

TRENDS IN THE MINIMUM INHIBITORY CONCENTRATIONS OF ERYTHROMYCIN, CLARITHROMYCIN, AZITHROMYCIN, CIPROFLOXACIN, AND TRIMETHOPRIM/SULFAMETHOXAZOLE FOR STRAINS OF *BORDETELLA PERTUSSIS* ISOLATED IN THE CZECH REPUBLIC, 1967–2015

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SUMMARY

Objective: The aim of the study was to determine trends in the minimum inhibitory concentrations (MICs) of erythromycin used as first-line therapy and alternative antibiotics against *Bordetella pertussis* (*B. pertussis*) strains isolated from patients with whooping cough in the Czech Republic (CR) in three periods from 1967 to 2015.

Methods: In total, 135 isolates from the years 1967–2015 were analysed. The strains were divided into three groups by the year of isolation: 1967–1999 (42 strains), 2004–2010 (43 strains), and 2011–2015 (50 strains). MIC of selected antibiotics (erythromycin, clarithromycin, azithromycin, ciprofloxacin, and trimethoprim/sulfamethoxazole) were obtained by the reference agar dilution method on Bordet Gengou Agar with 15% defibrinated sheep blood.

Results: The study set included 70 strains previously tested for MICs of erythromycin and four other antibiotics. In the three study periods, the MICs of the tested antibiotics for *B. pertussis* were nearly identical. All but a single strain, inhibited by erythromycin at a concentration of 0.03 mg/l, were inhibited by two concentrations of erythromycin and azithromycin (0.06 and 0.125 mg/l). Clarithromycin inhibited the strains from all three study periods at the following concentrations: 0.03, 0.06, and 0.125 mg/l. Any of the 135 strains was inhibited by ciprofloxacin at a single concentration of 0.06 mg/l and by trimethoprim/sulfamethoxazole at three concentrations (0.125, 0.25, and 0.5 mg/l).

Conclusion: The study set of 135 Czech strains of *B. pertussis* isolated in 1967–2015 appears to be homogeneous in terms of the MICs for five antimicrobials. The MICs remained in a narrow range of two to three low concentrations; the unimodal distribution of the MICs suggests the absence of resistance mechanisms. The highest MICs of erythromycin, clarithromycin, and azithromycin were equally 0.125 mg/l, that of ciprofloxacin was 0.06 mg/l, and that of trimethoprim/sulfamethoxazole was 0.5 mg/l. Over the study period of 55 years, the MICs of the study antibiotics remained in the same ranges.

Key words: *Bordetella pertussis*, MIC, erythromycin, clarithromycin, azithromycin, ciprofloxacin, trimethoprim/sulfamethoxazole

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INTRODUCTION

Pertussis, or whooping cough, is a disease caused by *Bordetella pertussis*. It affects all age categories and can be life-threatening, especially in infants and toddlers. After a positive downward trend in pertussis as a result of vaccination implemented in the late 1940s, cases of this infection started to emerge again, first in the Netherlands, despite the high vaccination coverage rates (1). A higher incidence of pertussis in both adolescents and adults was

reported in the USA (2), and a Canadian study revealed that one in five cases of chronic cough in these age categories is caused by *B. pertussis* (3).

Macrolides have been used for the treatment and prophylaxis of pertussis for more than 50 years. The first-line therapies for the treatment of *B. pertussis* infections are erythromycin or azithromycin (4–6). The use of alternative antibiotics is limited by the age of the patient: clarithromycin and trimethoprim/sulfamethoxazole can be safely used in children older than one month or two months

of age, and ciprofloxacin can only be given to persons over 18 years of age (5, 6). Despite the generally good clinical response to antibiotic therapy of *B. pertussis* infections, cases of therapy failure have been reported. A detailed analysis has shown low immunocompetence in patients, infected by a strain which was reliably inhibited *in vitro* by low concentrations of the respective antibiotics used for the treatment of such infections, to be the plausible cause of therapy failure (4, 5, 7).

First sporadic but alarming reports of *B. pertussis* strains resistant to erythromycin (and to other macrolides and azithromycin) have emerged since the mid-1990s. The first resistant strain came from Arizona (8), and was followed by isolates highly resistant to erythromycin from other areas in the USA (9, 10), Taiwan (11), France (12), and Iran (13). In some areas of China, a high incidence of *B. pertussis* strains with an erythromycin MIC of >256 mg/l was noticed (14, 15). The first data on the resistance to nalidixic acid and increased MICs to ciprofloxacin and four other fluoroquinolones in six strains of *B. pertussis* were reported from Japan (16).

In view of the globally increasing incidence of laboratory confirmed infections caused by *B. pertussis*, attention needs to be paid, at the national level, to the epidemiology and biological properties of this species, including resistance to first-line and alternative antibiotics. In 2014, the results of erythromycin, clarithromycin, azithromycin, ciprofloxacin, and trimethoprim/sulfamethoxazole MIC testing in 70 strains of *B. pertussis* (17) showed low MIC values of all antibiotics tested. The original set of strains was extended to include archived and surveillance isolates of *B. pertussis* to be tested for the MICs of erythromycin and four other antibiotics. In the present study, the MICs of selected antibiotics are compared between isolates from three time periods within 1967–2015.

MATERIALS AND METHODS

Bacteria

The National Reference Laboratory for Pertussis and Diphtheria collected 135 clinical isolates of *B. pertussis* from 1967 to 2015 within the national pertussis surveillance programme. All strains were tested for selected antibiotics, the minimum inhibitory concentrations were investigated.

B. pertussis strains were isolated from patients with a clinical disease from Prague (45 strains), South Bohemia Region (37 strains), South Moravia Region (four strains), Central Bohemia Region (two strains), Liberec Region (one strain), Moravia-Silesia Region (seven strains), Pardubice Region (one strain), Hradec Králové Region (three strains), and the Highlands Region (two strains). The origin of 33 strains was unknown. The age of patients fluctuated in a range from one month to 47 years, but was not indicated for 10 samples. Since 2007, *B. pertussis* isolates have been stored frozen at -70°C , the isolates from the period 1967–2004 were stored freeze-dried. *B. pertussis* strains grew on Charcoal Agar (Oxoid cz, Ltd) and were incubated at a temperature of $35 \pm 1^{\circ}\text{C}$ under normal atmospheric conditions for 96 hours. Identification to the species level were validated by *Bordetella pertussis* diagnostic serum (Remel Ltd, USA) in accordance with the manufacturer's instructions.

Minimum Inhibitory Concentration Testing

The agar dilution method was used for determination of the MICs of antibiotics (18).

Erythromycin, clarithromycin, azithromycin, ciprofloxacin as a representative of fluoroquinolones, trimethoprim, and sulfamethoxazole with known activities were provided by Sigma – Aldrich (Czech Republic) in the necessary form (substances). The initial concentrations of 1000 mg/l of erythromycin, clarithromycin, azithromycin, ciprofloxacin, and trimethoprim and a stock concentration of 19000 mg/l of sulfamethoxazole were prepared with the respective diluents and solvents (17, 18).

Medium

On the day of MIC testing, dehydrated Bordet Gengou Agar Base (Difco Lab. Inc., Becton-Dickinson) was used to prepare Bordet Gengou Agar (BGA) base in accordance with the manufacturer's instructions. After cooling to ca 52°C , the base was added with defibrinated sheep blood (LabMediaServis Ltd) to a final concentration of 15%. Liquid BGA with sheep blood (BGA-SB) was kept in a water bath at 52°C until used.

Cultivation Plates with Antibiotics

On the day of preparation of the plates with antibiotics, 12 working concentrations of double geometric series in a range of 0.02–40 mg/l were prepared from the starting concentration of each antibiotic, which was 10 times as high as the required final concentration. The definitive concentrations on the plates in a range of 0.002–4 mg/l for each antibiotic were acquired by combining 2 ml of the working concentration with 18 ml of BGA-SB. Before inoculation, the plates were 30 minutes pre-dried.

Inoculum

To test antimicrobial susceptibility, *B. pertussis* strains were plated on Charcoal Agar and incubated at $35 \pm 1^{\circ}\text{C}$ under normal atmospheric conditions for 72 hours. 35°C Mueller-Hinton (MH) broth and colonies of *B. pertussis* were used for inoculum preparation with final turbidity of a 0.5 McFarland standard, which corresponds to ca 5×10^8 cells/ml.

Plating and Incubation of Samples

Inocula of strains were plated using a 36-pin inoculator head onto the surface of each of 12 BGA-SB plates with antibiotics, arranged in ascending order of concentrations. For growth control, two antibiotics-free plates were added to each set (one before and the other after plating the inocula onto the plates with antibiotics). The final concentration at a pin imprint on BGA-SB plates was around 5×10^5 cells/ml. In each series, 34 strains of *B. pertussis* and the respective control strains were tested. After plated, the test plates were incubated in the inverted position under normal atmospheric conditions at $35 \pm 1^{\circ}\text{C}$ for 72 hours.

Quality Control

For the quality control of BGA without antibiotics, strain *B. pertussis* ATCC 12742 was used (19). For the quality control of

antibiotic dilutions in BGA-SB, two strains were employed with the indicated allowed MIC range and target MICs of antibiotics as specified by the EUCAST for Mueller Hinton Agar with 5% sheep blood (EUCAST QC). *Streptococcus pneumoniae* (*S. pneumoniae*) ATCC 49619 was used for the quality control of the dilutions of erythromycin, clarithromycin, azithromycin, and trimethoprim/sulfamethoxazole. To control ciprofloxacin (for which the reference values of MIC of *S. pneumoniae* ATCC 49619 are not available) *Staphylococcus aureus* (*S. aureus*) ATCC 29213 was employed (20).

Evaluation

After incubation, the concentration of the respective antibiotic which clearly inhibited the growth of the tested and control strains was read and designated as the MIC.

RESULTS

The study set included 70 strains previously tested for the MICs of erythromycin and four other antibiotics (17). The newly added strains were archived isolates from 1967–1999 (15 strains) and new isolates obtained after 2010 (50 strains). The study set was divided by the date of isolation into three groups comparable in size: 42 strains from 1967–1999, 43 strains from 2004–2010, and 50 strains from 2011–2015. No strain from 2000–2003 was available for analysis since the laboratory that collected the strains ceased activities in 1999, and the collection of strains was resumed in 2004.

All study strains, strain *B. pertussis* ATCC 12742 for the quality control of BGA, and both strains for the quality control of dilutions of antibiotics showed good growth on BGA-SB.

Table 1 summarizes the results of MICs of erythromycin, azithromycin, clarithromycin, and trimethoprim/sulfamethoxazole of control strain *S. pneumoniae* ATCC 49619 and those of ciprofloxacin of control strain *S. aureus* ATCC 29213.

rofloxacin of control strain *S. aureus* ATCC 29213. All MICs of antibiotics of the two control strains tested on BGA-SB were within the allowable range, and 17 of 30 (57%) MICs measured matched the EUCAST target MICs for the respective antibiotics (20).

On BGA-SB with antibiotics, the study strains were clearly inhibited by the respective concentrations of antibiotics, and thus the MICs were easy to read. Table 2 shows the MIC ranges, MIC₅₀, and MIC₉₀ of five antibiotics for 135 strains of *B. pertussis* isolated in three periods of time. All but one study strain, which was inhibited by a concentration of 0.03 mg/l of erythromycin, were inhibited by two concentrations of erythromycin and azithromycin (0.06 and 0.125 mg/l) and by three concentrations of clarithromycin (0.03, 0.06, and 0.125 mg/l), with the highest MICs of these similar antibiotics being equally 0.125 mg/l. The concentration inhibiting 90% of strains (MIC₉₀) was one dilution step of the double geometric series lower for erythromycin (0.06 mg/l) in comparison with clarithromycin and azithromycin (0.125 mg/l). All strains were inhibited by two concentrations of trimethoprim/sulfamethoxazole (0.125 and 0.25 mg/l). MIC₉₀ of all study antibiotics but ciprofloxacin, which inhibited any strain in a single concentration of 0.06 mg/l, was no more than one dilution step of the double geometric series higher than MIC inhibiting 50% of strains (MIC₅₀). In all three study periods, the MIC parameters (MIC range, MIC₉₀, and MIC₅₀) were equal or highly similar, and MIC distribution was unimodal, as can be seen from Figure 1 for erythromycin.

DISCUSSION

In view of the growth requirements and low growth rates of *B. pertussis*, the method of choice for its susceptibility testing is to determine MICs of antibiotics by the agar dilution method. The testing protocol is defined by the internationally recognized American Clinical and Laboratory Standards Institute (CLSI) (18). Nevertheless, for using this method for *B. pertussis* testing,

Table 1. Minimum inhibitory concentrations (MICs) of erythromycin, clarithromycin, azithromycin, and trimethoprim/sulfamethoxazole of control strain *Streptococcus pneumoniae* ATCC 49619 and ciprofloxacin MICs in strain *Staphylococcus aureus* ATCC 29213 on Bordet Gengou Agar with 15% of sheep blood

		Minimum inhibitory concentrations (mg/l)				
		<i>S. pneumoniae</i> ATCC 49619				<i>S. aureus</i> ATCC 29213
		Erythromycin	Clarithromycin	Azithromycin	Tri/sulfa*	Ciprofloxacin
Range of allowable MIC**		0.03–0.125	0.03–0.125	0.06–0.25	0.125–1.0	0.125–0.5
Target MIC**		0.06	0.06	0.125	0.25–0.5	0.25
Testing date	16 July 2014	0.06	0.06	0.125	0.5	0.5
	26 July 2014	0.06	0.06	0.06	0.5	0.5
	27 July 2014	0.06	0.03	0.06	0.5	0.5
	16 July 2015	0.06	0.06	0.125	1.0	0.5
	17 July 2015	0.125	0.06	0.125	1.0	0.5
	23 July 2015	0.06	0.03	0.125	0.5	0.5

MICs of antibiotics identical to the target ones are written in bold.

* trimethoprim/sulfamethoxazole in a ratio of 1 : 19

** on Mueller Hinton Agar with 5% of sheep blood

Table 2. Minimum inhibitory concentration (MIC) range, MIC₅₀ and MIC₉₀ of 135 strains of *Bordetella pertussis* recovered in three time periods within 1967–2015

Antibiotic	Time of isolation	No of strains	Range (mg/l)	MIC ₅₀ (mg/l)	MIC ₉₀ (mg/l)
Erythromycin	1967–1999	42	0.03–0.125	0.06	0.125
	2004–2010	43	0.06–0.125	0.06	0.125
	2011–2015	50	0.06–0.125	0.06	0.06
Azithromycin	1967–1999	42	0.06–0.125	0.06	0.125
	2004–2010	43	0.06–0.125	0.06	0.125
	2011–2015	50	0.06–0.125	0.06	0.125
Clarithromycin	1967–1999	42	0.03–0.125	0.06	0.125
	2004–2010	43	0.03–0.125	0.06	0.125
	2011–2015	50	0.03–0.125	0.06	0.06
Ciprofloxacin*	1967–1999	42	0.06	0.06	0.06
	2004–2010	43	0.06	0.06	0.06
	2011–2015	50	0.06	0.06	0.06
Tri/sulfa**	1967–1999	42	0.125–0.5	0.25	0.5
	2004–2010	43	0.25–0.5	0.5	0.5
	2011–2015	50	0.25–0.5	0.5	0.5

MIC₅₀: MIC which inhibits 50% of strains, MIC₉₀: MIC which inhibits 90% of strains

*all strains were inhibited by a single concentration of ciprofloxacin

**trimethoprim/sulfamethoxazole in a ratio of 1:19

the basic requirements for obtaining comparable results have not been formulated, i.e. type of culture medium, type of blood, blood concentration, and inoculum concentration. Some authors recommend using Mueller-Hinton Agar (MHA) with 5% of sheep blood (13, 22–24) intended for susceptibility testing of fastidious bacteria in accordance with the European institutions (21) for standardisation of methods. However, in our previous study, about half only of 70 strains of *B. pertussis* grew reliably on this culture medium, despite all required conditions being met (17). Poor growth was observed particularly in archived, over 30-year-old isolates. Therefore, to test susceptibility, a more appropriate medium, in terms of nutrients, Bordet Gengou Agar (BGA) with blood, was preferred as recommended by Hill et al. (25) and used also by others in MIC testing (14–16, 26, 27). All tested strains of *B. pertussis* grew well on this medium and MIC could be clearly read, regardless of type of antibiotic.

The set of 135 strains of *B. pertussis* presented in this study was analysed in two parts one year apart. The results for 70 strains

from part 1 have been published (17). In both parts, the MICs of the antibiotics tested for control strains *S. pneumoniae* ATCC 49619 and *S. aureus* ATCC 29213 were in the allowed range and over half of the MICs measured were equal to the EUCAST target (20). The quality control results support the reliability of our results of the MICs of antibiotics which are consistent with those reported by others on BGA (14–16, 26, 27) and with those obtained on MHA (13, 22–24). No MIC breakpoints of antibiotics have been established for *B. pertussis* to differentiate between susceptibility and resistance; nevertheless, the erythromycin concentration of 0.125 mg/l is considered as a cut-off between susceptible and non-susceptible strains (9, 25). As the highest MIC of the antibiotics for the study strains was lower than the respective breakpoints for *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Helicobacter pylori*, and other fastidious bacteria (21), it can be assumed that none of the study strains was resistant to erythromycin or other antibiotics tested. Resistance to erythromycin or fluoroquinolones in *B. pertussis* strains isolated worldwide is linked to a point mutation in the 23S rRNA gene (10, 12–15, 28) or gyrA gene (16). Unlike strains with other resistance mechanisms, strains with resistance linked to a point mutation are not expected to spread rapidly. Nevertheless, a study of *B. pertussis* isolates from 1970–2014 conducted in northern China found that 91.1% of isolates from 2013–2014 were highly resistant to erythromycin, clarithromycin, and azithromycin due to a point mutation (14). A contributor to the rapid spread of these resistant strains within one year was probably a massive consumption of azithromycin in China, associated, as observed previously, with high rates of resistance to azithromycin and macrolides in the respiratory pathogens *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* (29). China has also reported resistance to macrolides and azithromycin in *B. pertussis* isolates from two healthy school-age girls (28). Therefore, it can be assumed that under high selective pressure of an antibiotic, a single strain with resistance linked to a point mutation can spread rapidly, even endemically.

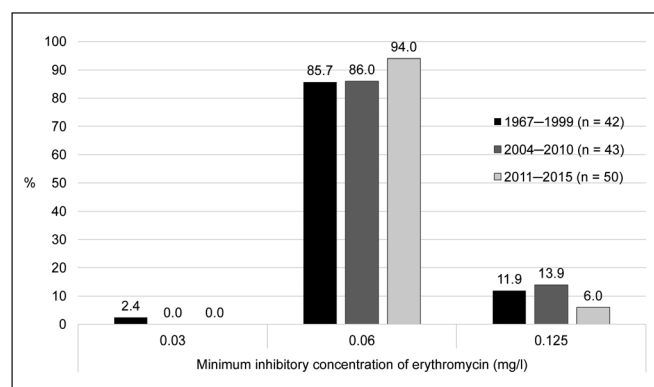


Fig. 1. Distribution (%) of the minimum inhibitory concentrations (MICs) of erythromycin for 135 strains of *Bordetella pertussis* isolated in 1967–2015 by study period.

CONCLUSION

Based on our results, the set of 135 strains of *B. pertussis* appears to be homogeneous in terms of the MICs of the antibiotics tested since all strains were inhibited by only two low concentrations and exceptionally by one or three low concentrations. The absence of any resistance mechanism in the study strains is also supported by the fact that the distribution of MICs of all antibiotics was unimodal. A wide variation in the numbers of isolates from different regions points to the fact that *B. pertussis* infections are vastly underdiagnosed in the Czech Republic. Therefore, it is necessary to improve the surveillance and collection of *B. pertussis* strains for MIC testing of erythromycin and alternative antibiotics in order to allow for early detection of resistance, since the consumption of macrolides and azithromycin in the Czech Republic is ever increasing (30).

Conflict of Interests

None declared

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