Expression of Efflux Pump Gene \( pmrA \) in Fluoroquinolone-Resistant and -Susceptible Clinical Isolates of \( S.\ pneumoniae \)

Laura J. V. Piddock,* Maggie M. Johnson, S. Simjee†, and L. Pumbwe

Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom

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Thirty-four ciprofloxacin-resistant (MIC ≥ 2 \( \mu \)g/ml) and 12 ciprofloxacin-susceptible clinical isolates of \( S.\ pneumoniae \) were divided into four groups based upon susceptibility to norfloxacin and the effect of reserpine (20 \( \mu \)g/ml). The quinolone-resistance-determining regions of \( parC, parE, gyrA, \) and \( gyrB \) of all ciprofloxacin-resistant clinical isolates were sequenced, and the activities of eight other fluoroquinolones, acriflavine, ethidium bromide, chloramphenicol, and tetracycline in the presence and absence of reserpine were determined. Despite a marked effect of reserpine upon the activity of norfloxacin, there were only a few isolates for which the activity of another fluoroquinolone was enhanced by reserpine. For most isolates the MICs of acriflavine and ethidium bromide were lowered in the presence of reserpine despite the lack of effect of this efflux pump inhibitor on fluoroquinolone activity. The strains that were most resistant to the fluoroquinolones were predominantly those with mutations in three genes. Expression of the gene encoding the efflux pump \( Pmra \) was examined by Northern blotting (quantified by quantitative competitive reverse transcriptase PCR) and compared with that of \( S.\ pneumoniae \) R6 and R6N. Within each group there were isolates that had high-, medium-, and low-level expression of this gene; however, increased expression was not exclusively associated with those isolates with a phenotype suggestive of an efflux mutant. These data suggest that there is another reserpine-sensitive efflux pump in \( S.\ pneumoniae \) that extrudes ethidium bromide and acriflavine but not fluoroquinolones.

Fluoroquinolone resistance in \( S.\ pneumoniae \) is usually due to mutations in the genes encoding the target topoisomerase enzymes. Mutations frequently occur in \( parC \), which encodes the A subunit of DNA topoisomerase IV, or \( gyrA \), which encodes the A subunit of DNA gyrase (for examples, see references 7 to 11). Mutations in the genes \( parE \) and \( gyrB \), encoding the B subunits of these proteins, are reported less frequently (e.g., reference 8). Resistance to norfloxacin can also be due to active efflux (2, 6). This mechanism usually gives rise to smaller increases in the MIC of norfloxacin than in the MIC of this agent for strains containing mutations affecting DNA topoisomerase IV and/or DNA gyrase. The MICs of some fluoroquinolones are decreased in the presence of reserpine (an efflux pump inhibitor), leading to the conclusion that this suggests an active efflux system (3, 4, 12). Four studies have described mutant \( S.\ pneumoniae \) strains with phenotypes suggestive of an efflux mutant (2, 6, 13, 16), and in 1999 Gill et al. (6) described a putative efflux pump for fluoroquinolones encoded by the gene \( pmrA \). This pump mediated low-level resistance to norfloxacin, ethidium bromide, and acriflavine.

There were several objectives of the present study: (i) to determine the level of expression of \( S.\ pneumoniae \) \( pmrA \) in wild-type and ciprofloxacin-resistant clinical isolates of \( S.\ pneumoniae \); (ii) to determine the susceptibility of these isolates to newer fluoroquinolones in the presence and absence of reserpine; (iii) to determine the DNA sequence of the quinolone-resistance-determining regions (QRDRs) of \( parC, parE, gyrA, \) and \( gyrB \) for all resistant isolates; and (iv) to determine whether expression of \( pmrA \) is associated with higher MICs of fluoroquinolones with or without a mutation(s) in a topoisomerase gene.

MATERIALS AND METHODS

Bacteria and growth conditions. \( S.\ pneumoniae \) M4 (NCTC 7465 type 1), \( S.\ pneumoniae \) M3 (NCTC 7466 type 2), and \( S.\ pneumoniae \) R6 and R6N (6) were used throughout as control strains. M3 and M4 produce a capsule whereas R6 and R6N do not. Strain R6N is a strain with an efflux phenotype derived from strain R6 transformed by Gill et al. (6) with DNA from IN27, a spontaneous norfloxacin-resistant laboratory mutant of ATCC 49619. Clinical isolates were obtained from a variety of sources: 16 isolates were from MRL Pharmaceutical Services (8); 15 isolates were from the Lung Investigation Unit, University Hospital, Birmingham, United Kingdom (12); and 13 clinical isolates were from the Centers for Disease Control and Prevention, Atlanta, Ga. (9). All strains were maintained at −80°C on Protect beads (Protect Bacterial Preservers, TSC Ltd., Heywood, United Kingdom) without antibiotic and grown overnight in brain heart infusion broth (Unipath, Basingstoke, United Kingdom) incubated at 37°C in 5% CO\(_2\). The solid medium was lososiest agar supplemented with 5% defibrinated horse blood. The identification of each species was confirmed by Gram stain and optochin sensitivity, and the presence of capsule was determined with the SlideX Pneumo-Kit (bioMeriéux SA). Thirty of the clinical isolates were shown to be capsule producers.

Antibiotics and susceptibility determination. The MIC of each antibiotic for each strain was determined by a standard agar doubling dilution method (1). All of the following antibiotics were gifts and were made up and used according to the manufacturers’ instructions: ciprofloxacin and moxifloxacin (Bayer AG, Leverkusen, Germany); sparfloxacin (Rhone DPC Europe, Paris, France); grepafloxacin (Glaxo Wellcome, London, United Kingdom); gatifloxacin (Grunenthal GmbH, Stolberg, Germany); clinafloxacin (Parke-Davis Warner Lambert, Ann Arbor, Mich.); levofloxacin (Aventis, Strasbourg, France); sitafloxacin (Daiichi, Tokyo, Japan); norfloxacin, tetracycline, chloramphenicol, and acriflavine (Sigma); and ethidium bromide (BDH). Reserpine (Sigma) was added to a final concentration of 20 \( \mu \)g/ml.

PCR and DNA sequencing. The QRDRs of \( gyrA \) (nucleotides [nt] 137 to 408), \( gyrB \) (nt 1096 to 1553), \( parC \) (nt 104 to 465), and \( parE \) (nt 981 to 1334) of each

* Corresponding author. Mailing address: Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom. Phone: 0121-414-6966. Fax: 0121-414-3454. E-mail: l.j.v.piddock@bham.ac.uk.
† Present address: U.S. Food and Drug Administration, Center for Veterinary Medicine, Laurel, MD 20708.
strain were amplified by PCR from a whole-cell lysate. The primers were designed with Primer computer software from the DNA sequences of each gene available in the EMBL database (GenBank accession numbers: parC and parE, X95717; gyrA, X95718; gyrB, Z67740). The DNA sequences of all amplimers were determined by MWG Biotech.

Expression of pmrA. Northern blotting was performed with RNA (20 μg) extracted with Trizol (Gibco BRL) and as described in the Amersham Gene Images kits. To remove any contaminating DNA, the samples were treated with DNase I (Roche Diagnostics; catalog no. 776785), and complete removal was verified by direct PCR with the RNA as a template. Sample-to-sample RNA uniformity was determined by examining 16S rRNA expression in parallel. The PCR was used to generate a 558-bp fragment of the structural gene for PmrA (GenBank accession no. AJ007367; nt 431 to 988), which was used as the probe. The intensities of each band on the Northern blot were determined by computer scanning and image analysis, and scores to the nearest full integer were assigned. The pixel area means values, or Adobe PhotoShop version 4.0 software, which gave band intensities as floating numbers.

The clinical isolates were divided into four groups based upon their susceptibility to norfloxacin and the effect of reserpine. Group 1 consisted of 14 isolates which required ≥16 μg of norfloxacin/ml for inhibition. In the presence of 20 μg of reserpine/ml the MIC of norfloxacin was reduced by fourfold or more. Group 2 consisted of four isolates which required 4 μg of norfloxacin/ml for inhibition and for which the MIC was also reduced with reserpine (by ≥4-fold). Group 3 consisted of 20 isolates which required 16 μg of norfloxacin/ml for inhibition but for which reserpine did not lower the MIC by more than 1 dilution. Group 4 consisted of eight isolates which were susceptible to norfloxacin (MIC ≤ 4 μg/ml) and for which reserpine did not lower the MIC by more than 1 dilution.

There were some highly resistant isolates in group 1 requiring 64 μg of ciprofloxacin/ml for inhibition (Table 1). While some of the isolates of this group remained susceptible to some of the other fluoroquinolones, only clinafloxacin and sitafloxacin remained active against all the isolates. Despite a marked effect upon the activity of norfloxacin by reserpine, there were only a few isolates for which the activity of another fluoroquinolone was enhanced by reserpine. Only for two isolates in this group were the MICs of ciprofloxacin lowered by fourfold. For the majority of isolates the MICs of acriflavine and ethidium bromide were lowered in the presence of reserpine. The MIC of chloramphenicol was unaffected by the presence of reserpine, and the MIC of tetracycline was lowered by only 1 dilution, if at all. Of interest, the isolates in group 1 were more susceptible to all fluoroquinolones than were those in group 3 (as shown by the MIC at which 50% of the isolates tested were inhibited [MIC₅₀]).

The four isolates of group 2 were susceptible to ciprofloxacin; however, the MIC of this agent was lowered by no more than 1 dilution in the presence of reserpine. In the presence of reserpine the MICs of all other fluoroquinolones were lowered by no more than 1 dilution. For three isolates the MIC of ethidium bromide was lowered by reserpine, and for one isolate the MIC of tetracycline was also reduced.

All isolates in group 3 required 16 μg or more of norfloxacin/ml for inhibition. Reserpine had a minimal effect upon these MICs. Four isolates were much more resistant to ciprofloxacin (MICs of 32 to 128 μg/ml) than were any of the other isolates and also much less susceptible, if not resistant, to all other fluoroquinolones in this study. Reserpine had no effect on the activity of any of the other fluoroquinolones for the isolates in this group. However, the MICs of acriflavine and ethidium bromide were lowered in the presence of reserpine despite the lack of effect of this efflux pump inhibitor on fluoroquinolone activity. The isolates that were most resistant to the fluoroquinolones were predominantly those with mutations in three genes.

The eight isolates in group 4 were all susceptible to norfloxacin and ciprofloxacin, and the MICs were lowered at most by only 1 dilution in the presence of reserpine. Reserpine had little or no effect upon the activities of any of the fluoroquinolones. However, as for the other groups the MICs of acriflavine and ethidium bromide were lowered by ≥4-fold in the presence of reserpine.

Mutations in the QRDRs of topoisomerase genes. The QRDRs of parC, parE, gyrA, and gyrB were determined for all control strains and those that were resistant to norfloxacin. The four control strains had wild-type DNA sequences for all four genes. All resistant strains harbored one or more mutations in the QRDR of one or more genes.

Ten of the isolates in group 1 contained a mutation in parC (most substituting phenylalanine for serine 79). Four isolates had a mutation in parE (three of which substituted valine for isoleucine 460). Eight isolates had a mutation in gyrA (all substituting phenylalanine for serine 81), and none had a mutation in gyrB. Four isolates contained a mutation(s) in only a single gene (two had a mutation in parC, and two had a mutation in gyrA). Six isolates had a mutation in two genes (one had a mutation in parC and parE, three had a mutation in parC and gyrA, and one had a mutation in parE and gyrA). Two isolates had a mutation in three genes (parC, parE, and gyrA). Seventeen of the isolates in group 3 contained a mutation in parC (11 substituting phenylalanine for serine 79). Eight isolates had a mutation in parE (two substituting asparagine for
Aspartate 435, five substituting valine for isoleucine 460, and one isolate having both substitutions). Fourteen isolates had a mutation in gyrB (10 substituting phenylalanine for serine 81 and 4 substituting tyrosine for serine 81), and none had a mutation in gyrA. Three isolates contained a mutation(s) in only a single gene (two had a mutation in parC, and one had a mutation in gyrA). Six isolates had a mutation(s) in two genes (four had a mutation in parC and gyrA, and two had a mutation in parE and gyrA). Seven isolates had a mutation in three genes (six had a mutation in parC, parE, and gyrA, and one had a mutation in parC, gyrA, and gyrB). Some isolates had two mutations in one gene.

In general the highest MICs of fluoroquinolones were seen for those isolates with multiple mutations in the topoisomerase genes. The most resistant isolate had mutations in three genes. For such isolates the activities of sitafloxacin and clinafloxacin were reduced to 0.5 μg/ml.

**Expression of pmra in clinical isolates.** Northern blotting was used to assess the expression of pmra in all control strains and clinical isolates. A band with heavy density was deemed to have high-level expression and was scored as 4; low-level expression was scored as 1, and medium-level expression was scored as 2 (Fig. 1). The lack of detectable expression was designated by 0. Strain R6N was shown to have high-level expression of pmra, confirming that R6N overexpresses this gene; wild-type strain R6 had low-level expression. Control strain M3 had medium-level expression, while strain M4 did not express pmra. It has since been shown elsewhere that strain M4 has a large deletion within this gene (L. Weigel, personal communication). Three clinical isolates also did not express pmra. Eight isolates expressed high levels of pmra mRNA. QC RT-PCR was used to quantify the expression of pmra by 18 isolates. Two strains which gave no detectable signal with Northern blotting expressed no pmra mRNA and 0.037 pg of pmra mRNA/μl, respectively. The isolates that gave a low signal (= 1) on Northern blotting were divided into three groups by QC RT-PCR: R6 and three isolates expressed 0.011 pg of pmra mRNA/μl, one isolate expressed 0.011 pg of pmra mRNA/μl, and one isolate expressed 0.011 pg of pmra mRNA/μl.

![Image](http://aac.asm.org/)

**FIG. 1.** Northern blot of pmra of clinical isolates of S. pneumoniae. Lane 1, R6; lane 2, R6N; lanes 3 to 12, selected clinical isolates showing range of expression of pmra.
mRNA/μl. Three strains (R6N and two isolates) with a high signal (=4) on Northern blotting expressed 1, 0.37, and 0.33 pg of \textit{pmrA} mRNA/μl, respectively. Four clinical isolates which were susceptible to norfloxacin and for which reserpine did not lower the MIC expressed high levels of \textit{pmrA} mRNA. Increased \textit{pmrA} expression was also not associated with those isolates that required the highest concentration of fluoroquinolones for inhibition (Fig. 2). In summary, within each group there were isolates that had high-, medium-, and low-level expression of this gene, and increased expression was not exclusively associated with those isolates with a phenotype suggestive of an efflux mutant.

DISCUSSION

Analyses of the DNA sequences of the QRDRs of \textit{parC}, \textit{parE}, \textit{gyrA}, and \textit{gyrB} revealed no novel mutations. Some isolates contained multiple mutations in one, two, or three genes; while the origins of these isolates are not fully known, it is clear that each bacterium must have been exposed to a fluoroquinolone on multiple occasions for these mutations to have accumulated, as it is extremely unlikely that such isolates could have emerged after a single exposure. To obtain a similar mutant in the laboratory would require at least three exposures to a fluoroquinolone.

Despite the grouping of the isolates based upon the effect of reserpine on norfloxacin activity, there was no clear association between the groups and \textit{pmrA} expression, as within each group there were isolates that had high-, medium-, and low-level expression of this gene. QC RT-PCR was found to be more accurate than was Northern blotting and, based upon expression of \textit{pmrA} mRNA, further divided the isolates into smaller groups. It had been anticipated that, as strain R6N was selected on the basis of low-level resistance to norfloxacin and the MIC for it was decreased in the presence of reserpine, isolates with a similar phenotype would also overexpress \textit{pmrA}. However, the isolates in this study have shown that this is not the case. In addition, the enhancing effect between norfloxacin and reserpine was seen even for isolates that expressed little or no \textit{pmrA} mRNA. For other bacterial species several efflux pumps have now been described, and so it is suggested that \textit{S. pneumoniae} also possesses more than one efflux pump and that reserpine inhibits another pump in addition to PmrA; this inhibition could give rise to the lower MICs of norfloxacin in the presence of this inhibitor. Reserpine may also interact with multiple efflux pumps with overlapping substrate profiles. It was also interesting that the majority of the clinical isolates were cross resistant to ethidium bromide and acriflavine and that the MICs of these agents were also lowered by fourfold or more by reserpine irrespective of norfloxacin susceptibility. These data suggest that \textit{S. pneumoniae}, irrespective of antibiotic susceptibility, possesses an efflux pump that is inhibited by reserpine, is constitutively expressed, and pumps out ethidium bromide and acriflavine. It is interesting that few of the MICs
of the newer fluoroquinolones were reduced by reserpine. The data from the present study suggest that the normal efflux pump(s) in wild-type S. pneumoniae and/or overexpression of pmrA does not contribute to resistance to fluoroquinolones other than norfloxacin. As the pneumococcal genome is now available (15) and it is clear that several putative efflux pump genes are present, identification of those involved in antibiotic transport can proceed.

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REFERENCES