Prevalence of *qnrS*-positive *Escherichia coli* from chicken in Thailand and possible co-selection of isolates with plasmids carrying *qnrS* and trimethoprim-resistance genes under farm use of trimethoprim

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ABSTRACT One hundred and twenty chicken samples from feces (n = 80), the carcass surface at slaughter at 2 meat chicken farms (n = 20), and retail chicken meat from 5 markets (n = 20) collected during 2018 and 2019 were examined for the prevalence of plasmid-mediated quinolone resistance (**PMQR**) in *Escherichia coli*. We detected *qnrS*-positive *E. coli* in a total of 74 samples from feces (n = 59), the carcass surface (n = 7), and retail meat (n = 8). These 74 *qnrS*-positive isolates were tested for antimicrobial susceptibility to determine the minimum inhibitory concentrations (**MICs**) of certain antimicrobials and genetically characterized. Ampicillin-resistance accounted for 71 of the 74 isolates (96%), followed by resistance to oxytetracycline (57/74; 77%), enrofloxacin (**ERFX**) (56/74; 76%), sulfisoxazole (**SUL**) (56/74; 76%), trimethoprim (**TMP**) (49/74; 66%), and dihydrostreptomycin (48/74; 65%). All farm-borne SUL- and TMP-resistant isolates except one were obtained from samples from farm A where a combination of sulfadiazine and TMP was administered to the chickens. Concentrations of ERFX at which 50 and 90% of isolates were inhibited were 2 μg/mL and 32 μg/mL, respectively. Diverse pulsed-field gel electrophoresis (**PFGE**) patterns of *Xba*I-digested genomic DNA were observed in the *qnrS*-positive isolates from fecal samples. Several isolates from feces and the carcass surface had identical *Xba*I-digested PFGE patterns. S1-nuclease PFGE and Southern blot analysis demonstrated that 7 of 11 *dfrA13*-positive fecal isolates carried both the *qnrS* and *dfrA13* genes on the same plasmid, and 2 of 3 *dfrA1*-positive isolates similarly carried both *qnrS* and *dfrA1* on the same plasmid, although the PFGE patterns of *Xba*I-digested genomic DNA of the isolates were different. These results suggest that the *qnrS* gene is prevalent in chicken farms via horizontal transfer of plasmids and may partly be co-selected under the use of TMP.

Key words: chicken, *Escherichia coli*, plasmid-mediated quinolone resistance, *qnrS*, trimethoprim

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INTRODUCTION

Antimicrobial resistance occurs as a result of antimicrobial usage and is a global problem to human and animal health (McEwen and Collignon, 2018). The inappropriate use of antimicrobial medications important to humans in animals and the in-feed use of these drugs are major concerns. Quinolones, including fluoroquinolones are categorized as "highest priority critically important" antimicrobials for human medicine (WHO, 2019) and have been used in food animals in many countries (Collignon et al., 2016; Roth et al., 2019), including poultry in Thailand (Nhung et al., 2016; Wangroongsarb et al., 2021).

Although chromosomal mutations in topoisomerase genes are well-recognized for quinolone resistance, plasmid-mediated quinolone resistance (**PMQR**) was first described in 1998 in a clinical isolate of *Klebsiella pneumoniae* (Martínez-Martínez et al., 1998) and additional PMQR genes in *Enterobacteriaceae* have been found in isolates from various species worldwide (Jacoby et al, 2014). However, PMQR potentially facilitates the selection of higher levels of quinolone resistance in the presence of quinolones (Poirel et al., 2006; de Toro et al., 2010; Nishikawa et al., 2019). PMQR genes in *Salmonella* isolates from chickens (Cavaco et al., 2007; Sinwat et al., 2015) and pigs (Luk-In et al., 2017) in

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Thailand have been reported. To the authors' knowledge, however, data are unavailable for PMQR genes in *Escherichia coli* from chickens, although isolation of quinolone-resistant *E. coli* has been reported in Thailand (Chaisatit et al., 2012; Trongjit et al., 2016). Because *E. coli* is a member of the normal microflora of the poultry intestine, the organism may reflect selection pressure due to antimicrobial use on farms (Ozaki et al, 2011).

To clarify prevalence of PMQR and several important antimicrobials categorized by WHO (2019) among *E. coli* isolates of meat chicken origin in Thailand, the antimicrobial susceptibility of isolates obtained from both the feces and swabs of carcass surfaces at slaughter from 2 chicken farms as well as samples from retail chicken meat were determined. Because a combination of sulfadiazine and trimethoprim (**TMP**) was used at one of the participating farms, possible association of the use of the drug and the prevalence of the PMQR gene was investigated using genetic analyses.

MATERIALS AND METHODS

Sample Collection

Cloacal swabs were obtained from 50 chickens in August 2018 and 20 chickens in March 2019 at farm A, and 10 chickens in March 2019 at farm B. A combination of sulfadiazine and TMP was orally administered to the chickens at farm A; the indication and dose were not available. In the slaughter facilities situated in close proximity to each farm, the surfaces of 10 carcasses each from farms A and B were sampled in August 2018 and March 2019, respectively, by rubbing with cotton applicators over a total area of 5 × 5 cm. Twenty pieces of chicken meat were obtained from 3 supermarkets and 2 fresh markets in July 2018, and 2 supermarkets and 2 fresh markets in May 2020. All the samples were kept at 4°C and during transportation to the laboratory for 4 to 6 h.

Bacterial Isolates

Cloacal swab samples were suspended in saline and a loopful of suspension was spread onto deoxycholate hydrogen sulfide lactose (**DHL**) agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with or without 0.05 μg/mL enrofloxacin (**ERFX**) and incubated at 37°C for 20 h. Each cotton applicator that was applied to a carcass was immersed in 3 mL heart-infusion broth and incubated at 37°C for 20 h. Approximately 3 g of each meat sample was added to 30 mL of heart-infusion broth and incubated as described above. The broth cultures were plated onto DHL agar and incubated as above. Two potential colonies per plate were picked and identified as *E. coli* using polymerase chain reaction (**PCR**) targeting the beta-glucuronidase gene (*uidA*) (McDaniels et al., 1996).

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (**MIC**s) of ERFX were determined using an agar dilution method based on Clinical Laboratory Standards Institute (**CLSI**) document M7-A8 (Clinical Laboratory Standards Institute 2009). *E. coli* ATCC 25922 was used for quality control. MICs have been interpreted using previously defined resistance breakpoints (2 μg/mL) (Kojima et al., 2009). One isolate harboring the *qnrS* gene (see RESULTS) was arbitrarily selected from each sample set. For these representative isolates, the MICs of other antimicrobials were determined based upon the following resistance breakpoints: ampicillin (**AMP**), 32 μg/mL; ceftiofur (**CTF**), 8 μg/mL; dihydrostreptomycin (**DSM**), 32 μg/mL; oxytetracycline (**OTC**), 16 μg/mL; chloramphenicol (**CHL**), 32 μg/mL; and trimethoprim (**TMP**), 16 μg/mL. For sulfisoxazole (**SUL**), 512 μg/mL was adopted as the breakpoint according to CLSI document M100-S20 (Clinical Laboratory Standards Institute 2010).

PCR Detection of Antimicrobial Resistance Genes

Isolates exhibiting an EFRX MIC >0.25 μg/mL were screened for eight PMQR genes using multiplex (*qnrD* and *oqxAB*) or simplex (*qnrS, qnrA, qnrB, qnrC, qepA*, and *aac(6')-Ib-cr*) PCR (Park et al., 2006; Robicsek et al., 2006b; Chmelnitsky et al., 2009; Ciesielczuk et al., 2013). For isolates resistant to TMP, 5 genes (*dfrA1, dfrA5, dfrA7, dfrA9*, and *dfrA13*) responsible for this resistance were screened using PCR with primer pairs described by Maynard et al. (2004). For isolates exhibiting CTF MICs above the breakpoint (8 μg/mL), PCR detection of CTX-M-type beta-lactamase genes was performed using specific primer sets (Saishu et al., 2014).

Pulsed-Field Gel Electrophoresis

Isolates positive for the *qnrS* gene were subjected to *Xba*I-digested pulsed-field gel electrophoresis (**PFGE**) as previously described (Ozaki et al., 2011).

Plasmid DNA Analysis

The chromosomal or plasmid location of *qnrS* and TMP-resistant genes was determined in isolates with antimicrobial resistance. Southern blot analysis was performed using S1 nuclease-digested genomic DNA of selected isolates separated by PFGE according to previously described methods (Shahada et al., 2011). DNA from the PFGE gel was transferred onto a Hybond-N+ membrane (Amersham Biosciences UK Ltd., Little Chalfont, UK) and PCR-amplified *qnrS, dfrA1, dfrA13*, or CTX-M group 4 beta-lactamase gene fragments from each of the *qnrS*-, *dfrA1*-, *dfrA13*-, or CTX-M group 4 beta-lactamase gene-positive isolates were labeled with digoxigenin using a DIG High Prime Labeling and Detection Starter Kit (Roche Diagnostics Corp., Indianapolis, IN) and used as a specific probe for each gene.

Statistical Analysis

Differences in the isolation rate of *qnrS*-positive *E. coli* between plates supplemented with and without 0.05 μg/mL ERFX were evaluated by application of chi-square test. Fisher's exact test was used to evaluate the prevalence of antimicrobial-resistant *E. coli* in fecal samples between each of the participating farms. Differences were considered significant at $P < 0.05$.

RESULTS

Isolation Rates of qnrS-Positive E. coli Using Plates Supplemented With or Without ERFX

Among the PMQR genes tested, only *qnrS* was detected in *E. coli* isolated from the samples. Of the 40 retail meat samples, *qnrS*-positive *E. coli* were isolated from 21 and 14 samples using DHL plates supplemented with and without 0.05 μg/mL ERFX, respectively (Table 1), and the isolation rate between each of the plates was not significantly different. In 9 samples, *qnrS*-positive *E. coli* were isolated from both ERFX-supplemented and nonsupplemented DHL plates. Additionally, *qnrS*-positive *E. coli* were isolated from 28 and 35 fecal samples collected in August 2018 using DHL plates supplemented with and without ERFX, respectively, and the isolation rate between each of the plates was not significantly different. Thus, DHL plates without ERFX were used thereafter for isolation of PMQR *E. coli* from fecal samples.

Prevalence of Antimicrobial Resistance Among qnrS-Positive E. coli Obtained During 2018 and 2019

A total of 74 samples obtained during 2018 and 2019 yielded *qnrS*-positive *E. coli* isolates (Table 2). One *qnrS*-positive isolate was arbitrarily selected from each sample set. A total of 74 *qnrS*-positive isolates were subjected to antimicrobial susceptibility tests using 8 drugs and genetic analysis. AMP-resistance was determined in 71 of the 74 isolates (96%). Resistance to other antibiotics included OTC (57/74; 77%), ERFX (56/74; 76%), SUL (56/74; 76%), TMP (49/74; 66%), and DSM (48/74; 65%). The rate of *qnrS*-positive *E. coli* isolates from fecal samples from farm A to isolates resistant to each antimicrobial was similar between the 2 sampling times. The total prevalence of resistance to DSM, SUL, and TMP among fecal isolates from farm A were significantly ($P < 0.05$) higher than those obtained among isolates from farm B (Table 2). We isolated *qnrS*-positive *E. coli* from 65 to 80% of the fecal samples collected at the 2 farms. Among the isolates, the ERFX MIC ranged from 0.5 to 64 μg/mL and concentrations at which 50% and 90% of isolates were inhibited (ERFX MIC50 and ERFX MIC90) were 2 μg/mL and 32 μg/mL, respectively. Although less than 50% of either the carcass surface samples yielded *qnrS*-positive *E. coli*, the ERFX MIC ranged from 0.5 to 16 μg/mL, ERFX MIC50 and ERFX MIC90 were 8 and 16 μg/mL, respectively. Seven of 8 *qnrS*-positive isolates from retail meat samples exhibited EFRX MICs of 1 or 2 μg/mL, with the MIC for the remaining isolate at 16 μg/mL.

Table 1. Isolation of plasmid-medicated quinolone resistance (PMQR)-positive *Escherichia coli* (*E. coli*) from DHL plates with or without enrofloxacin (ERFX).

	Number of samples that yielded *qnrS*-positive *E. coli*		
DHL[1] plates supplemented with ERFX	Retail meat (n = 40)	Carcass surface (n = 20)	Fecal samples collected in Aug 2018 (n = 50)
Yes	21	5	28
No	14	5	35
Both plates	9	3	25

[1]DHL, deoxycholate hydrogen sulfide lactose.

Antimicrobial Resistance Genes Other Than qnrS Among E. coli Isolates Obtained During 2018 and 2019

Of the 45 total TMP-resistant *E. coli* isolates from farm A, *dfrA1* and *dfrA13* were detected in 4 and 13 isolates, respectively, including one isolate from a carcass surface. Additionally, an isolate from retail meat was positive for the *dfrA13* gene and another isolate was resistant to CTF (MIC, 128 μg/mL) and harbored the CTX-M group 4 beta-lactamase gene.

qnrS-Positive E. coli Isolates Obtained From Retail Meat Samples in 2020

Eighteen *qnrS*-positive isolates obtained from retail meat samples in May 2020 were tested for antimicrobial susceptibility using only a limited number of drugs, and 4, 6, and 13 isolates were resistant to CTF, ERFX, and TMP, respectively. The ERFX MIC ranged from 0.5 to 64 μg/mL, and ERFX MIC50 and ERFX MIC90 were 1 and 32 μg/mL, respectively.

XbaI-Digested PFGE Patterns of qnrS-Positive E. coli Isolates

PFGE analysis revealed highly diverse patterns of *qnrS*-positive *E. coli* isolates obtained from farms A and B, although several isolates from each farm showed identical patterns, respectively (Supplementary Figure 1). Additionally, PFGE patterns of *qnrS*-positive *E. coli* isolates obtained from carcass surface samples from farm B were indistinguishable from those observed in a *qnrS*-positive fecal isolate from farm B. PFGE patterns of the retail meat isolates were different from each other.

Table 2. Prevalence of *qnrS*-positive *Escherichia coli* (*E. coli*) isolates and antimicrobial resistance of PMQR-positive isolates.

					No. of *qnrS*-positive isolates resistant to[2]:							
Sample type	Source	Sampling date	No. of samples	*qnrS*-positive[1]	AMP	CTF	DSM	CHL	OTC	ERFX	SUL	TMP
Feces	Farm A	Aug 2018	50	38	36	0	27[a]	12	35	30	37[a]	34[a]
	Farm A	Mar 2019	20	13	13	0	11[a]	4	13	10	13[a]	11[a]
	Farm B	Mar 2019	10	8	8	0	3[a]	0	6	5	0[a]	0[a]
Carcass surface	Farm A	Aug 2018	10	2	2	0	2	1	2	2	2	2
	Farm B	Mar 2019	10	5	5	0	1	1	1	4	1	1
Retail meat	Markets	Jul 2018	20	8	7	1	4	0	0	5	3	2
		May 2020	20	18	ND[3]	4	ND	ND	ND	6	ND	13

Abbreviations: AMP, ampicillin, CHL, chloramphenicol; CTF, ceftiofur; DSM, dihydrostreptomycin; ERFX, enrofloxacin; No., number; OTC, oxytetracycline; PMQR, plasmid-mediated quinolone resistance; SUL, sulfisoxazole; TMP, trimethoprim.

[a]Significant ($P < 0.05$) differences in the prevalence of antimicrobial-resistant *E. coli* in fecal samples between farms A and B.

[1]No. of samples that yielded *qnrS*-positive *E. coli*.

[2]One isolate from each sample set was subjected to antimicrobial susceptibility testing (see MATERIALS AND METHODS).

[3]Not done.

Plasmid DNA Analysis Using S1 Nuclease-Digested PFGE and Southern Blot Hybridization

The S1 nuclease-digested PFGE patterns of *qnrS*- and *dfrA13*-positive *E. coli* isolates contained various plasmids ranging from less than 50 kilobase pairs (kbp) to approximately 200 kbp (Table 3 and Supplementary Figures 2 and 3). Southern blot hybridization revealed that plasmids of approximately 200 kbp detected in 7 of the 11 *dfrA13*-positive fecal isolates from farm A that carried both the *qnrS* and *dfrA13* genes, although PFGE patterns of *Xba*I-digested genomic DNA of the 7 isolates differed from each other (Supplementary Figure 1). In 3 isolates from fecal samples, these genes were located on separate plasmids (Table 3 and Supplementary Figure 2). Plasmid DNA from the remaining isolate (Supplementary Figure 2, lane 23) was degraded and hybridization was not detected. Plasmids in the isolates obtained from retail meat and carcass surface samples from farm A carried both genes, although the sizes were not identical to those in the fecal isolates. In2 of 3 *dfrA1*-positive isolates, both the *qnrS* and *dfrA1* genes were located on the same plasmid (Table 3 and Supplementary Figure 3). Additionally, a retail meat isolate carried the CTX-M- group 4 beta-lactamase gene and *qnrS* on the same plasmid (Table 3 and Supplementary Figure 3).

DISCUSSION

The occurrence of *qnrS* in the present study is higher than that in previous reports on this gene in *E. coli* from chicken and meat originating worldwide (Li et al., 2014; Niero et al., 2018; Nishikawa et al., 2019; Seo and Lee, 2019). Additionally, in this study, diverse PFGE patterns were found in the *E. coli* isolates from fecal samples, suggesting that *qnrS*-positive *E. coli* were prevalent on the farms. Interestingly, the sizes of plasmids carrying the *qnrS* gene were similar in isolates with different PFGE patterns from farm A. These results were possibly due to transmission of the plasmids carrying this gene among *E. coli* at this farm, although detailed

Table 3. Plasmid location of antimicrobial resistance genes in *qnrS*-positive *Escherichia coli* isolates.

				Estimated size (kilo base pairs) of plasmid harboring:[1]			
Isolate no.	Sample type	Source	Sampling date	*qnrS*	*dfrA13*	*dfrA1*	CTX-M group 4[2]
1	Retail meat	Market	Jul 2018	150	150		
12	Feces	Farm A	Aug 2018	200	200		
19	Feces	Farm A	Aug 2018	200	200		
24	Feces	Farm A	Aug 2018	<49	50		
25	Feces	Farm A	Aug 2018	200	200		
28	Feces	Farm A	Aug 2018	<49 and 50	120		
30	Feces	Farm A	Aug 2018	200	200		
33	Feces	Farm A	Aug 2018	200	200		
45	Feces	Farm A	Aug 2018	200	200		
17	Carcass surface	Farm A	Aug 2018	60	60		
23	Feces	Farm A	Aug 2018	ND	ND		
49	Feces	Farm A	Mar 2019	200	200		
54	Feces	Farm A	Mar 2019	50	110		
10	Feces	Farm A	Aug 2018	80		80	
29	Feces	Farm A	Aug 2018	80		80	
38	Feces	Farm A	Aug 2018	210		ND	
4	Retail meat	Market	Jul 2018	250			250

[1]The size of plasmid harboring antimicrobial resistance genes was estimated by pulsed-field gel electrophoresis of S1 nuclease-digested genomic DNA of *Escherichia coli* and Southern blot hybridization with a probe prepared from the polymerase chain reaction amplicon using primer pairs specific for each of the genes (see Supplementary Figures 2 and 3).

[2]CTX-M group 4 beta-lactamase gene

characterization of these plasmids would be necessary to confirm this. More than half of the *qnrS*-positive *E. coli* isolates obtained from fecal samples demonstrated low-level resistance to quinolones as demonstrated by the ERFX MIC50 for these isolates being 2 μg/mL. The *qnrS* gene encodes protein QnrS that have been shown to protect *E. coli* DNA gyrase from quinolone inhibition at low concentrations (Jacoby et al., 2014). However, the selective pressure of fluoroquinolones may result in elevated resistance, which is caused by mutations to *gyrA* (Poirel et al., 2006; de Toro et al., 2010). Further studies for the isolates in this study are necessary to elucidate possible association of the presence of the *qnrS* gene with additional mechanisms for elevated resistance to fluoroquinolones including sequence analysis of the *gyrA* gene.

The presence of a low concentration (0.05 μg/mL) of ERFX in DHL agar plates was unlikely effective for isolation of *qnrS*-positive isolates because the isolation result from plates supplemented with and without ERFX for retail meat samples were contrary to that for fecal samples. High prevalence of a variety of *qnrS*-positive *E. coli* isolates on the participating farms may partly be a possible reason for the results for fecal samples that differences in the isolation rate between plates with and without ERFX were not significant.

PFGE patterns of *qnrS*-positive isolates from the carcass surface and fecal samples were identical to each other, suggesting that the carcasses may be contaminated with intestinal contents via meat processing. Moreover, *qnrS*-positive *E. coli* were isolated from retail meat samples, although it was not possible to trace whether the meat products from the farms participating in this study were sold in the markets where meat samples were collected. The prevalence of *qnrS*-positive *E. coli* in retail chicken meat obtained in May 2020 was almost twice what it was in July 2018. Sinwat et al. (2015) have reported that 5 of 80 *Salmonella* isolates from chicken meat collected from 2010 to 2013 in Thailand harbored the *qnrS* gene. Thus, continuous monitoring for contamination of chicken meat with *Enterobacteriaceae* carrying this gene is necessary. The present study additionally demonstrated that an isolate from a retail meat sample carried both *qnrS* gene and CTX-M group 4 beta-lactamase gene which is one of the genes encoding extended-spectrum beta-lactamases (**ESBL**s), on the same plasmid, although the source of the isolate was unknown. Plasmids carrying PMQR genes occasionally have genes encoding ESBLs, causing co-selection and therapeutic concerns (Robicsek et al., 2006a; Jacoby et al., 2014).

High prevalence of SUL- and TMP-resistance in isolates obtained from fecal samples from farm A might be associated with the use of the combination of sulfadiazine and TMP at this farm. Several isolates from fecal samples at this farm carried both a TMP-resistance gene (*dfrA1* or *dfrA13*, encoding dihydrofolate reductases) and *qnrS* on the same plasmid, suggesting that the *qnrS* gene may be partly co-selected under these conditions. Because more than 30 genes conferring resistance to TMP have been identified (Wüthrich et al., 2019) and only 4 of these were examined in this study, other genes not investigated here may be involved in TMP resistance in the isolates. The co-existence of TMP-resistance and PMQR genes, including *qepA* in *E. coli* of feline origin (Chen et al., 2014), *qnrB6* in *Klebsiella pneumoniae* and *Citrobacter freundii* of canine origin (Ma et al., 2009), and *qnrS* in avian pathogenic *E. coli* from broiler chickens (Yoon et al., 2020) have been reported. Chen et al. (2014) demonstrated that multidrug resistant plasmids harboring the *qepA* gene had disseminated in *E. coli* isolates from companion animals, food animals, and farm environments in China. In the present study, the prevalence of SUL- and TMP-resistance was low in *E. coli* isolates from farm B, although *qnrS*-positive isolates were obtained from this farm at a rate comparable to that in farm A. Thus, the prevalence of *E. coli* with PMQR may be caused by the use of quinolones in poultry in Thailand (Nhung et al., 2016; Wangroongsarb et al., 2021) and the association of the combined use of sulfadiazine and TMP at farm A with the selection of *E. coli* with TMP resistance and PMQR is likely to be limited. Because only 2 farms participated in the present study, it is important to conduct large-scale studies to evaluate the prevalence of PMQR in *E. coli* originating from chickens in Thailand. High prevalence of TMP-resistance in *qnrS*-positive isolates (13/18) from retail chicken meat samples obtained in May 2020 may be taken into consideration.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

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