Effect of pH on the *in Vitro* Activity of and Propensity for Emergence of Resistance to Fluoroquinolones, Macrolides, and a Ketolide

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Abstract

Antibiotic activity against common respiratory pathogens can be affected by the pH of the medium (*in vitro*) or the bodily fluid (*in vivo*) in which bacteria are present. The ionized fraction of an antibiotic is not able to efficiently penetrate bacterial or mammalian membranes, reducing the quantity of molecules able to exert their antibacterial effect resulting in elevated MIC values This study shows that the activity of macrolide antibiotics is particularly sensitive to acidic conditions, whereas a ketolide and fluoroquinolones are much less affected. Furthermore, induction of spontaneous and multistep macrolide resistance is greatly increased in acidic medium. In contrast, telithromycin and moxifloxacin did not induce resistance at any pH. Antibiotics which are less likely to induce resistance *in vitro* may also be less likely to induce the development of resistance in patients with respiratory tract infections.

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Introduction

The *in vitro* activity and *in vivo* pharmacokinetics of antibacterial agents are sensitive to the pH of the bacterial growth medium or body fluids, respectively. It is generally accepted that only the nonionized fraction of an antibiotic penetrates biological membranes and reaches its intracellular target or the site of infection [1].

Macrolides and ketolides (14-, 15-, or 16-membered rings) contain a basic sugar with a tertiary, ionizable amine group (pK_a range of 7.7–9.0) [2, 3]. Consequently, in acidic medium, macrolide molecules are ionized which slows the entry of the molecule into bacterial cells, resulting in an increase in the MIC of the antibiotic [4–8].

Azithromycin, in contrast to erythromycin, clarithromycin, and roxithromycin, has a second basic, tertiary amine on the aza group (pKa 9.5) [3, 9]. This second ionizable group has a higher pK_a, increasing the probability that one of the amino groups is ionized such that azithromycin will have difficulty entering the cell. Therefore, the activity of azithromycin is particularly sensitive to changes in pH [4, 5].

Telithromycin differs from the classical macrolides by the replacement of the neutral sugar, α-L-cladinose, at position 3 of the erythronolide ring with a keto group and an additional C_{11}-C_{12} carbamate residue on the erythronolide ring. Because of these structural modifications, telithromycin has three pK_a values, i.e. two ionizable groups (pK_a 1 and 2) within the C_{11}-C_{12} side chain and a third one within the sugar at position 5 (pK_1 [pyridinium] = 3.0; pK_2 [imidazole] = 5.1; pK_3 [dimethylamine] = 8.7). Consequently, a reduction or an increase in pH results in ionization of telithromycin [10, 11].

Most fluoroquinolones (e.g. ciprofloxacin and levofloxacin) contain two ionizable groups (pK_a range of 6.0 to 8.8) with isoelectric points ranging from 6.8 to 7.1. Thus, fluoroquinolones are in their least ionized state at physiological pH which allows for a major fraction of fluoroquinolone molecules to freely enter bacterial and mammalian cells [12, 13]. Moxifloxacin has pK_a values of 6.4 and 9.5 and an isoelectric point of 7.9, such that an acidic pH results in ionization of this fluoroquinolone [14].

This study sought to further investigate the effect of pH on various commonly prescribed antibiotics (macrolides, ketolides, and fluoroquinolone). Antimicrobial activity and induction of antibiotic resistance were examined in several bacterial species in response to several different pH conditions.

Materials and Methods

Susceptibility Testing

Minimal inhibitory concentrations (MICs) were determined according to DIN 58940 by the broth dilution method using Mueller Hinton (MH) broth and a final inoculum of 10^5 colony-forming units (CFU) [15]. *Streptococcus pneumoniae* was cultivated in

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brain-heart infusion (BHI) broth supplemented with 10% serum and was incubated in ambient air; *Haemophilus influenzae* was grown on BHI agar supplemented with 1% hemoglobin and IsoVitalex TM enrichment. The pH of the media was adjusted by adding either 1 M HCl or NaOH after autoclaving. The macrolides tested include erythromycin (ERY), roxithromycin (ROX), clarithromycin (CLA), and azithromycin (AZI) as well as the ketolide telithromycin (TEL). Levofloxacin (LFX) and moxifloxacin (MFX) were the fluoroquinolones tested.

Bacterial Strains

For each species studied, one recent clinical isolate (designated as K1 for each species) and one American Type Culture Collection (ATCC) strain were used. The ATCC strains were *Staphylococcus aureus* ATCC29213, *S. pneumoniae* ATCC49619, *Streptococcus pyogenes* ATCC19615, *H. influenzae* ATCC49247 and *Moraxella catarrhalis* ATCC43617.

Development of Resistance

Spontaneous Emergence of Resistance. Fluoroquinolone-resistant variants were isolated by spreading an inoculum of 10^9–10^{10} CFU/ml over Iso-sensitest agar plates incorporating the study drugs at 2 × and 4 × the MICs of the individual test organisms. Following an overnight incubation at 37 °C, the frequency of spontaneous resistance was determined.

Multistep Emergence of Resistance. To characterize the emergence of multistep drug resistance, a serial broth-dilution procedure was employed. Bacteria were grown overnight in MH or BHI broth containing 2-fold dilutions of the antibiotics ranging from 1,024 to 0.01 mg/l (similar to MIC determinations). From the culture containing the highest drug concentration permitting visible bacterial growth (i.e. 0.5 × MIC), an aliquot was transferred as a 1:20 dilution to inoculate a second set of serial antibiotic dilutions. After overnight incubation, the dilution procedure was repeated again over a total period of 7 days. After completion of the serial transfers, bacteria with the highest MICs were subcultured daily on drug-free agar in order to assess the stability of antibiotic resistance.

Results

Effect of pH on MIC

The *in vitro* activities of levofloxacin and moxifloxacin were marginally affected by acidification or alkalinization of the growth media for all five bacterial species tested. The MICs of levofloxacin and moxifloxacin were increased by only one titration step for all species tested, except for moxifloxacin in *M. catarrhalis* which was unaffected (Table 1).

For the all the macrolides and the ketolide tested, a reduction in pH resulted in elevated MICs. The MICs of azithromycin and roxithromycin rose by > 5 titration steps so that the MICs for *S. aureus*, *S. pneumoniae*, *S. pyogenes*, and *H. influenzae* ranged from 4 to > 64 mg/l. Although the MICs of telithromycin were not as markedly affected as those for the macrolides, they were generally increased by approximately 3 to 4 titration steps. All the macrolides and the ketolide exhibited greatest *in vitro* activity at an alkaline pH (Table 1).

Table 1
Susceptibility testing (MIC in mg/ml) at various pH values.

Bacterial strain	MIC for MFX at: pH6/pH7/pH8	MIC for LFX at: pH6/pH7/pH8	MIC for ERY at: pH6/pH7/pH8	MIC for ROX at: pH6/pH7/pH8	MIC for CLA at: pH6/pH7/pH8	MIC for AZI at: pH6/pH7/pH8	MIC for TEL at: pH6/pH7/pH8
S. aureus ATCC	0.25/0.12/0.06	1.0/1.0/0.5	4.0/0.5/0.12	6/0.5/0.12	8.0/0.5/0.12	> 64/2.0/0.5	0.5/0.12/0.06
S. aureus K1	0.12/0.06/0.06	1.0/0.5/0.5	2.0/0.25/0.06	4.0/0.25/0.03	1.0/0.25/0.12	64/1.0/0.03	0.25/0.06/0.06
S. pneumoniae ATCC	0.25/0.12/0.06	1/0.5/0.5	4.0/0.12/0.06	16.0/0.25/0.06	1.0/0.12/0.06	8.0/0.25/0.06	0.12/0.03/0.01
S. pneumoniae K1	0.25/0.12/0.06	1/0.5/0.5	2.0/0.12/0.06	8.0/0.25/0.03	1.0/0.12/0.06	4.0/0.25/0.06	0.12/0.03/0.01
S. pyogenes ATCC	0.25/0.12/0.12	1/0.5/0.5	0.25/0.06/0.03	16/0.25/0.12	1.0/0.12/0.12	8.0/0.25/0.12	0.12/0.03/0.03
S. pyogenes K1	0.12/0.06/0.06	0.5/0.75/0.25	0.12/0.03/0.03	8.0/0.25/0.06	0.5/0.06/0.06	4.0/0.25/0.06	0.25/0.03/0.03
H. influenzae ATCC	0.06/0.03/0.03	0.03/0.017/0.015	> 64/8/8	> 64/32/16	> 64/16/8	32/2/1	16/4/2
H. influenzae K1	0.03/0.015/0.035	0.015/0.006/0.006	64/4/1	> 64/16/8	32/8/4	16/2/0.5	82/1
M. catarrhalis ATCC	0.06/0.06/0.06	1.0/0.5/0.5	1.0/0.12/0.06	4.0/0.5/0.12	1.0/0.12/0.06	0.5/0.06/0.03	0.12/0.06/0.03
M. catarrhalis K1	0.06/0.06/0.06	1.0/0.5/0.5	0.5/0.06/0.03	2.0/0.25/0.12	0.5/0.06/0.03	0.25/0.03/0.01	0.12/0.03/0.01

Table 2
Spontaneous emergence of resistance at various pH values.

Bacterial strain	Spontaneous resistance frequency (at 2xMIC)						
	MFX pH6/pH7/pH8	LFX pH6/pH7/pH8	ERY pH6/pH7/pH8	ROX pH6/pH7/pH8	CLA pH6/pH7/pH8	AZI pH6/pH7/pH8	TEL pH6/pH7/pH8
S. aureus ATCC	7.9x10^{-8}/8.1x10^{-8}/ 4.0x10^{-9} [a]	6.5x10-8/4.0x10^{-9} [a]/ 7.6x10^{-8}	[b]/2.3x10^{-7}/ 4.1x10^{-7}	[b]/7.2x10^{-7}/ 2.1x10^{-8}	[b]/3.1x10^{-8}/ 8.2x10^{-8}	[b]/3.2x10^{-6}/ 4.8x10^{-7}	2.1x10^{-8}/3.2x10^{-8}/ 2.7x10^{-9} [a]
S. aureus K1	5.2x10^{-8}/9.1x10^{-8}/ 4.2x10^{-9} [a]	7.2x10-8/4.2x10^{-9} [a]/ 1.0x10^{-9}	[b]/8.6x10^{-6}/ 9.3x10^{-6}	[b]/6.4x10^{-6}/ 1.9x10^{-7}	[b]/5.1x10^{-8}/ 1.4x10^{-8}	[b]/7.4x10^{-6}/ 6.5x10^{-7}	9.4x10^{-7}/5.1x10^{-8}/ 3.2x10^{-9} [a]
S. pneumoniae ATCC	8.7x10^{-8}/2.2x10^{-9}/ 4.0x10^{-9} [a]	8.8x10-8/4.0x10^{-9} [a]/ 1.2x10^{-9}	[b]/9.3x10^{-7}/ 1.2x10^{-8}	[b]/1.1x10^{-8}/ 1.3.6x10^{-8}	[b]/1.5x10^{-7}/ 1.5x10^{-7}	[b]/1.3x10^{-6}/ 6.7x10^{-8}	7.6x10^{-8}/4.0x10^{-9} [a]/ 7.0x10^{-9} [a]
S. pneumoniae K1	8.3x10^{-8}/1.5x10^{-9}/ 4.0x10^{-9} [a]	8.0x10-8/4.0x10^{-9}/ 3.2x10^{-9} [a]	[b]/6.8x10^{-7}/ 6.3x10^{-7}	[b]/1.5x10^{-7}/ 1.5x10^{-7}	[b]/4x10^{-8}/ 4x10^{-8}	[b]/2.1x10^{-6}/ 5.8x10^{-8}	8.2x10^{-8}/4.0x10^{-9} [a]/ 4.0x10^{-9} [a]
S. pyogenes ATCC	5.2x10^{-8}/9.1x10^{-8}/ 4.2x10^{-9} [a]	5.2x10-8/9.1x10^{-8}/ 4.2x10^{-9} [a]	[b]/8.4x10^{-7}/ 9.2x10^{-7}	[b]/1.1x10^{-8}/ 2.9x10^{-8}	[b]/1.2x10^{-7}/ 6.4x10^{-8}	[b]/1.8x10^{-6}/ 9.5x10^{-7}	6.9x10^{-8}/3.2x10^{-9} [a]/ 3.2x10^{-9} [a]
S. pyogenes K1	5.2x10^{-8}/9.1x10^{-8}/ 4.2x10^{-9} [a]	5.2x10-8/9.1x10^{-8}/ 4.2x10^{-9} [a]	[b]/7.8x10^{-7}/ 8.9x10^{-7}	[b]/1.8x10^{-7}/ 7.5x10^{-8}	[b]/2.5x10^{-7}/ 3.5x10^{-8}	[b]/1.9x10^{-6}/ 2.7x10^{-8}	7.3x10^{-8}/3.7x10^{-9} [a] /3.7x10^{-9} [a]
H. influenzae ATCC	5.2x10^{-8}/9.1x10^{-8}/ 4.2x10^{-9} [a]	1.0x10-9/4.0x10^{-9} [a]/ 1.0x10^{-9}	n.d.	n.d.	[b]/4x10^{-4}/ 5.9x10^{-6}	[b]/6.2x10^{-6}/ 6.8x10^{-6}	6.7x10^{-7}/3.2x10^{-8}/ 9.2x10^{-8}
H. influenzae K1	5.2x10^{-8}/9.1x10^{-8}/ 4.2x10^{-9} [a]	1.7x10-8/6.9x10^{-8}/ 6.1x10^{-8}	n.d.	n.d.	[b]/2.4x10^{-4}/ 8.7x10^{-5}	[b]/3.7x10^{-6}/ 4.9x10^{-6}	3.8x10^{-7}/4.1x10^{-8}/ 1.3x10^{-9}
M. catarrhalis ATCC	5.2x10^{-8}/9.1x10^{-8}/ 4.2x10^{-9} [a]	9.2x10-8/1.0x10^{-9}/ 7.8x10^{-8}	[b]/3.2x10^{-6}/ 7.1x10^{-6}	[b]/7.6x10^{-6}/ 2.5x10^{-9}	[b]/3.1x10^{-7}/ 5.8x10^{-8}	[b]/5.9x10^{-6}/ 3.4x10^{-8}	4.7x10^{-8}/6.1x10^{-8}/ 1.4x10^{-9}
M. catarrhalis K1	5.2x10^{-8}/9.1x10^{-8}/ 4.2x10^{-9} [a]	8.2x10-8/8.8x10^{-8}/ 9.3x10^{-8}	[b]/2.6x10^{-6}/ 4.9x10^{-6}	[b]/2.0x10^{-9}/ 2.1x10^{-9}	[b]/1.1x10^{-7}/ 2.9x10^{-8}	[b]/8.5x10^{-6}/ 2.9x10^{-7}	2.6x10^{-8}/5.8x10^{-8}/ 9.3x10^{-8}

[a] no resistant mutants selected; [b] entire population developed resistance; n.d.: not done

Spontaneous Resistance Frequencies

The frequencies of bacterial resistance to moxifloxacin and levofloxacin were low for all the bacterial species studied and were only slightly affected by the pH of the media. There was a trend towards lower resistance frequencies at pH 8.0 for moxifloxacin and pH 7.0 for levofloxacin, which corresponds to their respective isoelectric points (Table 2).

The bacterial resistance frequencies to the macrolides were strongly pH-dependent. At neutral pH they ranged from approximately 1×10^{-7} to 5×10^{-8}; however, at acidic pH the entire population of bacteria developed resistance. Resistance frequency was reduced by approximately 10-fold compared to neutral pH when bacteria were grown in alkaline media (Table 2). Compared to the macrolides, telithromycin had a 100-fold lower propensity for resistance development which was almost unaffected by different pH values; however, there was a slight trend towards a higher propensity for resistance development at the acidic pH (Table 2). Comparable data were obtained when the spontaneous resistance frequencies were determined at 4× MIC (data not shown).

Multistep Emergence of Resistance

The serial broth-dilution method for induction of multistep mutational resistance development resulted in high-level resistance against all antibiotics tested except moxifloxacin during the 6-day study period. For the fluoroquinolones, induction of resistance was not influenced by the pH of the growth media. The MICs of moxifloxacin generally rose by one to two dilution steps, whereas the MICs of levofloxacin rose from 4- to 16-fold (Table 3).

In general the MICs of the macrolides studied rose by 16- to 133-fold during the 6-day study period. The increase in MICs occurred at all the pH conditions tested; however, the increases tended to be more pro-

nounced at the acidic pH (Table 3). The MIC for azithromycin was particularly high (> 64 mg/l) for the *S. aureus* strains grown at pH 6.

The MICs of telithromycin were affected species specifically; for *S. aureus* the MIC rose more than 10-fold upon subculture in acidic media in particular, but tended to be less marked in alkaline media. The MICs for *S. pneumoniae* remained unchanged upon serial subculture in neutral medium, and rose 25-fold at a pH of 8.0. In acidic medium, however, telithromycin MICs for *S. pneumoniae* rose > 66-fold (Table 3).

In general, the susceptibilities of *S. pyogenes* were similarly affected as those of *S. pneumoniae* upon serial exposure to subinhibitory concentrations (data not shown). *H. influenzae* and *M. catarrhalis* could not be studied under these experimental conditions as a consequence of poor growth or no growth at acidic pH values from day 2–3 onward.

Table 3
Multistep emergence of resistance at various pH values.

Bacterial strain	pH	MFX MIC at: day 0/day 3/ day 6	LFX MIC at: day 0/day 3/ day 6	ERY MIC at: day 0/day 3/ day 6	ROX MIC at: day 0/day 3/ day 6	CLA MIC at: day 0/day 3/ day 6	AZI MIC at: day 0/day 3/ day 6	TEL MIC at: day 0/day 3/ day 6
S. aureus ATCC	7	0.12/0.5/0.5	1.0/4.0/16	0.5/11/8	0.5/2/16	0.5/2./32	2.0/8/16	0.12/1/2
S. aureus K1	7	0.06/0.12/0.12	1.5/2.0/16	0.25/2/16	0.25/2/32	0.25/4/16	1.0/8/16	0.06/2/8
S. pneumoniae ATCC	7	0.12/0.25/0.25	0.5/2.0/8.0	0.12/1/4	0.25/1/4	0.12/2./32	0.25/1/2	0.03/0.03/0.06
S. pneumoniae K1	7	0.12/0.12/0.25	0.5/2.0/16	0.12/0.5/2	0.25/2/8	0.12/4./32	0.25/2/8	0.03/0.03/0.03
S. aureus ATCC	6	0.25/0.10/0.5	1.0/8.0/16	4.0/16/> 64	16/> 64/> 64	8.0/32/>64	> 64/> 64/> 64	0.5/2.0/8.0
S. aureus K1	6	0.12/0.25/0.25	1.0/4.0/16	2.0/16/> 64	4/16/> 64	1.0/4/32	> 64/> 64/> 64	0.25/2.0/4.0
S. pneumoniae ATCC	6	0.25/0.5/0.5	1.0/4.0/16	4.0/16/> 64	16/> 64/> 64	16/> 64/> 64	8/32/> 64	0.12/1.0/8.0
S. pneumoniae K1	6	0.25/0.25/0.25	1.0/4.0/16	2.0/8/> 64	8/32/> 64	8/> 64/> 64	4/16/> 64	0.12/2.0/16
S. aureus ATCC	8	0.06/0.12/0.25	0.5/2.0/8.0	0.12/0.5/4	0.12/0.5/8.0	0.12/0.5/4.0	0.5/1.0/4.0	0.06/0.25/2.0
S. aureus K1	8	0.06/0.12/0.12	0.5/4.0/16	0.06/0.25/2	0.03/0.25/2.0	0.12/0.5/4.0	0.03/0.12/0.5	0.06/0.25/1.0
S. pneumoniae ATCC	8	0.06/0.12/0.12	0.5/1.0/4.0	0.06/0.5/2	0.06/0.25/2.0	0.06/0.25/2.0	0.06/0.25/1.0	0.01/0.12/0.25
S. pneumoniae K1	8	0.06/0.12/0.12	0.5/1.0/8.0	0.06/1.0/4	0.03/0.12/1.0	0.06/0.5/2.0	0.06/0.25/1.0	0.01/0.06/0.25

Discussion

The *in vitro* activity of antibacterial agents is routinely studied under well-defined and standardized methods. For most bacterial species, the disc diffusion or MIC tests are performed in ambient air. However, for testing *S. pneumoniae* and *H. influenzae* CO_2 is required for incubation. The addition of 5% CO_2 to the incubation atmosphere results in an acidification of the medium; the pH is reduced from 7.3 to 6.5 [16, 17]. Thus, the question is whether such a CO_2-triggered shift in pH values in the media mirrors physiological conditions. On the one hand, there is no direct correlation between CO_2 and the pH of fluids like sinus secretions [18] or exhaled breath; in condensate [19], on the other hand, the mean value of in- and expiratory CO_2 is approximately 4%, which corresponds to the amount of CO_2 added to the incubation atmosphere for bacterial respiratory tract infection (RTI) pathogens. The mean pH values found in the exhaled breath condensate of healthy subjects was 6.15 as compared to a pH value of 5.88 of stable cystic-fibrosis (CF) patients; in CF patients with an infective exacerbation, the mean pH value was 5.32 [19]. In contrast to the acidic environment in lung tissue or epithelial lining fluid, the pH values in the ears are alkaline. In patients suffering from otitis media mean pH values of 7.7 to 8.2 were measured. A summary of the pH values measured in various body fluids is provided in table 4 [18–37]. Thus, depending on the body site studied, the pH at the focus of infection is either acidic or alkaline. Consequently, evaluation of the effect of acidic or alkaline pH values on the activity of antibacterials is of clinical relevance.

The determination of the pH of fluids from the respiratory tract is both a helpful diagnostic tool and

Table 4
pH values in respiratory secreta and otitis.

		pH		
Sample	**Disease/infection**	**Mean**	**Range**	**Reference**
Exhaled breath condensate	Healthy control	6.15		[18]
	Stable CF	5.88		[18]
	CF, infective exacerbation	5.32		[18]
Epithelial lining fluid	Fatal lung	6.2		[19–23]
	Healthy adults		6.5–7.5	[19–23]
Sinus secretion	Healthy adults	6.5	5.7–7.2	[24]
	Healthy adults	7.4		[25]
	Healthy adults	7.5	7.2–7.9	[26]
	Chronic sinusitis	7.5	6.8–8.1	[26]
	Acute sinusitis	7.5	6.2–8.2	[27]
	Chronic sinusitis	7.4	6.2–8.4	[27]
	Sinusitis		7.7–7.9	[28]
Effusion in otitis media	Acute otitis media	7.7	6.7–8.4	[27]
	Secretory otitis media	8.2	7.3–8.8	[27]
	Chronic otitis media	8.1	7.8–8.6	[27]
Aspirated purulent secretion	Maxillary sinusitis	6.8	5.8–7.3	[17]
Aspirated non-purulent secretion	Maxillary sinusitis	7.4	6.7–8.0	[17]
Mucus of the nose	Healthy adults	7.56		[26]
	Supportive sinusitis		5.8–6.5	[26]
Nasal secretion	Healthy adults		6.4–7.8	[29–31]
Bronchi	Healthy adults		6.5–7.5	[20]
Exudative pleural fluid	Tuberculosis		7.0–7.3	[32]
Parapneumonic effusion	Hospitalized CAP			
	a. complicated	6.8	5.8–7.5	[33]
	b. uncomplicated	7.4	6.4–8.0	[33]
	Bacterial pneumonia			
	a. complicated	6.8	6.2–7.2	[34]
	b. uncomplicated	7.3	7.1–7.5	[34]
	HAP		6.8–7.2	[35]
	Tuberculosis		7.1–7.4	[35]
	Empyema	6.9	6.8–7.3	[36]
	Benign cases	7.4	7.3–7.5	[36]

an appropriate aid in the management of RTIs. *Fine* et al. [38] have developed a scoring system based on aggregate patient characteristics, like demographic factors, comorbidities, physical and laboratory findings, including pH determinations, which helps to guide clinical decisions in treatment of community-acquired pneumonia.

This study clearly indicates that MICs of macrolides for the major RTI pathogens increased parallel to a decrease in pH values; in contrast, the MICs were generally lowest at an alkaline pH. On average, MICs of all the macrolides tested increased 10-fold in an acidic environment. Azithromycin was more affected than the other macrolides due to the presence of a second basic group in its structure, making it more likely to be ionized compared to the other macrolides.

Consequently, as a reduction in pH results in a significant increase in MICs, the propensity for the emergence of a resistant phenotype is also increased. In an acidic environment entire bacterial populations displayed a resistant phenotype. This phenomenon is most likely due to a loss of antibacterial activity at the acidic pH since the increased MICs remained stable only when subcultured in acidic media but not upon subculture at a neutral pH.

Thus, based on the interpretive MIC categories of these macrolides for the species tested, these bacteria must be classified as intermediately susceptible or resistant at a pH of 6.0. This finding is in agreement with published data [4–7, 39–45]. Importantly, the propensity for macrolide resistance development is increased when bacteria were

grown under acidic conditions. It is tempting to speculate that the rapid emergence of *in vitro* resistance triggered by the macrolides tested at an acidic pH is mirrored by clinical findings demonstrating the development of resistance in the oropharyngeal flora. For example, a single course of erythromycin triggered a substantial increase in the proportion of erythromycin-resistant oral streptococci [46]. The selection of resistant oral streptococci was studied in healthy volunteers following the oral administration of 1.5 g erythromycin and a second dose of 0.5 g 6 hours later, as in routine dental surgery. Two days later mean counts of oral streptococci resistant to 1, 4, and 64 mg/l erythromycin were 23%, 17%, and 6%, respectively. Highly macrolide-resistant streptococcal species were *Streptococcus sanguis*, *Streptococcus mitis* and *Streptococcus salivarius*; the majority of isolates were resistant to 256 mg/l of all macrolides and clindamycin.

The emergence and persistence of macrolide resistance in the oropharyngeal flora of patients with coronary artery disease was studied [65]. This study was part of an intervention study in cardiothoracic surgery to study the effects of clarithromycin on the presence of *Chlamydia pneumoniae* in cardiovascular tissue. In a randomised, double-blind study, the patients received a daily dose of 500 mg slow release clarithromycin or placebo for 2 weeks on average. On the one hand, nasal carriage of *S. aureus* was significantly reduced in the clarithromycin group. On the other hand, however, *Haemophilus parainfluenzae* carriage was not affected. In contrast, clarithromycin resistance (MIC ≥ 32 mg/l) increased significantly and persisted for at least 8 weeks. In addition, clarithromycin had a clear effect on the distribution of the different macrolide resistance genes in the oropharyngeal streptococci and staphylococci. A significant rise in the *ermB* gene was detected in the streptococci, as was a significant rise in the *ermC* gene in staphylococci [65].

Likewise, a short course of clarithromycin caused a substantial development of resistance in the oropharyngeal flora, e.g. clarithromycin resistance in *Haemophilus* spp. increased up to 70% and remained stable for 50 days after exposure [47]. Following a 10-day course of 500 mg clarithromycin, significant decreases in susceptibilities of alpha-hemolytic streptococci, intestinal enterococci, and enterobacteria were observed with post-exposure MICs rising to > 128 mg/l which remained stable for 14 days [48]. A further illustration of induction of macrolide resistance comes from an Australian aboriginal community given azithromycin for trachoma control. Macrolide resistance in *S. pneumoniae* rose from 1.9% before treatment to 54.5%, 34.5% and 5.9% at 2 weeks, 2 months and 6 months after treatment, respectively [49]. Taken together, the results of this *in vitro* study and the clinical data discussed above suggest that the potential for macrolide induction of resistance in the oropharyngeal flora should be systematically monitored.

The antibacterial activity of telithromycin is less markedly affected by a shift of pH values. This is most likely due to the pronounced *in vitro* activity of telithromycin mediated by the C_{11}–C_{12} carbamate residue which carries two ionizable NH_2 groups with pK_a values of 3.0 and 5.1. Consequently, it was nearly impossible to induce telithromycin resistance in both pneumococci tested; in the staphylococci, the frequency of resistant bacteria selection was approximately $3 – 5 \times 10^{-8}$ at pH 7 and remained unchanged in an acidic or alkaline environment. This is in agreement with previously published data [50–52]. In contrast, a dramatic rise in telithromycin MICs (220-fold increase) was noted if inducibly macrolide-resistant *S. pneumoniae* strains were exposed to acidified medium [53–55].

Studies have shown that ketolide resistance is more likely to develop in "primed" bacteria. Macrolide susceptible strains of *S. pneumoniae* require daily passages on subinhibitory concentrations for approximately 3 weeks to develop telithromycin resistance, whereas strains with a preexisting *erm* gene require only 3 to 6 passages and strains with a preexisting *mef* gene require 7 [50]. In another study, high level, stable telithromycin resistance developed in erythromycin A resistant strains (*erm*- or *mef*-resistant strains) after only two passages [56]. In a study of staphylococci, telithromycin and cethromycin (an investigational ketolide) selected for constitutively expressed mutants when an isolate harboring an inducibly expressed *erm* gene was exposed to telithromycin [57–60]. These findings indicate that ketolides rapidly select for constitutively expressed macrolide resistance in streptococci and staphylococci carrying inducibly expressed *erm* or *mef* genes despite their low MICs as determined by routine procedures.

Several reports have demonstrated that telithromycin has decreased activities against *S. pneumoniae* and *S. pyogenes* with *erm*-mediated macrolide resistance [50, 51, 61, 62]. It is known that some *erm* gene-carrying *S. pneumoniae* isolates are heterogeneously resistant to telithromycin; this heterogeneous population can easily be transformed into homoclones being resistant to 10 mg telithromycin/l. Homoclones remained macrolide and ketolide resistant after ten passages on drug free agar in these studies [63].

The clinical significance of these findings is at present unknown. It is likely that the risk for resistance selection is highest in infections with a high population density. In contrast to the macrolides and the ketolides tested, the *in vitro* activity of the two fluoroquinolones and the propensity for fluoroquinolone resistance development in the major RTI pathogens was not affected by a shift in pH values. This is in agreement with previously published data [45, 64], demonstrating that the MICs of moxifloxacin for gram-positive bacteria as well as *H. influenzae* and *M. catarrhalis* were marginally affected by pH shifts.

Moxifloxacin, in contrast to levofloxacin, had a very low propensity to induce resistance in all bacteria tested. The high potency of moxifloxacin is most likely responsible for its ability to not select for resistance. It is possible that this *in vitro* phenomenon may also be true *in vivo*, making moxifloxacin an excellent treatment for respiratory tract infections while preventing fluoroquinolone resistance.

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