ANTIMICROBIAL AGENTS

Susceptibility of *Streptococcus pyogenes* to azithromycin, clarithromycin, erythromycin and roxithromycin *in vitro*

G. J. VAN ASSELT*, J. H. SLOOS, R. P. MOUTON, C. P. A. VAN BOVEN
and J. A. M. VAN DE KLUNDERT

Department of Medical Microbiology, University Hospital Leiden, PO Box 9600, 2300 RC Leiden, The Netherlands

Summary. The susceptibility of 180 clinical isolates of *Streptococcus pyogenes* from six regions of The Netherlands to the macrolide antibiotics azithromycin, clarithromycin, erythromycin and roxithromycin was analysed. The results of a microbroth MIC method, the E-test method and a disk diffusion assay were compared, and the MBC determined. In addition, the susceptibility to erythromycin of 436 clinical isolates of *S. pyogenes* from the Leiden region was determined. The microbroth MIC₉₀s of azithromycin, clarithromycin, erythromycin and roxithromycin for group A streptococci were ≤ 0.5 mg/L. Erythromycin had the lowest MIC₉₀ (0.09 mg/L). The MIC data obtained with the E-test method suggested that clarithromycin and erythromycin had slightly higher anti-streptococcal activity than azithromycin and roxithromycin *in vitro*. MICs obtained with the E-test were lower than those found with the microbroth method. Only minor discrepancies were observed among the three methods. The MBC₅₀ for both clarithromycin and erythromycin was 0.75 mg/L and 5.0 mg/L for azithromycin and roxithromycin. None of the 180 strains and two of the collection of 436 strains (0.5%) were resistant to erythromycin and the other macrolides tested; MICs ranged from 1 to 16 mg/L. The erythromycin-resistant strains showed an inducible type of macrolide-lincosamide-streptogramin B (MLS) resistance.

Introduction

The incidence of severe *Streptococcus pyogenes* infections including rheumatic fever has increased in the last decade;^{1,2} Some of these group A streptococcal infections can have a fatal outcome due to the production of pyrogenic exotoxin.² However, most group A streptococcal infections appear to be no more severe than in previous years.³ Because persistence of *S. pyogenes* in the throat can lead to the development of rheumatic fever in some cases, eradication of these bacteria after pharyngotonsillitis is considered to be important.^{4–6}

Penicillin is still the drug of choice for treatment of infections with group A streptococci, but despite the uniform sensitivity to penicillin of group A streptococci, failures in the penicillin treatment of pharyngotonsillitis may occur.⁷ This can be due to the phenomenon of penicillin tolerance in the streptococci^{8,9} or to β -lactamase production by the sur-

rounding flora.^{10,11} Erythromycin, a bacteriostatic drug, is the alternative treatment for group A streptococcal infections if penicillin fails. It is also useful for infections caused by many gram-positive organisms in patients allergic to penicillin.

Erythromycin, a well established, safe and effective antimicrobial agent, has been the subject of renewed interest over the last decade because of its activity against respiratory tract pathogens, such as *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Chlamydia* spp. New macrolide antibiotics are now available with an expanded spectrum and better pharmacological properties than erythromycin.^{12–14} Mainly because of the improved gastrointestinal tolerance of azithromycin, clarithromycin and roxithromycin, compared to erythromycin, these macrolides could be used as alternatives to penicillin. Some of these new macrolides are becoming the antibiotics of first choice in some clinics for treatment of respiratory tract, ear and throat infections, because of their activity against *Haemophilus influenzae*.¹¹ Because of this increased use in empirical therapy, the effect of various macrolides in group A streptococcal infections is of interest. Few reports exist on this.¹⁵

Resistance of group A streptococci to erythromycin

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*Present address and address for correspondence: Department of Medical Microbiology, Westeinde Hospital, Lijnbaan 32, 2512 VA Den Haag, The Netherlands.

has been found in several countries, and rates range from 0.5 to 60%. The highest rates were in Japan¹⁶ and certain areas in Finland.¹⁷ Data from The Netherlands has not been published yet.

The purpose of the present study on group A streptococci was: to determine both the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of erythromycin, azithromycin, clarithromycin and roxithromycin for group A streptococci; to compare the results of the traditional microbroth MIC determination and the recently available E-test and to correlate both with the outcome of the disk diffusion assay; and to estimate the rate of erythromycin resistance in The Netherlands.

Materials and methods

Strains

Two separate collections of clinical isolates were examined: one collection comprised 180 group A streptococci from throat and pus cultures performed in hospital laboratories in six areas of The Netherlands (30 strains each from Leiden, Den Haag, Rotterdam, Utrecht, Deventer and Enschede). These unrelated clinical isolates were collected consecutively during a period of several months in 1993. The second collection comprised 436 clinical isolates of group A streptococci, obtained from different clinical specimens in the period 1988–1990 in the Leiden region.

β -Haemolytic colonies were identified as group A streptococci by a latex agglutination test (Streptex; Wellcome Diagnostics, Dartford). The strains were maintained in glycerol broth at -70°C until all isolates were collected. Strains from one laboratory were stored after lyophilisation. Stocks of group A streptococci were subcultured twice on to defibrinated sheep blood 5% agar (Oxoid and BioTrading) before susceptibility testing. *Enterococcus faecalis* strain ATCC^T 29212 (erythromycin MIC 2 mg/L) and *S. pyogenes* strain Otto (# 350; erythromycin MIC > 64 mg/L), obtained from H. Seppälä (Finland), were included as controls.

Antibiotics

Antibiotic preparations were obtained from their respective manufacturers: azithromycin from Pfizer Inc., New York, USA; clarithromycin and erythromycin from Abbott Laboratories, Abbott Park, IL, USA; roxithromycin from Roussel Uclaf, Paris, France. E-test strips of azithromycin, clarithromycin, erythromycin and roxithromycin were obtained from AB Biodisk, Solna, Sweden. Disks of azithromycin, clarithromycin and erythromycin were supplied by BBL (BBL Sensi-Disc, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA); disks of roxithromycin from Oxoid (Unipath Limited, Basingstoke, Hampshire). Disks contained 15 μg of antibiotic. Disks of penicillin (10 μg) were obtained

from BBL and tetracycline (30 μg) and clindamycin (10 μg) from Oxoid.

MIC and MBC determinations

The MICs of azithromycin, clarithromycin, erythromycin and roxithromycin for the 180 isolates were determined by a broth microdilution method. Serial two-fold dilutions of antimicrobial drug solutions were added to Mueller-Hinton broth (pH 7.3) to achieve concentrations, after addition of the cells, from 0.0039 mg/L to 4.0 mg/L for all macrolides. For resistant strains the range was extended to 128 mg/L. A volume of 0.05 ml/well of each antimicrobial agent was introduced into the wells of the microdilution plates. Plates were stored at -20°C until used. The plates were inoculated by adding 0.05 ml to yield a final inoculum of 10^5 cfu/well (10^6 cfu/ml). The plates were covered and incubated in air at 37°C for 18–20 h; the MICs, defined as the lowest concentration of each antibiotic that completely inhibited visible growth, were read visually after the incubation period.

To determine the MBC, 1 μl of the MIC dilutions from each well was applied with a multipoint inoculator to the surface of agar plates containing sheep blood 5% v/v. The plates were incubated for 24 h in air at 37°C . The MBC was defined as the lowest antibiotic concentration that did not yield visible colonies, i.e., killed > 99.9% of the initial inoculum. All tests were repeated once on a separate occasion. Both values were taken into account.

The MIC susceptibility breakpoints were 1 mg/L for erythromycin,¹⁸ 2 mg/L for azithromycin and roxithromycin,^{17,19} and 1 mg/L for clarithromycin.²⁰

E-test method

The collection of 180 isolates was evaluated by means of the E-test method, according to the instructions of the manufacturer. The E-test yields a quantitative MIC determination by means of a strip containing an antibiotic gradient. The performance of the E-test is comparable to a disk diffusion assay. E-test strips were placed on sheep blood agar plates that had been inoculated with a cotton swab dipped into a bacterial suspension with a turbidity equivalent to that of a 0.5 McFarland standard. Plates were incubated in air at 37°C for 18–24 h. The MIC was read at the point of intersection of the zone edge with the E-test strip. All tests were repeated once on a separate occasion. Both values were taken into account.

Agar disk diffusion assay

The disk diffusion method was performed on blood agar base supplemented with sheep blood 5%, with both Oxoid and BBL disks. For 180 strains, the disks of azithromycin, clarithromycin, erythromycin and roxithromycin were placed on plates inoculated to achieve semi-confluent growth. This was obtained by applying a 10-fold dilution of a bacterial suspension, equivalent to a turbidity of 0.5 McFarland standard,

to the plates with a cotton swab. After incubation for 18–24 h in air at 37°C, the diameters of the inhibition zones were measured. All tests were repeated once on a separate occasion. Both values were taken into account.

The collection of 436 strains was tested for susceptibility to penicillin, erythromycin and tetracycline by the disk diffusion method as described above. For strains with an erythromycin zone diameter < 27 mm, MICs were determined by the broth microdilution method, as described above, to distinguish between resistant and moderately susceptible strains. Strains were considered resistant when the inhibition zone was < 18 mm.²¹

Disk induction test

Erythromycin-resistant strains were subjected to a disk induction test with erythromycin as inducer of clindamycin resistance. In this test, the distance between disks was 20–30 mm. After incubation for 18–24 h at 37°C, blurring of the zone around the clindamycin disk was considered to be evidence of inducible resistance. Resistance to erythromycin without blurring of the clindamycin zone of inhibition indicated constitutive resistance.

Results

The in-vitro activities (MICs) of the four macrolides against 180 group A streptococci assessed by the microbroth and E-test methods are summarised in the table. All strains were susceptible to erythromycin. In the disk diffusion assay, the mean of the zone diameters for azithromycin was 27 mm (range 23–42), for clarithromycin 31 mm (27–46), for erythromycin 31 mm (27–43) and for roxithromycin 28 mm (24–42). The data for the three methods indicated that either erythromycin or clarithromycin was the most active. In view of its MBC₅₀, the bactericidal activity of clarithromycin and to a lesser extent erythromycin was reached at a lower concentration than that of azithromycin and roxithromycin (table). The MICs of the macrolides obtained by the E-test were lower than those obtained by the microbroth method. Most of the MIC values obtained by the microbroth and E-test methods ranged within three dilution steps. The MICs of the great majority of strains were within narrow limits, as were those produced by the disk diffusion

Figure. Disk agar susceptibility test (disk approximation) of a group A streptococcus with inducible MLS resistance. CC, clindamycin (10 µg); E, erythromycin (15 µg).

assay. Because of this narrow range, regression analysis was not considered useful. Susceptibility tests were reproducible: for ≥ 95 % of strains the outcome of the repeated experiments was within two dilution steps, except for the microbroth MIC tests for clarithromycin. The values of the repeated MBC experiments differed by more than two dilution steps for 27 % of strains. The discrepant MIC and MBC determinations were excluded from the analysis.

The mean MIC of erythromycin against *E. faecalis* ATCC^T 29212 was 2.57 mg/L (range 2.0–3.0). These data corroborated the MIC given by the ATCC. The mean MIC of azithromycin against the enterococcus was 8.00 mg/L (range 6.0–12.0); for clarithromycin it was 3.21 mg/L (0.5–6.0) and for roxithromycin, 17.14 mg/L (8.0–24.0).

No major differences in activity were found when strains that originated from six regions in The Netherlands were compared (data not shown).

Table. MIC and MBC for 180 erythromycin-susceptible group A streptococci from The Netherlands

Macrolide	MIC (mg/L) by E-test			MIC and MBC (mg/L) by microbroth method					
	range	MIC ₅₀	MIC ₉₀	MIC range	MIC ₅₀	MIC ₉₀	MBC range	MBC ₅₀	MBC ₉₀
Azithromycin	0.024–0.500	0.158	0.250	0.06–0.63	0.12	0.31	0.09–≥ 8.0	5.00	≥ 8.0
Clarithromycin	0.016–0.079	0.032	0.047	0.05–0.31	0.09	0.16	0.05–≥ 8.0	0.75	≥ 8.0
Erythromycin	0.016–0.125	0.056	0.094	0.05–0.31	0.06	0.09	0.03–≥ 8.0	0.75	≥ 8.0
Roxithromycin	0.032–0.345	0.158	0.250	0.06–0.63	0.12	0.31	0.09–≥ 8.0	5.00	≥ 8.0

The 436 consecutive isolates of group A streptococci from the Leiden region were all penicillin susceptible. For erythromycin the MIC₉₀ was 0.12 mg/L. Two of these strains exhibited low-level resistance to erythromycin as well as the 14-membered macrolides, clarithromycin and roxithromycin, and the 15-membered macrolide, azithromycin; the MIC ranged from 1 to 16 mg/L. The control strain obtained from H. Seppälä yielded MICs and MBCs for azithromycin, clarithromycin, erythromycin and roxithromycin of ≥ 256 mg/L.

Both low-level erythromycin-resistant strains were susceptible to clindamycin but not azithromycin. The disk approximation test revealed induction of clindamycin resistance by erythromycin; the zone of inhibition of clindamycin was reduced on the erythromycin side of the disk (figure).

The two erythromycin-resistant strains were also tetracycline resistant. The overall tetracycline resistance rate for the 436 strains was 22.7%. This was constant in each year of the study period.

Discussion

The in-vitro anti-streptococcal activities of azithromycin and roxithromycin were similar; a similar situation was demonstrated for the more active macrolides, clarithromycin and erythromycin. For many of the isolates, all macrolides tested did not exhibit bactericidal activity within the concentration range used (MBC₉₀ ≥ 8.0 mg/L). The bactericidal concentrations of clarithromycin and erythromycin (MBC₅₀) were the lowest of all the macrolides tested. On the basis of their MIC and MBC clarithromycin and erythromycin were the most active macrolides against group A streptococci in these tests.

When the E-test method was compared with the microbroth MIC method no major discrepancies were found. The slightly lower MIC values obtained by the E-test method than the microbroth method could be explained by the use of different media with different pH conditions (sheep blood agar *versus* Mueller-Hinton broth). Both the disk test and the E-test method are applicable in a diagnostic laboratory for susceptibility screening of group A streptococci for the macrolides tested in this study.

The non-homogeneous or granular suspensions of some of the isolates may have played a role in the variations in MBCs. This, together with the small volume of inoculum (1 μ l) deposited by the multipoint inoculator, could have caused some of the differences and small variations in MBC; in the definition of the MBC a single surviving cfu determines the MBC. Because of this sampling error it is recommended that the sample volume should be at least 10 μ l.

No erythromycin-resistant group A streptococci were found among the 180 strains from 1993, whereas the level of resistance for strains from the Leiden region isolated between 1988 and 1990 was 0.5%. This

low rate of resistance is in agreement with the cumulative data recorded during 1990–1992 in seven regional laboratories in The Netherlands.²²

The two erythromycin-resistant strains had an inducible, rather than a constitutive, type of macrolide-lincosamide-streptogramin B (MLS) resistance. They proved to be susceptible to clindamycin but not to the 15-membered macrolide, azithromycin.^{23, 24} Furthermore, these strains showed induction of clindamycin resistance by erythromycin, as demonstrated by the disk induction test (figure). The occurrence of inducible MLS resistance is in accordance with reports by other European and North American groups. Constitutive MLS resistance was first demonstrated in Japan¹⁶ and only recently in Finland.¹⁷ Constitutive MLS-resistant strains are generally resistant to all macrolides, as was the reference strain obtained from H. Seppälä.^{25, 26} According to several authors^{23, 27, 28} resistance to erythromycin and tetracycline may be genetically linked, but in this study no association between tetracycline and erythromycin resistance was found; 27.7% of strains from the Leiden region were tetracycline-resistant. All but two of these isolates were susceptible to erythromycin, indicating that for these strains resistance to erythromycin and tetracycline is not linked. This is in agreement with other findings.^{17, 29, 30}

In Japan, Australia and Finland, the high incidence of resistance to erythromycin is due to a high usage of antibiotics. The rate of resistance of group A streptococci to erythromycin tends to decrease with decreasing macrolide use.^{16, 31} The use of macrolides in animal feed has also led to selection of resistant organisms capable of transfer of their genetic material to group A streptococci.³² The low prevalence of resistant strains in this study may have resulted from a more restricted use of the macrolides. The rate of erythromycin-resistant group A streptococci ranges from 0.5 to 8% in Europe and North America.^{29, 33–35} In some areas of Australia, Finland and Japan, resistance rates of 17.6%, 54% and 60%, respectively, have been found.^{16, 30, 31}

All macrolides tested in this study had a sufficient bacteriostatic activity against group A streptococci, although the MIC and MBC data were slightly in favour of clarithromycin and erythromycin. Expansion of the spectrum of the new macrolides offers an advantage over erythromycin.^{12, 13} The pharmacokinetics of the new macrolides are characterised by improved oral bioavailability, increased tissue penetration and persistence, and longer clearance times compared with those of erythromycin.^{14, 36} The frequency of adverse effects of erythromycin, such as dose-related epigastric distress, may be reduced with the new macrolides.

The relative preference for the new macrolides in therapy for group A streptococcal infections depends on their in-vitro activity, comparative pharmacokinetics and, most importantly, clinical studies. In pharyngitis studies, in which one of the new macrolides

is compared with penicillin, clinical cure or improvement is often the same for the two groups.^{37,38} However, penicillin treatment may possibly result in higher rates of successful eradication of group A streptococci from the throat.³⁹ Furthermore, there is as yet no convincing evidence that a course of a macrolide will prevent acute rheumatic fever.⁴⁰ In view of the lack of bactericidal action of the macrolides and the development of resistance under conditions of unrestricted use of these drugs, penicillin is still the therapy of first-choice for group A streptococcal infections. The macrolides may be used for this in the case of penicillin allergy in patients and penicillin tolerance in the streptococci.

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