phenotypic and genotypic AMR patterns on pig farms. This has to be interpreted with caution especially given the relatively small sample size of our study (6 chickens, 6 pig farms), all subjected to considerable AMR selection pressure, and the potential existence of confounding factors that were not included in the analyses. Unfortunately the fact that in all farms antimicrobials were used intensively (i.e. no 'negative controls' were available) makes it difficult to elucidate this relationship. Also in all farms antimicrobials were present in commercial feeds, but it was not possible to determine exactly which antimicrobials are likely to confound this association. In addition, AMR bacteria may also be acquired from external sources, and potentially transfer to current animals from animals kept in the building during the previous cycle, ('carry-over'). Because of the impossibility of anticipate farmer behavior, we believe that such studies should be preferably addressed in experimental farm settings where antimicrobial exposure (i.e. quinolones, macrolides) is well controlled.

Among *E. coli* isolates from pigs, a lower prevalence of AMR was found in finishers compared with younger pigs for ciprofloxacin, gentamicin and MDR. It has been suggested that this may be a reflection of the fitness cost of resistant organisms in the intestinal tract (30). For chickens, an overall lower prevalence of AMR and MDR was found among chicks sampled on arrival to the farm, and an increase was detected for all antimicrobials during production. However levels of fully resistance against ciprofloxacin and ampicillin on arrival were particularly high (46.7% and 36.7% respectively). Unfortunately, information about potential antimicrobial usage in hatcheries was not gathered. In poultry farms, resistant bacteria may be introduced through vertical transmission from parental flocks or contamination in the hatchery environment (31). The presence of resistance in new-born piglets is likely to reflect lateral transmission from the sows (32). In addition to AMU, husbandry practices, inadequate cleaning and disinfection, and the types of feed used may all contribute to AMR (7, 32).

In spite of the limited small sample size (12 farms), our study provides accurate baseline information on AMU, as well as on the dynamics of phenotypic and genotypic resistance among *E. coli* over the different stages of production in emerging pig and poultry production systems in southern Vietnam. We recommend that the future research should focus in the simultaneous detection of large numbers of AMR genes of greater concern, especially those coding for resistance to critically important antimicrobials and that are carried on 'mobile' genetic elements. We also recommend that longitudinal study should capture quantitative changes in the resistome in the sample matrix (as opposed to individual colonies). Although such technologies are currently very costly, they will probably become affordable in the near future. *E. coli* showed high prevalence of resistance against last line antimicrobial (colistin) as well as critically important antimicrobials (ciprofloxacin and gentamicin) for human medicine. We strongly

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386 recommend that these important antimicrobials should be restricted for treatment of clinical
387 disease, and not used for prophylaxis.

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397 **Conflict of interest**

398 The authors declare that they have no competing interests.

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Table 1: Amounts of antimicrobials (in mg per kg of live weight) present in commercial feeds and estimated consumption of in-feed antimicrobials in each of the 9 farms investigated (Details of antimicrobials in feed were not available for farms C2, P1 and P4). Period 1= Early phase of production; Period 2= Late phase of production.

Product No.	Period of use	Antimicrobial	Concentration of antimicrobial in feed (mg/kg)	Estimated consumption of in-feed antimicrobials								
				Chicken farms					Pig farms			
				C1	C3	C4	C5	C6	P2	P3	P5	P6
1	1 and 2	Avilamycin OR	10	28.5	28.5	28.5	57.0	28.5				
		Enramycin	10	28.5	28.5	28.5	57.0	28.5				
2	1 and 2	Enramycin	10		28.5	28.5	28.5					
3	1 and 2	Colistin* OR	180						702.0			
		Florfenicol OR	30						117.0			
		Kitazamycin OR	110						429.0			
		Tiamulin OR	120						468.0			
		Amoxicillin*	150						585.0			
4	1 and 2	Avilamycin OR	10						39.0			
		Kitasamycin OR	110						429.0			
		Tiamulin	120						468.0			
5	1 and 2	Tylosin OR	40							156.0		
		Enramycin OR	20							78.0		
		Colistin*	150							585.0		
6	1 and 2	Chlotetracycline OR	50							195.0		
		Neomycin OR*	65							253.5		
		Enramycin OR	20							78.0		
		Virginiamycin	10							39.0		
7	1 and 2	Chlotetracycline OR	50							95.0		
		Virginiamycin OR	10							39.0		
		Lincomycin	20							78.0		
8	1 and 2	Chlotetracycline OR	100								390.0	
		Bacitracin	50								195.0	
9	1	Tylosin OR	40									156.0
		Enramycin OR	20									78.0
		Amoxicillin*	300									1,170.0
10	1	Tylosin OR	40									156.0
		Enramycin OR	20									78.0
		Colistin*	150									585.0
11	1 and 2	Chlotetracycline OR	50									195.0
		Lincomycin OR	20									78.0
		Virginiamycin OR	10									39.0
		Enramycin	20									78.0

*Antimicrobials considered to be 'critically important' for human medicine according to WHO criteria.

513 **Table 2**: Amounts of antimicrobials (in mg per kg of live weight) administered for prophylactic and therapeutic purposes in chicken
514 and pig farms. Each line represents one antimicrobial component.

Antimicrobial agent	Antimicrobial class	Chicken farms						Pig farms					
		C1	C2	C3	C4	C5	C6	P1	P2	P3	P4	P5	P6
Neomycin*	Aminoglycoside							11.7					
Kanamycin*										11.1			
Gentamicin*						26.7			16.2	6.7		5.9	14.3
Amoxicillin	β-lactam				40.0	13.3				25.1		22.3	
Ampicillin										17.8			
Cephalexin	Cephalosporin						5.6						
Ceftiofur											9.5		
Tylosin	Macrolide	23.0					5.6		12.2				
Tilmicosin			6.8	10.0									
Spiramycin*									12.5				23.8
Chloramphenicol	Phenicol								6.5				
Florfenicol			11.3	16.7								11.1	
Colistin*	Polypeptide				5.3	1.8				1.4			3.3
Enrofloxacin	Fluoroquinolone				66.7		11.1			23.3	19.1		40.6
Norfloxacin*						53.3					1.8		4.9
Doxycycline	Tetracycline	46.0	5.6	8.3		26.7	5.6					5.5	
Oxytetracycline								11.7					
Total		69.0	23.7	35.0	112.0	121.8	27.9	23.4	47.4	85.4	30.4	44.8	86.9

515 *Antimicrobials considered to be 'critically important' for human medicine according to WHO criteria.

516 **Table 3**: Estimation of the level of agreement between resistance phenotype and genotype in *E.*
517 *coli* isolates.

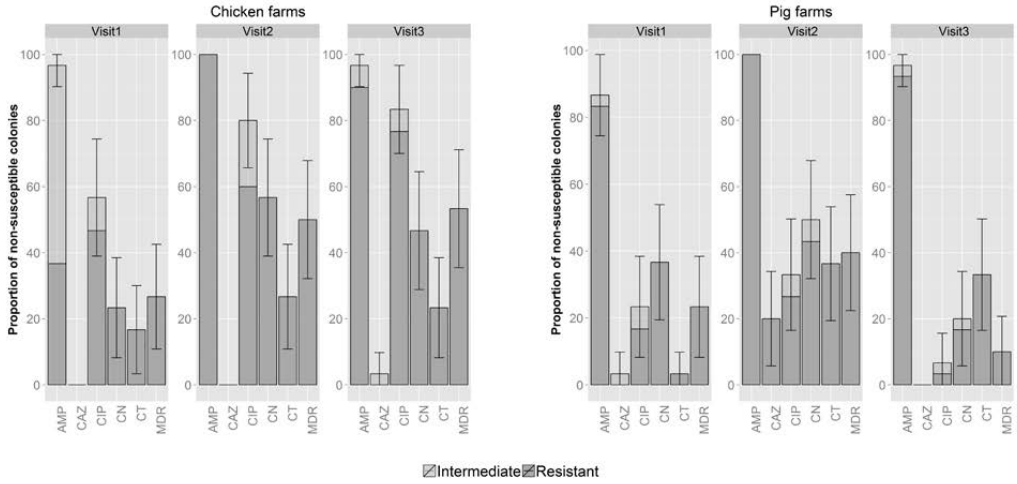
Antimicrobial	Gene carried	Chicken						Pig					
		κ	a	b	c	d	p_{value}	κ	a	b	c	d	p_{value}
Ciprofloxacin	*qnrA*	0.07	20	48	4	18	0.150	-0.07	2	17	8	63	0.835
	qnrB	N.C.	0	66	0	24	N.C.	N.C.	0	19	0	71	N.C.
	qnrS	0.02	19	50	5	16	0.368	0.12	6	13	33	38	0.127
	aac(6')-Ib-cr	N.C.	0	66	0	24	N.C.	0.15	2	17	0	71	0.055
	qepA	N.C.	0	66	0	24	N.C.	N/C	0	19	0	71	N.C.
	$gyrA_{single}$	0.01	22	44	8	16	0.50	0.24	7	12	10	61	0.012
	$gyrA_{double}$	0.43	39	27	0	24	<0.001	0.73	12	7	0	71	<0.001
Gentamicin	*aadA*	0.33	28	11	18	33	<0.001	0.13	29	3	43	15	0.030
	strA/B	0.26	26	13	17	34	<0.001	0.19	10	22	8	50	0.011
Colistin	*mcr-1*	1.0	20	0	0	70	<0.001	0.84	17	5	0	68	<0.001

518 a= positive genotype, positive phenotype; b= negative genotype, positive phenotype; c= positive
519 genotype, negative phenotype; d= negative genotype, negative phenotype; N.C.= not calculated.

520 **Table 4**: Associations between antimicrobial use and phenotypic and genotypic resistance
521 among *E. coli* isolates from pigs. Only significant (p< 0.05) risk ratios (RR) are presented.

Exposure	No. of farms using antimicrobial	Outcome	RR	95%CI	p_{value}
Exposure, antimicrobial use in Period 1; Outcome, resistance in second sampling					
Quinolones	3	CT	11.0	2.71-44.66	<0.001
Cephalosporins	1	CT	4.17	1.49-11.71	0.007
Quinolones	3	CIP	9.0	1.84-44.07	0.007
Cephalosporins	1	CIP	5.0	1.71-14.6	0.003
Cephalosporins	1	*strA/B*	5.0	1.34-18.61	0.016
Quinolones	3	*gyrA**	4.50	1.23-16.46	0.023
Cephalosporins	1	*gyrA**	4.17	1.49-11.71	0.007
Exposure, antimicrobial use over the whole production; Outcome, resistance in third sampling					
Tetracyclines	1	*gyrA**	10.0	2.71-36.96	<0.001
β-lactams	2	*gyrA**	5.0	1.02-24.52	0.047
Phenicols	1	*gyrA**	20.0	4.38-91.42	<0.001

522 CI= confidence interval; CIP= ciprofloxacin; CT= colistin; RR= Risk ratio.*Single or double mutation.



523

524 **Figure 1**: Prevalence of AMR among 180 *E. coli* (30 isolates/visit/host species) from 12 farms in
525 Tien Giang, Vietnam (2013-2014). The error bars indicate 95% confidence intervals. AMP=
526 ampicillin; CAZ= ceftazidime; CIP= ciprofloxacin; CN= gentamicin; CT= colistin; MDR=
527 multidrug resistance.
528

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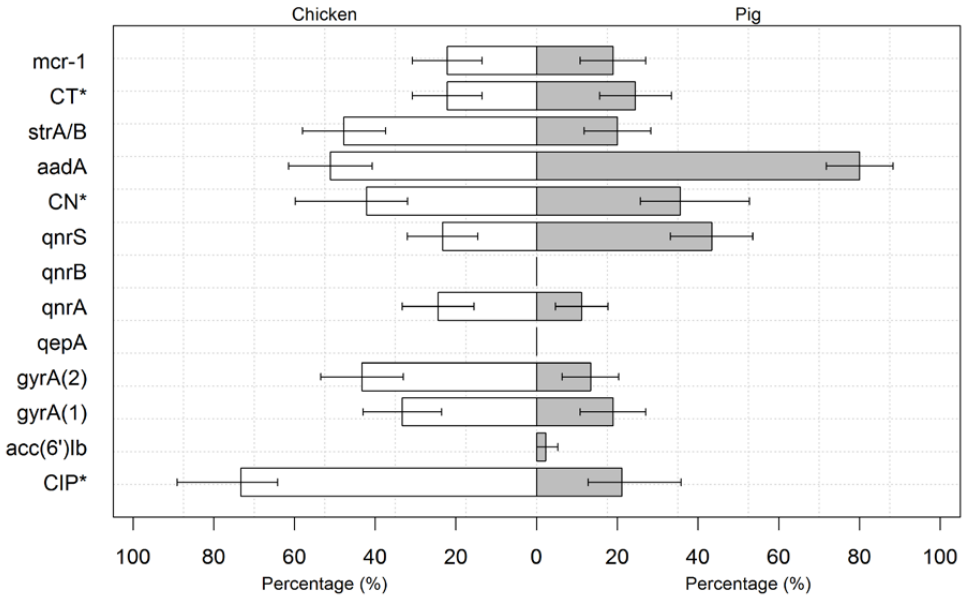

530

531 **Figure 2**: Prevalence of antimicrobial resistance genes among 180 *E. coli* isolates recovered
532 from 12 farms in Tien Giang, Vietnam (2013-2014). *Phenotypic prevalence of antimicrobial
533 resistance. CIP= ciprofloxacin; CN= gentamicin; CT= colistin; (1)= single mutation, (2)= double
534 mutation. The error bars indicate 95% CI.
535