Antimicrobial drug resistance at the human-animal interface in Vietnam

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Chapter 7: Zoonotic transmission of the *mcr-1* colistin resistance gene from non-intensive poultry farms in Vietnam

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CHAPTER 7

Abstract

We investigated the consequences of colistin use in backyard chicken farms in Vietnam, for the presence of mcr-1 in fecal samples from chickens and humans. Detection of mcr-1 in chicken samples was associated with colistin use, whereas mcr-1-carrying bacteria in humans was associated with exposure to mcr-1-positive chickens.

Keywords: colistin resistance, chicken, farmer, mcr-1, plasmid

CHAPTER 7

Introduction

Colistin resistance has emerged and has been increasing gradually [1]. In addition to the known chromosomally mediated resistance mechanisms, a plasmid-mediated colistin resistance gene named *mcr-1* was discovered in China and subsequently elsewhere around the world [2, 3]. The *mcr-1* gene was first detected in *Escherichia coli* and *Klebsiella pneumoniae* isolated from pigs, chicken, retail meat, pork and hospitalized patients, with the prevalence of 28% in chickens, in China. The discovery of the *mcr-1* gene was followed by a wealth of reports indicating its global geographic spread; its presence on various plasmids in multiple bacterial species originating from a variety of environmental and animal sources; and its likely circulation for at least several decades [3].

Usage of colistin in animal production has been suggested as the most likely contributing factor to the emergence of the *mcr-1* gene [2]. However, unbiased studies that demonstrate an epidemiological link between the use of colistin in agriculture and the prevalence of *mcr-1* carrying bacteria in the community are lacking. Such studies require an integrated one-health approach and are crucial for the design of interventions to control the spread of the *mcr-1* gene [4].

Whereas its use in humans is negligible [5], colistin is one of the most commonly used antimicrobial drugs in animal production in Vietnam, including household farms [6] which are common in developing countries worldwide and are characterized by low levels of investment and bio-containment [7]. We investigated the consequences of colistin usage in such non-intensive poultry farms for the prevalence of colonization with *mcr-1* carrying bacteria and the risk of onward transmission to humans by molecular epidemiological analyses in chickens, their farmers and unexposed populations in a defined geographical area of southern Vietnam.

Methods

Study population

This study was performed as a part of a cross-sectional observational study of antimicrobial drug usage and antimicrobial resistant *E. coli* colonization in household farms and the community [8]. The target population included non-intensive chicken farms and their farmers in three districts in

Tien Giang province, representing approximately 45% of the total chicken population in the Mekong Delta, southern Vietnam. A total of 204 chicken farms and 204 chicken farmers, defined as an adult person (≥ 18 years old) responsible for raising the chickens, who were not hospitalized in the previous 4 weeks, were randomly selected as described previously [8, 9]. In brief, sampling was stratified by farm size (10 – 200 chickens, 'household farms'; 201 – 2000 chickens, 'small size farms') and by district (My Tho city, Cho Gao district and Chau Thanh district) (total 6 strata). The sample size was calculated based on requirements for determining the prevalence of *E. coli* resistance against a number of different antimicrobials in each district. Age- and sex-matched individuals who are not involved in poultry farming were randomly selected from the same district as the farmers (N=204), as well as from the provincial capital (N=102) using the population census provided by the Preventive Medicine Centre (PMC) in Tien Giang [10].

Written informed consent was obtained from all participants prior to recruitment. The study was approved by the Department of Health in Tien Giang, the Peoples' Committee of Tien Giang Province, and the Oxford University Tropical Research Ethics Committee (OxTREC, No. 48/11).

Data collection

Data on human antimicrobial drug usage during the month prior to the study visit, including the product's commercial name, packaging format, dosage, and duration of usage, was collected for all participants as well as for all household members by medicine cabinet surveys, using a structured questionnaire containing both open and closed questions (supplementary material 1). Data on antimicrobial usage for chickens was similarly collected during interviews with the farmers, using a questionnaire as published previously [8]. The medicine cabinet survey has been shown to be efficient in getting data on antimicrobial drugs usage in the community [11]. Usage of an antimicrobial drug was defined as the reported usage in the previous month and/or the presence of the antimicrobial drug in the medicine cabinet. All questionnaires were administered by staff from Sub-Department of Animal Health (SDAH) and PMC for chicken and human antimicrobial usage, respectively.

Sample collection

To avoid seasonal effects, farm and household visits were evenly distributed over the period of March 2012 and April 2013. Faecal samples from chickens were collected using boot-swabs or hand-held gauze swabs, as described previously [8]. These sampling methods yield a faecal sample representative of the entire chicken population for each farm and thus do not provide information on individual chickens. Such a sampling approach is adequate given the limited confinement of the chicken in the farms [8]. Rectal swab samples from human participants were obtained using Faecalswab (Copan, Italy).

The sample collection was conducted by combined sampling teams from Tien Giang SDAH and PMC. Samples were stored and transported at 4^{o}C to the laboratory at the Oxford University Clinical Research Unit in Ho Chi Minh City and cultured within 24 hours after collection.

Sample analysis

Buffered peptone water (225 mL) was added to each chicken faecal sample in different container and was manually shaken. A volume of 1 mL from each container was diluted 1:1000 in saline solution. Human rectal swabs were vortexed to release and suspend the sample in the liquid transport medium and then 100 µL was diluted 1:100 in saline solution. This dilution resulted in growth of >100 separate colonies after a volume of 50 µL of each diluted sample was plated onto MacConkey agar without and supplemented with nalidixic acid [16 mg/L], ceftazidime [2 mg/L], and gentamicin [8 mg/L], and incubated overnight at 37^{0}C. A sweep from the full growth was collected and stored in glycerol at - 20^{o}C, after, five randomly selected *E. coli*-like colonies from MacConkey agar without antimicrobials and two from each of three antimicrobial-supplemented agars, were picked and sub-cultured for identification and antimicrobial susceptibility testing (AST). AST was performed using disc diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines and breakpoints (supplementary material 2) [12]. From each subject, all isolates with a unique phenotypic antimicrobial susceptibility pattern were stored for further analyses.

Detection of the *mcr-1* gene

To assess the prevalence of the *mcr*-1 gene in faecal samples, all MacConkey sweep samples were screened by conventional PCR as described previously [2]. The PCR-based *mcr-1* gene detection in a sweep sample is a targeted metagenomic approach to detect the gene within a sample enriched for viable Gram-negative microorganisms. Positive control *E. coli* DNA and negative controls were included in each run.

To subsequently confirm the presence of the *mcr-1* gene in *E. coli*, we determined the presence of the *mcr-1* gene in 200 *E. coli* isolated from chickens and humans, which were randomly selected from the total collection of 3160 stored *E. coli* isolates using PCR (supplementary material 2). To detect a potential association of the *mcr-1* gene with the presence of other emerging resistance mechanisms, in particular the production of extended spectrum beta-lactamases (ESBL), we additionally included 122 ESBL-producing *E. coli* isolates. These included 38 ESBL-producing *E. coli* isolated from chickens, which is the total number of ESBL-producing *E. coli* isolates found in chicken faecal samples in the study; and 84 ESBL-producing *E. coli* isolated from human samples, randomly collected and stratified by human sampling group. All *mcr-1* positive *E. coli* isolates were tested for colistin susceptibility using E-test and interpreted according to EUCAST breakpoints [13].

Characterization of *mcr-1*-positive *E. coli* isolates

Whole-genome sequencing was performed for all *mcr-1* positive *E. coli* isolates. Bacterial DNA was extracted from fresh pure cultures using either the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) or the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Library preparation was done in accordance with the manufacturer's instructions (Illumina, San Diego, CA, USA) and sequenced using Illumina MiSeq technology with 150 paired-end settings. Data was cleaned and analysed using an analysis pipeline as described in the Supplementary Material 2.

Data analyses

The prevalence of faecal colonization with *mcr-1* carrying bacteria was adjusted for the stratified survey design by assigning a stratum-specific sampling weight (supplementary material 2).

We built logistic regression models to investigate risk factors associated with faecal colonization with *mcr-1* carrying bacteria in chicken farms and human participants. The list of variables that were tested in the univariable analyses is shown in the Supplementary Material 3. Based on their biological plausibility and a p value <0.15 in the univariable analyses, variables were considered for multivariable analysis and were included using a step-wise forward approach [14]. Variables were retained in the final models if the p-value was <0.05. All interactions between final significant variables were tested. All statistical analyses were performed using R packages 'epicalc', 'survey' and 'adegenet' (http://www. r-project.org).

Results

Prevalence of faecal colonization with *mcr-1* carrying bacteria

Of a total of 204 chicken faecal samples, collected from 204 farms, and of 510 human faecal specimens, 188 and 440 MacConkey sweeps were available for *mcr-1* screening by PCR, respectively. The remaining sweep samples were either missing (16 chicken and 45 human samples), were not available because the primary faecal culture on MacConkey agar did not show any growth (7 human samples), or could not be tested because the culture after storage did not show any growth (18 human samples). Characteristics of farms and human participants for which a sweep sample was available were similar when compared to characteristics for farms and participants without sweep sample (supplementary material 4).

The adjusted prevalence of faecal colonization with *mcr-1* carrying bacteria was 59.4% (95% CI 47.9 – 71.0) in chicken faecal samples and 20.6% (95% CI 15.9 – 25.2) in human samples (Table 1), with much higher prevalence in farmers and rural individuals (25.2 and 17.6%, respectively) than in individuals living in the city (9.1%). The adjusted prevalence for small scale and household size farms was similar (Table 1).

Among 200 randomly selected *E. coli* isolates, *mcr-1* was detected in 10/78 (12.8%) chicken *E. coli* isolates, in 2/50 (4.0%) *E. coli* isolates from faecal samples of farmers and in none of 72 *E. coli* isolates from non-farming individuals. Similarly, *mcr-1* was detected in 9/38 (23.7%) and 1/44 (2.3%) of ESBL-producing *E. coli* isolated from chickens and farmers respectively.

Table 1. Prevalence of faecal colonization with *mcr-1*-carrying bacteria in chicken and humans in Tien Giang Province,Vietnam (2012 – 2013)

Source	Prevalence of faecal colonization with *mcr-1*-carrying bacteria	
	No of positive sweeps/ Total (%)	Adjusted prevalence (95% CI)
All chicken farms	93/188 (49.5)	59.4 (47.9 – 71.0)
Household chicken farms	53/94 (56.4)	59.5 (47.9 – 71.1)
Small scale chicken farms	40/94 (42.6)	47.9 (35.4 – 60.3)
All human participants	84/440 (19.1)	20.6 (15.9 – 25.2)
All farmers	45/179 (25.1)	25.2 (18.3 – 32.0)
Farmer exposed to *mcr-1*-negative chicken	16/91 (17.6)	15.5 (7.7 – 23.3)
Farmer exposed to *mcr-1*-positive chicken	29/88 (33.0)	34.7 (23.9 – 45.5)
Rural persons	31/173 (17.9)	17.6 (11.6 – 23.7)
Urban persons	8/88 (9.1)	9.1 (3.1 – 15.1)

The usage of colistin

Colistin was used in 39/204 (19.1%) of the chicken farms. *mcr-1* carrying bacteria were detected in 23/39 (59.0%) chicken samples obtained from farms that used colistin compared with 70/149 (47.0%) of chicken samples from farms where colistin was not used (p=0.25). None of the human participants reported the use of colistin (supplementary material 5)

Characterization of *mcr-1* carrying isolates

The 22 *mcr-1*-carrying *E. coli* isolates all showed reduced susceptibility to colistin (MIC 3-4 mg/L) and belonged to 13 different sequence types (STs) as determined by MLST (Table 2). The most frequent sequence type was ST156 including 5 isolates from chicken samples of 4 different farms. SNPs-based phylogeny of the core genomes showed little genomic similarity between isolates except for isolates belonging to the same MLST sequence type (Figure 1). Analysis of the acquired resistance genes, reflecting the accessory genome, showed a large variation in resistance gene content, with only the *tet(A)* gene, coding for tetracycline resistance, present in all genomes (supplementary material 6). Carbapenemase-encoding genes were not detected.

The *mcr-1* gene was located in genomic contigs (partially assembled plasmid sequences) which sizes ranged from 2761 to 120156 bp (average: 31018 bp). No other antibiotic resistance genes could be identified on the *mcr-1* carrying contigs. A replication origin could be located for 5

contigs, allowing identification of the plasmid incompatibility groups IncHI2 (1 isolate), IncI2 (2 isolates), and combined IncHI2 and IncHI2A (2 isolates).

The sequence of the transposon ISAplI, initially described as carrying the *mcr-1* gene, was identified in 18 out of 22 contigs carrying the *mcr-1* gene, with 100% similarity to the published sequence [2].

Risk factors associated with faecal colonization with *mcr-1* carrying bacteria

We investigated risk factors for faecal colonization with *mcr-1* carrying bacteria separately for small-scale and household farms since a joint model resulted in convergence. This convergence was mainly due to the inflated sampling weight that was assigned to the household chicken farms. Results of univariable analysis of risk factors are shown in supplementary material 3. Multivariable analysis identified the presence of chicken less than 20.5 weeks old and the usage of colistin as independent risk factors for faecal colonization with *mcr-1*-carrying bacteria in chickens (OR=21.3 and OR=5.8, respectively) in small-scale farms (Table 2). We were unable to identify the potential risk factor associated with faecal colonization with *mcr-1* carrying bacteria in household farms.

Among human participants, farmers who were exposed to *mcr-1* positive chicken showed a significantly increased risk of colonization with *mcr-1*-carrying bacteria (OR=5.3, Table 3) compared to urban individuals not involved in chicken farming, in contrast to rural individuals not exposed to chicken or farmers with *mcr-1* negative chickens.

Figure 1: Phylogenetic analyses of *mcr-1*-positive *E. coli* isolated from chicken farms and farmers in Vietnam (2012 – 2013)

Maximum likelihood tree for 22 mcr-1-carrying E. coli isolated from 15 chicken faecal samples and 3 human faecal swab samples based on a whole genome SNPs comparison using CSI Phylogeny 1.2, with E. coli SE15 as the reference strain. Red color indicates isolate from farmer; blue color indicates isolates from chicken. A total of 74585 SNPs were concatenated for pairwise comparison (difference between pairs: 0 to 32267). Branch length corresponds to the number of nucleotide substitutions per site. Corresponding multi-locus sequence types (ST) are indicated next to the isolates' names. Circles indicate isolates sharing the same ST. Isolates pairs CG05C.C1/CG05C.C2 (1 SNP difference), CG48C.A2/CG48C.G2 (1 SNP and 1 antimicrobial resistance gene difference), CT48C.C1/CT48C.C2 (4 SNPs and 3 antimicrobial resistance genes difference) and CT67C.C1/CT67C.C2 (0 SNPs difference) are phenotypically different but originate from the same sample and are therefore likely to be highly related or identical.

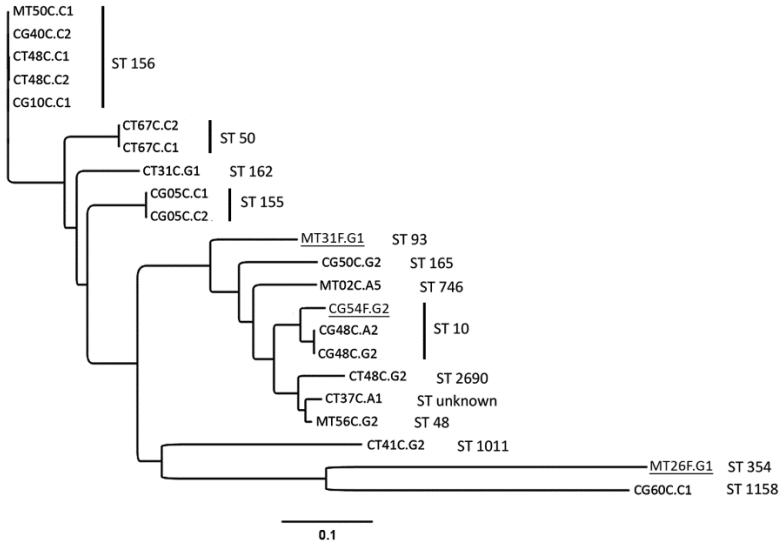

Table 2. Risk factors associated with faecal colonization with *mcr-1*-carrying bacteria in small-scale chicken farms (N = 94) and in humans (N = 440) in Vietnam (2012 – 2013)

Variables	Number of subjects	Number of *mcr-1* positive subjects	OR	95% CI	p-value
Small scale chicken farms (N = 94)*					
Age of the chickens					
< 20.5 weeks old	47	32	21.3	5.8-78.5	<0.001
≥ 20.5 weeks old	47	8	ref	ref	ref
Usage of colistin	21	14	5.1	1.4-18.8	0.017
Humans (N = 440)†					
Participant group					
Urban persons‡	88	8	ref	ref	ref
Rural persons‡	173	31	2.1	0.9-5.0	0.075
Farmers exposed to *mcr-1* negative chicken	91	16	1.8	0.7-4.7	0.205
Farmers exposed to *mcr-1* positive chicken	88	29	5.3	2.2 – 12.7	<0.001

*Intercept: - 2.15(SEM±0.57)

†Intercept: - 2.3(SEM±0.37)

‡ Not involved in poultry farming

CHAPTER 7

Discussion

We show that colonization with *mcr1* carrying bacteria of chickens is associated with colistin usage while colonization of humans is associated with exposure to *mcr-1* positive chickens. These findings suggest that the usage of colistin is the main driver for the observed very high *mcr-1* prevalence (59.4%) in faecal samples from chickens, with onward zoonotic transmission explaining the high prevalence (25.2%) in farmers. While our cross-sectional study design may preclude the demonstration of direct transmission of the *mcr-1* gene between chickens and humans, such transmission was reported for colistin resistant *E. coli* from a domesticated pig to humans [15, 16] as well as between companion animals and humans [17].

We found that younger chickens were more likely to be colonized with *mcr-1* carrying bacteria. This observation may be explained by the more intensive usage of antimicrobial drugs in younger chicken as indicated by the higher median of treatment incidence in young chickens (74.0; IQR [0 - 278]) compared to older chickens (46.3; IQR [0 - 124]). However, our study was insufficiently powered to detect such association in multivariable analysis. In addition, the gastrointestinal tract of younger chickens may be colonized by antimicrobial resistant bacteria more readily compared to older chickens [18]. Data from a previous study showed that the *mcr-1* gene could be detected even in one-day-old chicks which suggests a possibility of vertical transmission from chicken parent flocks [19].

The prevalence of *mcr-1*-carrying *E. coli* isolates ranged from 12.8% to 23.7% in chicken faecal samples and from 2.3% to 4.0% in human samples. The lower detection rate of the *mcr-1* gene in *E. coli* isolates compared to sweep samples may indicate the presence of this gene in *E. coli* strains other than those selected or bacterial species other than *E. coli* capable of growing on MacConkey agar. The percentage of *mcr-1*-carrying *E. coli* isolates in chickens in our study was similar to recently published data from Vietnam and China, but much higher than data from other countries such as Japan (0%), France (1.8%) and Brazil (3.0%) [2, 20-22]. However, such comparisons should be interpreted with caution because of differences in sample size and the number of *E. coli* isolates selected per sample, as well as sampling methods.

Mcr-1-positive isolates in our study also carried multiple other resistance genes, including genes encoding for extended-spectrum beta-lactamases (supplementary material 6). The proportion of

isolates carrying *mcr-1* gene among ESBL-producing *E. coli* was similar to a random selection of *E. coli* (p=0.22), indicating that the *mcr-1* gene is widespread among *E. coli* isolated from chickens independent of the presence of ESBL genes.

We found that the *mcr-1* gene was associated with at least three plasmid backbones belonging to the incompatibility groups IncI2, IncHI2 and IncHI2A which were also discovered in previous studies [2, 20, 23, 24]. The spread of the *mcr-1* gene on different plasmid types might explain its successful spread in different *E. coli* clones. Additionally, we identified the presence of the ISApl1 transposon in 81.8% (18/22) of our isolates. As this genetic element is involved in horizontal gene transfer [25] it is likely to be a key factor in explaining the presence of the *mcr-1* gene in different plasmid types identified within a limited time frame in a restricted geographic area.

Given the potentially serious consequences of the spread of the *mcr-1* gene from food-producing animals to humans and the association between usage of colistin in animals and the presence of *mcr-1*, prudent usage of antimicrobial drugs in animal production should be enforced globally, including in non-intensive farming settings such as 'backyard' farms, the most common farming setting worldwide.

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