Genotypic Diversity of Ciprofloxacin Nonsusceptibility and Its Relationship with Minimum Inhibitory Concentrations in Nontyphoidal Salmonella Clinical Isolates in Taiwan

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Abstract: This study analyzed the genetic diversity of ciprofloxacin (CIP) nonsusceptibility and the relationship between two major mechanisms and minimum inhibitory concentrations (MICs) of CIP in nontyphoidal Salmonella (NTS). Chromosomal mutations in quinolone resistance-determining regions (QRDRs) and plasmid-mediated quinolone resistance (PMQR) genes were searched from ResFinder, ARG-ANNOT, and PubMed for designing the sequencing regions in gyrA, gyrB, parC, and parE, and the 13 polymerase chain reactions for PMQR genes. We found that QRDR mutations were detected in gyrA (82.1%), parC (59.0%), and parE (20.5%) but not in gyrB among the 39 isolates. Five of the 13 PMQR genes were identified, including oqxA (28.2%), oqxB (28.2%), gyrS (18.0%), aac(6’)-Ib-cr (10.3%), and qnrB (5.1%), which correlated with the MICs of CIP within 0.25–2 µg/mL, and it was found that oqxAB contributed more than qnr genes to increase the MICs. All the isolates contained either QRDR mutations (53.8%), PMQR genes (15.4%), or both (30.8%). QRDR mutations (84.6%) were more commonly detected than PMQR genes (46.2%). QRDR mutation numbers were significantly associated with MICs (p < 0.001). Double mutations in gyrA and parC determined high CIP resistance (MICs ≥ 4 µg/mL). PMQR genes contributed to intermediate to low CIP resistance (MICs 0.25–2 µg/mL), thus providing insights into mechanisms underlying CIP resistance.

Keywords: ciprofloxacin nonsusceptibility; minimum inhibitory concentrations; plasmid-mediated quinolone resistance; nontyphoidal Salmonella
1. Introduction

In 2017, the World Health Organization listed fluoroquinolone (FQ)-resistant Salmonella spp. as priority 2 (high) pathogens for which novel antibiotics are urgently required [1]. The resistance of nontyphoidal Salmonella (NTS) to ciprofloxacin (CIP) has been increasing worldwide for the past two decades [2–8]. CIP is one of the most commonly prescribed FQs as the second-line antibiotic for medical use when narrow-spectrum antibiotics are ineffective [9]. However, one or a combination of mutations within quinolone resistance–determining regions (QRDRs) can cause FQ resistance either by changing the drug-binding affinity of two bacterial type II topoisomerases, namely DNA gyrase (encoded by \textit{gyrA} and \textit{gyrB}) and DNA topoisomerase IV (encoded by \textit{parC} and \textit{parE}), or by reducing the intracellular drug concentration through either decreased uptake or increased efflux; in addition, FQ resistance can occur due to the production of drug-modifying enzymes, target-protection proteins, or efflux pumps by plasmid-mediated quinolone resistance (PMQR) genes [9–11]. These molecular mechanisms are not mutually exclusive and can be accumulative.

Genotypic features of CIP nonsusceptibility caused by QRDR mutations and PMQR genes in NTS human isolates can vary with time and country. In a large survey conducted in Taiwan during 1999–2008, four PMQR genes \textit{oqxAB} (16.1%), \textit{qnrS} (4.8%), \textit{qnrD} (3.2%), and \textit{aac(6\prime)-lb-cr} (1.6%) were identified as resulting in CIP nonsusceptibility. High quinolone resistance could be attributable to \textit{gyrA} mutations Ser83Phe/Asp87Asn (80.6%) and Ser83Phe/Asp87Gly (16.7%) [12]. A large clinical survey in Spain during 2004–2008 revealed \textit{gyrA} mutations (mainly Asp87 and Ser83 substitutions) in 80% and \textit{parC} mutations in 5% (Thr57 substitution) of 105 human CIP-nonsusceptible NTS isolates, with only one strain carrying \textit{qrs}1 without QRDR mutations [3]. Another study conducted in Switzerland during 2005–2011 reported the substitution of Ser83Phe in \textit{gyrA} and Ser80Ile in \textit{parC} in all 16 CIP-resistant Salmonella human isolates, but PMQR genes were detected only in four CIP-intermediate strains [13]. Several recent studies have reported an association of PMQR genes with the CIP nonsusceptibility of NTS isolates [11,14–17]. In Ghana during 2016–2018, \textit{qnrS} was found in two of five CIP-intermediate NTS human isolates harboring \textit{gyrA} mutation in Ile203Ser [14]. A study in the United States during 2008–2014 detected \textit{qnrB} (61.1%), \textit{qnrS} (27.8%), \textit{qnrA} (5.6%), and \textit{aac(6\prime)-lb-cr} (4.2%) in 24% of NTS human isolates with a minimum inhibitory concentration (MIC) of CIP of >0.25 \(\mu\)g/mL and susceptibility to nalidixic acid [15]. By contrast, \textit{gyrA} and \textit{qnrA} mutations were noted in 95.2% and only 4.8%, respectively, of CIP-nonsusceptible NTS isolates obtained from Korean patients in 2016 [16]. However, \textit{qnrS} was the most common PMQR gene identified in a recent Korean study that reported a single PMQR gene (\textit{qnrA, qnrB, or qnrS}) and two PMQR genes (\textit{qnrS and aac(6\prime)-lb-cr or qnrA and qnrB}) present in 64.7% and 8.8% of CIP-nonsusceptible Salmonella strains, respectively [17]. Thus far, QRDR mutations in \textit{gyrA} and \textit{parC} have been more frequently observed than those in \textit{gyrB} and \textit{parE}; however, PMQR genes additively contribute to FQ resistance with considerably varying incidences [11]. How interplay occurs between multiple mechanisms in FQ resistance remains obscure [9].

In this study, we investigated the presence of QRDR mutations and PMQR genes through molecular biology in CIP-nonsusceptible NTS clinical isolates representatively sampled from different regions of Taiwan, and analyzed the prevalence of the detected genetic loci and their relationship with MICs.

2. Results

2.1. Concomitant Resistance to Ampicillin and Ceftriaxone in the CIP-Nonsusceptible NTS Isolates

In our study, 34 (87%) and 2 (5.1%) of the 39 CIP-nonsusceptible isolates were resistant to ampicillin and ceftriaxone, respectively.

2.2. Detected Genomic Point Mutations in Three QRDR Genes

A total of nine reported mutations in the QRDR with eight amino acid substitutions (codons 248, 259, and 260 in \textit{gyrA}; codons 170, 238, 239, and codon 250 in \textit{parC}, and codon 1372 in \textit{parE}) were detected in the 39 CIP-nonsusceptible NTS isolates (Table 1). Among
the 39 NTS isolates, QRDR mutations were present in gyrA of the 32 (82.1%) isolates, parC of the 23 (59.0%) isolates, and parE of the 8 (20.5%) isolates but not in gyrb of any isolate. No QRDR mutation or PMQR gene was present in more than 50% of the CIP-nonsusceptible NTS isolates except for two QRDR mutations, namely Thr57Ser in parC (58.9%) and Ser83Phe in gyrA (53.8%). Other reported QRDR mutations in gyrA, gyrB, and parC in Table S1 were not detected.

2.3. Detected Five PMQR Genes
A total of five known PMQR genes were identified in the 39 CIP-nonsusceptible NTS isolates (Table 1), namely aac(6′)-Ib-cr (10.3%; Supplementary Materials Figure S1A), oqxA (28.2%; Figure S1B) and oqxB (28.2%; Figure S1C) simultaneously and qnrB (5.1%; Figure S1D), and qnrS (18.0%; Figure S1E). The other eight PMQR genes, namely qepA, qnrA, qnrC, qnrD, qnrAS, qnrSM, qnrVP, and qnrVV, were not detected in all the 39 CIP-nonsusceptible NTS isolates (Figure S2).

2.4. Distribution of Detected QRDR Mutations and PMQR Genes
Of the 39 CIP-nonsusceptible NTS isolates, we observed that 12 (30.8%) isolates contained both QRDR mutations and PMQR genes, 21 (53.8%) isolates contained QRDR mutations only, and 6 (15.4%) isolates contained PMQR genes only (Figure 1). All the isolates contained either the reported QRDR mutations or known PMQR genes. Any QRDR mutation was detected in 33 (84.6%) of the 39 isolates, whereas any PMQR gene was noted in 18 (46.2%) of the 39 isolates (Table S2).

2.5. Relationship between Genetic Mechanisms and the MIC of CIP
In Group 1, a total of eight (20.5%) isolates with a high MIC of 32 μg/mL exhibited double QRDR mutations individually in gyrA and parC, and a single mutation in parE. In Group 2, 10 (25.6%) isolates with an MIC of 4–16 μg/mL had double QRDR mutations individually in gyrA and parC (Table 2). In Group 3, most of the 14 (35.9%) isolates with an MIC of 8 μg/mL had a single QRDR mutation in gyrA or/and in parC together with PMQR genes, except for three isolates in Types VI and IX that harbored only PMQR genes. In Group 4, seven (17.9%) isolates with an MIC of 0.25–0.5 μg/mL had a single QRDR mutation in gyrA or/and parC, presence of PMQR genes alone, or a single QRDR mutation in parC with qnrB.
Table 1. Genomic point mutations of the four QRDR genes and presence of PMQR genes in the 39 CIP-nonsusceptible NTS isolates.

| Isolate ID | gyrA   | parC   | parE   | PMQR Genes |
|------------|--------|--------|--------|------------|
|            | C248T  | C248A  | C249A  | C260G      | C170G    | A238C    | G239T    | G250A      | T1372C    | aac(6')-Ib-cr | oqxA | oqxB | qnrB | qnrS |
| C01        | +      | –      | –      | +          | +        | +        | –        | –          | +          | –            | –    | –    | –    | –    |
| C02        | +      | –      | –      | +          | +        | +        | –        | –          | –          | –            | –    | –    | –    | –    |
| C03        | +      | –      | –      | +          | +        | +        | –        | –          | –          | –            | –    | –    | –    | –    |
| C04        | +      | –      | –      | +          | +        | +        | –        | –          | +          | –            | –    | –    | –    | –    |
| C05        | +      | –      | +      | –          | +        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C06        | +      | –      | –      | +          | +        | +        | –        | –          | –          | –            | –    | –    | –    | –    |
| C07        | +      | –      | –      | +          | +        | +        | –        | –          | +          | –            | –    | –    | –    | –    |
| C08        | –      | +      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C09        | –      | +      | –      | –          | –        | –        | –        | –          | +          | –            | –    | –    | –    | –    |
| C10        | –      | +      | –      | –          | –        | –        | –        | –          | +          | –            | –    | –    | –    | –    |
| C11        | +      | –      | –      | +          | –        | –        | +        | +          | –          | –            | +    | +    | +    | +    |
| C12        | +      | –      | –      | +          | –        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C13        | –      | +      | –      | –          | –        | –        | –        | –          | +          | –            | –    | –    | –    | –    |
| C14        | –      | +      | –      | –          | –        | –        | –        | –          | +          | –            | –    | –    | –    | –    |
| C15        | +      | –      | –      | +          | –        | –        | +        | –          | –          | –            | +    | +    | +    | +    |
| C16        | +      | –      | –      | +          | –        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C17        | –      | +      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C18        | +      | –      | –      | +          | –        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C19        | +      | –      | –      | +          | –        | –        | –        | –          | +          | –            | –    | –    | –    | –    |
| C20        | –      | +      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C21        | –      | +      | –      | –          | –        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C22        | +      | –      | +      | –          | +        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C23        | –      | +      | –      | –          | –        | –        | +        | +          | –          | –            | –    | –    | –    | –    |
| C24        | +      | –      | +      | –          | +        | –        | +        | +          | –          | –            | –    | –    | –    | –    |
| C25        | –      | +      | –      | –          | +        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C26        | –      | –      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C27        | –      | –      | –      | –          | –        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C28        | +      | –      | +      | –          | +        | –        | –        | –          | +          | –            | –    | –    | –    | –    |
| C29        | –      | +      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C30        | –      | –      | –      | –          | –        | –        | –        | –          | –          | –            | –    | –    | –    | –    |
| C31        | +      | –      | –      | +          | –        | –        | +        | +          | –          | –            | +    | +    | +    | +    |
| C32        | +      | –      | –      | +          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C33        | –      | +      | –      | –          | +        | –        | –        | –          | +          | –            | +    | +    | +    | +    |
| C34        | –      | –      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C35        | –      | +      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C36        | –      | –      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C37        | +      | –      | –      | +          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C38        | –      | –      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |
| C39        | –      | –      | –      | –          | –        | –        | –        | –          | –          | –            | +    | +    | +    | +    |

Total: 39  21  10  10  9  23  9  8  1  8  4  11  11  2  7

Percentage  53.8%  25.6%  25.6%  23.0%  58.9%  23.0%  20.5%  2.6%  20.5%  10.3%  28.2%  28.2%  5.1%  18.0%
Table 2. Grouping of the ciprofloxacin nonsusceptibility profiles according to MICs and combination types of QRDR mutations and PMQR genes in the 39 NTS isolates.

| Group (Isolate No.) | MIC (µg/mL)/Isolate ID | Combination Type | QRDR Mutations | PMQR Genes |
|---------------------|------------------------|-----------------|----------------|-------------|
| 1                   | 32/C01, C03, C04, C06, C07, C16, C18, C21 | I               | gyrA           | parC parE   |
|                     |                        |                 | Ser83Phe       | Thr57Ser    |
|                     |                        |                 | Asp87Gly       | Ser80Arg    |
|                     |                        |                 | ParE           | Ser458Pro   |
| 2                   | 16/C12, C15, C28       | II              |                | --          |
|                     | 8/C19, C22, C24, C25   |                 | Ser83Phe       |                |
|                     | 4/C11                  |                 | Asp87Asn       |                |
|                     |                        |                 | Thr57Ser       |                |
|                     |                        |                 | Ser80Ile       |                |
| 3                   | 8/C05                  | III             | Ser83Phe       |                |
|                     |                        |                 | Asp87Asn       |                |
|                     |                        |                 | Thr57Ser       |                |
|                     |                        |                 | Glu84Lys       |                |
|                     |                        |                 |                | --          |
| 4                   | 8/C02                  | IV              | Ser83Phe       |                |
|                     |                        |                 | Asp87Gly       |                |
|                     |                        |                 | Thr57Ser       |                |
|                     |                        |                 | Ser80Arg       |                |
| 1                   | 2/C29                  | V                | Asp87Asn       |                |
|                     |                        |                 |                | --          |
|                     |                        |                 |                | qnrS        |
|                     |                        |                 |                | qrxA, qrxB  |
| 2                   | 2/C26, C27             | VI               |                | --          |
|                     |                        |                 |                | qnrS        |
|                     |                        |                 |                | qrxA, qrxB  |
| 3                   | 1/C33, C35             | VII              | Ser83Tyr       |                |
|                     |                        |                 |                | qnrS        |
|                     |                        |                 |                | qrxA, qrxB  |
| 4                   | 1/C34                  | IX               |                | --          |
|                     |                        |                 |                | qnrS        |
| 5                   | 0.5/C31, C37           | X                | Ser83Phe       |                |
|                     |                        |                 |                | qnrS        |
| 5                   | 0.5/36                 | XI               |                | --          |
|                     |                        |                 |                | qnrS        |
|                     | 0.5/C32                | XII              |                | --          |
|                     | 0.25/C30               |                 |                | aac(6')-Ib-cr|
|                     |                        |                 |                | qnrB        |
| 4                   | 0.25/C39               | XIII             | Ser83Phe       |                |
|                     |                        |                 |                |                |
|                     | 0.25/C38               | XIV              | --             | --          |
|                     |                        |                 | Thr57Ser       |                |
|                     |                        |                 |                | qnrS        |

Without QRDR mutations, the presence of either of the three PMQR genes resulted in higher CIP resistance in the two isolates with an MIC of 2 µg/mL in Type VI compared with the presence of one or two PMQR genes in the other four isolates with MICs of 0.25–1 µg/mL in Types IX, XII, and XI (Table 2). The PMQR gene aac(6')-Ib-cr was present only in three CIP-intermediate isolates in Types XI and XII, whereas another PMQR gene qnrB was present in only two CIP-intermediate isolates in Types XI and XIV (Group 4, Table 2). The other PMQR, gene qnrS, was detected in the seven isolates with MICs of 0.25–2 µg/mL.

The 39 collected clinical isolates were classified into four groups according to four different ranges of MICs (Table 3), and also classified into three groups according to QRDR mutation numbers (5, 4, and 0–3 mutations). Statistical analysis showed significantly positive associations between three groups of QRDR mutation numbers and four ranges of MICs (p < 0.001, Table 4).
Table 3. The number distribution of different MICs among four groups.

| Grouping by MICs No. of Isolates | Number of Different MICs (µg/mL) |
|----------------------------------|----------------------------------|
| 1 (32 µg/mL) | 0.25 0.5 1 2 4 8 16 32 |
| 2 (4–16 µg/mL) | 0 0 0 0 0 0 0 0 |
| 3 (1–2 µg/mL) | 0 0 0 0 0 0 0 0 |
| 4 (0.25–0.5 µg/mL) | 0 0 0 0 0 0 0 0 |
| Total | 39 3 4 11 3 1 6 3 8 |

Table 4. Cross tabulation of QRDR mutation numbers and MIC groups.

| Grouping by QRDR Mutation No. | Groups (MICs) |
|-------------------------------|---------------|
|                              | 1 (32 µg/mL) | 2 (4–16 µg/mL) | 3 (1–2 µg/mL) | 4 (0.25–0.5 µg/mL) |
| 1 (5 mutations) Case No. (%) | 8 (100) * | 0 (0) | 0 (0) | 0 (0) |
| 2 (4 mutations) Case No. (%) | 0 (0) | 10 (100) * | 0 (0) | 0 (0) |
| 3 (0–3 mutations) Case No. (%) | 0 (0) | 0 (0) | 14 (100) * | 7 (100) * |
| Total Case No. | 8 | 10 | 14 | 7 |

*p < 0.001, Fisher’s exact test.

3. Discussion

CIP nonsusceptibility can more accurately reflect the genuine clinical situation than CIP resistance in FQ-treated patients with salmonellosis. A study conducted in 2003 reported that both Typhi and non-Typhi *Salmonella* isolates exhibited resistance to nalidixic acid with decreased susceptibility and clinical response to FQs [18]. Before 2012, all Enterobacteriaceae shared common MIC and disk diffusion breakpoints for different FQs in the CLSI 2011. However, the CLSI M100 2012 re-evaluated the interpretive criteria for the susceptibility of extraintestinal *Salmonella* isolates to CIP and adopted new *Salmonella*-specific breakpoints as used in the Table 5 of this study. This information facilitates clinicians in deciding the maximal dosage and duration of FQs, or prescribing alternative antibiotics for patients infected by CIP-intermediate isolates [19]. CIP nonsusceptibility should be carefully evaluated because even a minor increase in the MIC of quinolone could unfavorably affect the treatment response [18,20,21]. In our study, the percentage of the CIP-nonsusceptible NTS isolates with ampicillin resistance (87%) was higher than that reported in a study conducted in Ethiopia (58.6%) [22]. Altogether, CIP nonsusceptibility and its increased co-resistance to other antibiotics indicate the worsening problems of treatment failure and delayed clinical responses in *Salmonella*.

The random detection or sequencing of hotspot genes limits the investigation in the genotypic diversity of genetic loci associated with CIP nonsusceptibility in NTS. The location and number of QRDR mutations and PMQR genes may contribute to the intensity of CIP resistance that is reflected by a quantitative change in the MIC. Therefore, we classified our 39 CIP-nonsusceptible NTS isolates into four groups, according to ranges of MICs, and 14 types, based on the combination of QRDR mutations and PMQR genes. Our grouping analysis demonstrated that *gyrA* and *parC*, present in 21 (53.8%) of the 39 isolates, were the major QRDR genes with genomic mutations accounting for CIP nonsusceptibility, particularly leading to high MICs. These two QRDR genes, harboring at least two mutations, resulted in a high MIC of ≥4 µg/mL for CIP, and 17 of the 18 (95%) isolates had an MIC of ≥8 µg/mL. In contrast, only intermediate resistance to CIP was observed in isolates harboring a single mutation in *gyrA* and *parC* individually and a single mutation in *gyrA* (Types X and XIII). The other QRDR gene, *parE*, exerted an additive synergistic effect on CIP resistance with *gyrA* and *parC*. A single mutation in *parE*...
led to a four-fold increase in the MIC by up to 32 μg/mL, compared with the isolate that harbored double mutations individually in gyrA and parC (Type I vs. Type IV). However, the add-on effect of PMQR genes on gyrA in elevating MICs of CIP was not as strong as that on parC and parE. When only one single mutation was individually present in gyrA and parC (Type X), an additional effect of qnrS (Type VII) increased the MIC of CIP by two-fold, thus increasing the level of resistance from intermediate to high. The additional effect of two and three PMQR genes increased the MICs of CIP to 1–2 μg/mL from 0.25 μg/mL when compared with only one single mutation in gyrA (Types VIII and V vs. Type XIII). The effect of CIP nonsusceptibility caused by parC was weaker than that caused by gyrA despite the coexistence of one qnr gene (Type XIV vs. Type VII). Furthermore, PMQR genes (oqxAB, qnrS, and aac(6′)-Ib-cr) exerted an additive synergistic effect on increasing CIP nonsusceptibility to resistance when only one single QRDR mutation was present in gyrA and/or parC (Type X vs. Types V, VII, and VIII). Furthermore, aac(6′)-Ib-cr exerted a cumulative effect on that of oqxA and oqxB in CIP resistance (Type V vs. VIII; Table 2). The effect of different resistance mechanisms on susceptibility to CIP based on data from Escherichia coli indicated that two gyrA mutations and one parC mutation caused a 60-fold change in the MIC of CIP, and one gyrA mutation caused a 10–16-fold change in the MIC of CIP; however, one parC mutation did not increase the MIC of CIP [9]. Our results showed a similar effect of simultaneous mutations in gyrA and parC on the MIC of CIP, but mutations in parC alone induced CIP nonsusceptibility. Furthermore, the presence of PMQR genes increased the MICs of CIP in the descending order of qnr (>30-fold change), oqxAB (16-fold change), and aac(6′)-Ib-cr (4-fold change) in E. coli [9]. Unlike E. coli, our five detected PMQR genes in NTS did not show a large difference in their CIP MICs between 0.25 and 2 μg/mL, and oqxAB contributed more than qnr genes to increasing CIP MICs. PMQR genes alone generally confer only low-level CIP nonsusceptibility compared with QRDR mutations.

The prevalence, number, and genomic loci of mutations in QRDR genes were correlated with their MICs. QRDR mutations in gyrA (82.1%) were more frequently observed than those in parC (59%) or parE (20.5%) in our 39 CIP-nonsusceptible NTS human isolates, and concurrent double mutations in both gyrA and parC coexisted in 18 strains (50%) highly resistant to CIP (Table 2). After in vitro exposure to FQs, compared with parC, gyrA was more inclined to undergo mutation in Salmonella spp., with the most frequent mutations observed in Asp87Asn and Asp87Tyr [23]. In addition, the predominance of gyrA with a rare report of gyrB was observed in other studies; however, the prevalence of parC and parE varied in human CIP-nonsusceptible NTS isolates. In our study, the most prevalent mutation in gyrA was Ser83Phe, followed by Ser83Tyr, Asp87Asn, and Asp87Gly [24]. One of the commonly detected mutations in parC at Thr57Ser was detected in Salmonella strains obtained from Finnish travelers without mutations in gyrA, gyrB, or parE [26]. The mutation Tyr57Ser in parC was also detected in 29 isolates with an MIC of >0.06 μg/mL in Hong Kong. Isolates with a single gyrA mutation were less resistant to FQs than those with an additional parC mutation (Tyr57Ser or Ser80Arg) [27]. In our study, Thr57Ser was the most prevalent mutation of parC in all the 23 strains, with their MICs increased to 4–32 μg/mL when a second parC mutation with double gyrA mutations coexisted. In accordance with a recent study, the parC mutation at Thr57Ser was detected in Salmonella pork isolates with the lowest (0.008-0.06 μg/mL) and highest MICs (0.025–2 μg/mL) of CIP being dependent on the type of gyrA mutation; high resistance to CIP (MIC: 32–64 μg/mL) was noted in all strains...
habiting multiple mutations in both gyrA and parC [28]. Accumulation of topoisomerase mutations leads to stepwise increases in resistance in S. enterica species, from mutations in GyrA at codons Ser83 and Asp87 to additional mutations in the same or a different target enzyme; other mechanisms (e.g., increased efflux or presence of PMQR genes) can result in high resistance levels [29,30].

The maximum diversity of PMQR genes depends on the number of PMQR genes selected for PCR in collected Salmonella isolates. To date, the detection of PMQR mechanisms usually requires up to six PCRs [21]. To detect the number of PMQR mechanisms, recent studies conducted in Korea, Taiwan, and the United States identified PMQR genes in human salmonellosis by performing five [17], eight [12], and nine PCRs, respectively [15]. To the best of our knowledge, this is the first study to perform the highest number of PCRs for identifying as many as 13 PMQR genes in NTS that detected six CIP-nonsusceptible isolates harboring only PMQR genes without mutations in the QRDR. A recent review article concluded that PMQR genes generally lead to only low-level quinolone resistance that does not exceed the clinical breakpoint [11]. However, we found that the presence of the three PMQR genes oqxA, oqxB, and qnrS in two isolates and the single gene qnrS in one isolate exhibited a phenotype of CIP resistance with a higher MIC of 1–2 µg/mL. Our new finding indicated that PMQR alone without QRDR mutations conferred a considerable level of quinolone resistance exceeding the clinical breakpoint.

PMQR genes usually conferred decreased susceptibility to FQs, but accelerated the selection of mutants with high quinolone resistance [31], and their actual prevalence varied widely from <1% to >50% depending on resistance mechanisms and bacterial species [11]. A recent study using WGS or PCR detected PMQR genes qnrB, qnrS, and qnrA in 94% of 72 CIP-intermediate but nalidixic-susceptible NTS isolates [15]. In a Finnish study, qnrS and qnrA were the only two PMQR genes detected in CIP-nonsusceptible S. enterica strains [26]. Similarly, qnrS, qnrA, and qnrB were the three most common PMQR genes of 34 S. enterica strains in South Korea [17]. Apart from these three studies, our study and another study conducted in Taiwan both demonstrated that oqxA, oqxB, and qnrS were the three most common PMQR genes detected in quinolone-nonsusceptible NTS isolates; eleven NTS isolates with oqxAB in our study were all CIP resistant with MICs of 1–2 µg/mL, whereas a plasmid carrying oqxAB was identified in nine CIP-resistant Salmonella isolates with no mutation in gyrA and an MIC of 2–4 µg/mL [12]. The acquisition of an IncH1-type plasmid harboring oqxAB upregulated the chromosomal efflux pump genes acrB, acrA, tolC, and yceE that enable the survival of S. Typhimurium under the lethal concentrations of CIP [32]. The simultaneous existence of both oqxAB and aac(6′)-lb-cr causing a 4-fold increase in the MIC or oqxAB and a single gyrA mutation was sufficient to develop CIP resistance (MIC: 1 µg/mL) [33]. In addition, 98% of oqxAB-positive and <60% of oqxAB-negative S. Typhimurium strains harbored mutations in gyrA or parC [33]. By contrast, our study results revealed that 75% of oqxAB-positive and 85.7% of oqxAB-negative NTS isolates harbored mutations in gyrA or parC. Therefore, oqxA and oqxB were determined as predominant PMQR genes with geographical characteristics in Taiwan.

PMQR genes play an important role in the CIP nonsusceptibility of NTS. In E. coli, aac(6′)-Ib-cr itself resulted in low-level CIP resistance, but could act additively in gyrA-bearing plasmids to generate high-level CIP resistance [34]. In our study, only three CIP-nonsusceptible NTS isolates harbored aac(6′)-Ib-cr that coexisted with either the QRDR mutation Asp87Asn in gyrA or the PMQR gene qnrS/qnrB, indicating a subordinate role of aac(6′)-Ib-cr in CIP resistance. By contrast, CIP-resistant Salmonella Litchfield isolates with a MIC of 1 µg/mL harbored aac(6′)-Ib-cr and qnrB [15], suggesting that additional factors responsible for the tuning of CIP resistance. In previous studies, qnr genes were frequently associated with CIP nonsusceptibility and low-level resistance in Salmonella, including qnrS1 in Salmonella isolates with MICs of 0.125–0.25 µg/mL [17], qnrD in one CIP-nonsusceptible Salmonella isolate with a MIC of 0.5 µg/mL, qnrS in three CIP-resistant Salmonella isolates with MICs of 1–4 µg/mL [12], and qnrS1 alone to reduce susceptibility to CIP MICs of 0.25–1 µg/mL in the absence of gyrA mutation [35]. Acquisition of qnrS1
is often associated with a single gyrA mutation in S. Typhimurium, and combination of qnrS1 and other PMQR genes is observed in other serotypes [36]. In our study, we found one CIP-resistant NTS isolate (MIC: 1 μg/mL) with qnrS alone in the absence of a QRDR mutation and other PMQR genes, suggesting the crucial role of qnrS in CIP resistance. WGS detected qnrB19 only but no QRDR mutations in CIP-resistant S. enterica serovar Isangi nonhuman isolates [37]. In our study, the co-existence of qnrB and aac(6’)-Ib-cr as well as qnrB with mutations in parC contributed to low-level CIP nonsusceptibility. Overall, PMQR genes confer CIP resistance alone or synergistically with other genetic determinants.

4. Materials and Methods

4.1. Bacterial Strains and Serotyping

A total of 39 CIP-nonsusceptible NTS clinical isolates were obtained from different regions of Taiwan between 2010 and 2016, including 34 (7%) of 488 NTS isolates from northern, central, southern, and eastern Taiwan collected in the Taiwan Surveillance of Antibiotic Resistance from NHRI during 2010 to 2016, and 5 NTS isolates from TMU-SHH during 2012 to 2016 (Table 5). The acquisition and utilization of these clinical isolates were approved by the Joint Institutional Review Board of TMU (TMU-JIRB No. N201602020) and the Biosafety Committee of Taipei Medical University Shuang Ho Hospital (No. BSL-2-0048). WGS was performed using MiSeq (Illumina, San Diego, CA, USA) in 9 of the 39 isolates, and serotypes were obtained through multilocus sequence typing.

Table 5. The 39 clinical isolates of CIP-nonsusceptible NTS and their antibiotic susceptibility to three antibiotics according to the CLSI guideline 2020.

| Isolate ID | Year | Region  | Serotype | Disc Inhibition Test | CIP  | AMP  | CRO  | CIP  | MIC (µg/mL) |
|------------|------|---------|----------|---------------------|------|------|------|------|-------------|
| C01        | 1998 | S       | –        | R R S               | 32   | >16  | ≤1   | CRO  |
| C02        | 1998 | C       | Schwarzengrund | R R S               | 8    | >16  | ≤1   | CRO  |
| C03        | 1998 | C       | Schwarzengrund | R R S               | 32   | >16  | ≤1   | CRO  |
| C04        | 1998 | S       | –        | R R S               | 32   | >16  | ≤1   | CRO  |
| C05        | 1998 | S       | –        | R S S               | 8    | ≤4   | ≤1   | CRO  |
| C06        | 1998 | S       | –        | R S S               | 32   | ≤4   | ≤1   | CRO  |
| C07        | 2000 | C       | Schwarzengrund | R R S               | 32   | >16  | ≤1   | CRO  |
| C08        | 2000 | E       | –        | R R S               | 1    | >16  | ≤1   | CRO  |
| C09        | 2000 | E       | –        | R R S               | 1    | >16  | ≤1   | CRO  |
| C10        | 2000 | E       | –        | R R S               | 1    | >16  | ≤1   | CRO  |
| C11        | 2000 | E       | –        | R R S               | 8    | ≤1   | ≤1   | CRO  |
| C12        | 2000 | E       | –        | R R S               | 16   | >16  | ≤1   | CRO  |
| C13        | 2000 | N       | –        | R R S               | 1    | >16  | ≤1   | CRO  |
| C14        | 2000 | C       | Typhimurium | I R S               | 1    | >16  | ≤1   | CRO  |
| C15        | 2000 | S       | –        | R R S               | 16   | >16  | ≤1   | CRO  |
| C16        | 2002 | S       | –        | R R S               | 32   | >16  | ≤1   | CRO  |
| C17        | 2002 | S       | –        | R S S               | 1    | ≤4   | 1    | CRO  |
| C18        | 2002 | N       | –        | R R R               | 32   | >16  | 8    | CRO  |
| C19        | 2002 | E       | –        | R R S               | 32   | >16  | ≤1   | CRO  |
| C20        | 2002 | E       | –        | R R S               | 1    | >16  | ≤1   | CRO  |
| C21        | 2002 | E       | –        | R R S               | 32   | >16  | ≤1   | CRO  |
| C22        | 2002 | E       | –        | R R S               | 8    | ≤1   | ≤1   | CRO  |
| C23        | 2002 | C       | –        | R S S               | 1    | ≤4   | ≤1   | CRO  |
| C24        | 2002 | N       | –        | R R S               | 8    | >16  | ≤1   | CRO  |
| C25        | 2002 | C       | Choleraesuis | R R S               | 8    | >16  | ≤1   | CRO  |
| C26        | 2010 | S       | –        | R S S               | 2    | ≤4   | ≤1   | CRO  |
| C27        | 2012 | E       | –        | R R S               | 2    | >16  | ≤1   | CRO  |
| C28        | 2012 | S       | –        | R R S               | 16   | >16  | ≤1   | CRO  |
| C29        | 2012 | C       | Typhimurium | R R S               | 2    | >16  | ≤1   | CRO  |
| C30        | 2012 | C       | Typhimurium | I R S               | 0.25 | >16  | ≤1   | CRO  |
| C31        | 2012 | C       | –        | I R S               | 0.5  | >16  | ≤1   | CRO  |
| C32        | 2012 | C       | –        | I R S               | 0.5  | >16  | ≤1   | CRO  |
Table 5. Cont.

| Isolate ID | Year | Region | Serotype | Disc Inhibition Test | MIC (µg/mL) |
|------------|------|--------|----------|---------------------|-------------|
|            |      |        |          | CIP * AMP † CRO ‡   |              |
| C33        | 2014 | C      | Enteritidis | R R S               | 1 > 16 ≤ 1 |
| C34        | 2014 | C      | –        | R R S               | 1 > 16 ≤ 1 |
| C35        | 2016 | N (SHH)| –        | I R S               | 0.5 > 16 ≤ 1 |
| C36        | 2015 | N (SHH)| –        | I R S               | 0.5 > 16 > 32 |
| C37        | 2014 | N (SHH)| Albany   | I R S               | 0.25 > 16 ≤ 1 |
| C38        | 2016 | N (SHH)| –        | I R S               | 0.25 > 16 ≤ 1 |
| C39        | 2013 | N (SHH)| –        | I R S               | 0.25 > 16 ≤ 1 |

N: northern, C: central, S: south, E: eastern, SHH: Shuang Ho Hospital; R: resistant, I: intermediate; –: not done; Amp: ampicillin, CIP: ciprofloxacin, CRO: ceftriaxone; * Disc diameters: ≥ 31 mm (S), 21–30 mm (I), and ≤ 20 mm (R) and MIC: ≤ 0.06 µg/mL (S), 0.12–0.5 µg/mL (I), and ≥ 1 µg/mL (R) for CIP-susceptibility; † Disc diameters: ≥ 17 mm (S), 14–16 mm (I), and ≤ 13 mm (R) and MIC: ≤ 8 µg/mL (S), 16 µg/mL (I), and ≥ 32 µg/mL (R) for AMP-susceptibility; ‡ Disc diameters: ≥ 23 mm (S), 20–22 mm (I), and ≤ 19 mm (R) and MIC: ≤ 1 µg/mL (S), 2 µg/mL (I), and ≥ 4 µg/mL (R) for CRO-susceptibility.

4.2. Antibiotic Susceptibility Test

The antibiotic susceptibility of CIP, ampicillin (AMP), and ceftriaxone (CRO) was determined using the disc inhibition test and by calculating their MICs according to the interpretive criteria provided in the Clinical and Laboratory Standards Institute (CLSI) guideline 2020 [38]. Antibiotic susceptibility was determined by measuring the diameters of inhibition zones and the MICs using the microdilution method for CIP and BD Phoenix (BD Biosciences, Franklin Lakes, NJ, USA) for AMP and CRO (Table 5).

4.3. Searching Mutations and Genes Associated with Quinolone Resistance in Three Databases

Genetic loci associated with quinolone resistance, including genomic mutations and plasmid genes, were thoroughly searched from ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/database.php, accessed on 31 May 2018), ARG-ANNOT (https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/arg-annot-2/, accessed on 31 May 2018), and PubMed (https://www.ncbi.nlm.nih.gov/pubmed, accessed on 31 May 2018). A total of 12 reported genomic mutations in QRDR (Table S1) and 13 PMQR genes (qnrA, qnrS, qnrB, aac(6′)-ib-cr, qepA, qnrC, qnrD, aoxA, aoxB, qnrAS, qnrSM, qnrVP, and qnrVV) were found to be associated with quinolone resistance.

4.4. Sequencing for the Detection of Genomic Mutations in the QRDR

Genomic DNA was isolated from the bacterial cultures of the 39 CIP-nonsusceptible NTS isolates using the bacterial genomic DNA purification kit (GeneMark, Taichung, Taiwan) according to the manufacturer’s instructions. According to the mutation profiles of gyrA, gyrB, parC, and parE (Table S1), the sequences of primer pairs were designed to generate PCR amplicons containing these genomic mutations in the four QRDR genes (Figure S3). DNA fragments corresponding to the QRDR genes of these strains were amplified through PCR using the designed primer pairs (Table S3A). In the GeneAmp PCR System 2700 Thermal Cycler (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), 10 ng/µL of template DNA was amplified in a 40-µL reaction solution containing 1 µM of each primer, 5 U of DreamTag DNA polymerase (Thermo Fisher Scientific, Waltham, USA), 63 µM of each deoxynucleoside triphosphate (Protech Technology Enterprise Co., Ltd., Taipei, Taiwan), and PCR buffer (Thermo Fisher Scientific, Waltham, MA, USA), with initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C (gyrB) or 58 °C (gyrA, parC, and parE) for 30 s, and extension at 72 °C for 1 min, followed by the final extension at 72 °C for 7 min. Subsequently, the PCR products were purified using the gel/PCR DNA fragment extraction kit (Geneaid, New Taipei City, Taiwan) and sequenced using the ABI 3730 XL DNA Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) for examining the reported genetic mutations of gyrA, gyrB, parC, and parE in the QRDR.
4.5. PCRs for the Detection of the 13 PMRQ Genes

Plasmid DNA was isolated from the bacterial cultures of the 39 CIP-nonsusceptible NTS isolates using a plasmid DNA purification kit (Protech Gene-Spin MiniPrep Purification Kit, Taipei, Taiwan) according to the manufacturer’s protocol. PCR was performed using specific primers designed with the help of BLAST (Table S3B). In the GeneAmp PCR System 2700 (Applied Biosystems), 10 ng/µL of template DNA was amplified in a 40-µL reaction solution containing 1 µM of each primer, 5 U of DreamTag DNA polymerase (Thermo Fisher Scientific), 63 µM of each deoxynucleoside triphosphate (Protech Technology Enterprise Co., Ltd., Taipei, Taiwan), and PCR buffer (Thermo Fisher Scientific), with initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C (qnrS and qnrB), 55 °C (qnrA, qepA, qnrC, qnrD, qnrAS, qnrVP, and qnrVV) or 60 °C (aac(6′)-Ib-cr, oqxA, oqxB, and qnrSM) for 30 s, followed by extension at 72 °C for 1 min and the final extension at 72 °C for 7 min. Subsequently, 6 µL of the amplified PCR product was electrophoresed through a 1.3% agarose gel containing 1× of SYBR Safe DNA Gel Stain (Invitrogen, Life Technologies, Carlsbad, CA, USA) in 1× TBE buffer. Gel electrophoresis was performed at 100 V for 30 min to separate the genes by their molecular weights, and the PCR products were visualized under ultraviolet light using the Alphalmager Mini Imaging System (ProteinSimple, San Jose, CA, USA). In addition to the available isolates (qnrS in C26; oqxA, oqxB, and qnrB in C29; and qnrD, qnrA and aac(6′)-Ib-cr in another two CIP-susceptible isolates) carrying these seven PMRQ genes, we generated one recombinant S. Typhimurium SL1344 strain carrying a synthetic DNA fragment (synthesized by BioBasic, Markham, ON, Canada), containing parts of sequences from qepA, qnrC, qnrAS, qnrSM, qnrVP, and qnrVV (Figure S4) and used it as the positive control in the PCR detection of these genes.

4.6. Statistical Analysis

The associations between categorical variables in QRDR mutation numbers and different ranges of MICs were analyzed using the chi-square test and Fisher’s exact test. Statistical analysis was performed using Statistical Package for Social Science (SPSS) version 21.0. A p value of <0.05 was considered statistically significant.

5. Conclusions

This present study demonstrated that QRDR mutations, although not predominant, were more common than PMQR genes in CIP-nonsusceptible NTS in Taiwan. Only two genetic loci, Thr57Ser in parC and Ser83Phe in gyrA, were detected in more than 50% of ciprofloxacin resistant NTS isolates. The grouping analysis showed significant positive association between QRDR mutation numbers and MICs (p < 0.001). Two QRDR mutations in gyrA and parC determined high CIP resistance with MICs of ≥4 µg/mL, whereas PMQR genes contributed to intermediate to low CIP resistance with MICs of 0.25–2 µg/mL, thus providing insights into mechanisms underlying CIP resistance.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.390/antibiotics10111383/s1, Figure S1: PCR detection of the PMQR genes aac(6′)-Ib-cr (A), oqxA (B), oqxB (C), qnrB (D), and qnrS (E) in the 39 CIP-nonsusceptible NTS isolates (white arrows indicate the presence of PMQR genes; bp: base pair, MK: marker, SL: Salmonella Typhimurium SL1344), Figure S2: PCR detection of the PMQR genes qepA (A), qnrA (B), qnrC (C), qnrD (D), qnrAS (E), qnrSM (F), qnrVP (G), and qnrVV (H) in the 39 CIP-nonsusceptible NTS isolates (bp: base pair, MK: marker, SL: Salmonella Typhimurium SL1344), Figure S3: Schematic of the generation of PCR amplicons comprising the reported mutations in the four QRDR genes of CIP-nonsusceptible NTS clinical isolates, Figure S4: Design map of the synthesized DNA fragment containing parts of the selected six PMQR gene sequences, ligated with plasmid pUC57 using the restriction enzymes Hin dIII and Mlu I for cloning into S. Typhimurium SL1344 as the recombinant strain, as the positive control of the six PMQR genes, Table S1: Mutational profiles of gyrA, gyrB, parC, and parE related to CIP resistance, Table S2: Distribution of QRDR mutations and PMQR genes in the 39 CIP-nonsusceptible NTS isolates, Table S3: Sequences of primer pairs for PCR amplicons for the four QRDR genes gyrA,
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