Antimicrobial susceptibility testing of Finnish *Bordetella pertussis* isolates collected during 2006-2017

Authors
Emilia Lönnqvist[a], Alex-Mikael Barkoff[a], Jussi Mertsola[b] and Qiushui He[a,c]

Affiliations
[a] *Institute of Biomedicine, Research Center for Cancer, Infections and Immunity, University of Turku, Turku, Finland*
[b] *Department of Pediatrics and Adolescent Medicine, Turku University Hospital, Turku, Finland*
[c] *Department of Medical Microbiology, Capital Medical University, Beijing, China*

Corresponding author: Prof Qiushui He, Institute of Biomedicine, Research Center for Cancer, Infections and Immunity, University of Turku, Kiinamyllynkatu 13, 20520 Turku, Finland (Tel. +358 2 333 7429, email: qiushui.he@utu.fi)

Abstract

Objectives

Macrolides, such as azithromycin and erythromycin, are the first line drug for (prophylactic) treatment of pertussis. However, *Bordetella pertussis* resistant to macrolides has been recently reported from several countries. To date the molecular mechanism of *B. pertussis* resistance to erythromycin has been associated with an A2047G mutation in the genome, which alters the erythromycin-binding site at domain V of the 23S rRNA of the 50S ribosomal subunit. The purpose of this study was to screen for macrolide, quinolone, or trimethoprim-sulfamethoxazole resistant strains among Finnish *B. pertussis* isolates.

Methods

In this study, antimicrobial susceptibility testing was performed on 148 *B. pertussis* strains isolated in Finland during 2006-2017. All the isolates were analysed with the allele-specific PCR for detection of macrolide resistance associated mutation A2047G in the 23S rRNA gene. The *gyrA* gene was sequenced for detection of the A260G mutation associated with quinolone resistance. For phenotyping, a random selection was made by selecting every third isolate (a total of N=50) for testing by the minimum inhibitory concentration method for erythromycin and azithromycin (E-test, bioMérieux) and single disk diffusion (inhibition zone size) of quinolone nalidixic acid (NAL, OXOID, United Kingdom), and trimethoprim-sulfamethoxazole (TMP/SMX, OXOID, United Kingdom).

Results

Neither the macrolide resistance associated mutation A2047G nor the quinolone resistance associated mutation A260G were detected in any of the isolates. The minimum inhibitory concentration of azithromycin and erythromycin ranged between 0.016 and 0.19 µg/ml and 0.016 and 0.25 µg/ml respectively. The size of the inhibition zone surrounding the nalidixic acid disk ranged between 22 and 27 mm in diameter. The inhibition zone surrounding the TMP/SMX disk ranged between 24 and 37 mm in diameter. Isolates resistant to any of the tested antibiotics were not identified.

Conclusions

The allele specific PCR is a simple and useful tool for screening of *B. pertussis* resistance to macrolides. All Finnish isolates tested were sensitive to macrolide, quinolone and TMP/SMX.

Since resistant *B. pertussis* is increasingly reported, regular screening should be considered in the national reference laboratories.

Key words: *Bordetella pertussis*; Pertussis; Antimicrobial susceptibility testing; Macrolide; Quinolone, Antibiotic; Finland

1. Introduction

Bordetella pertussis causes a respiratory infection in human known as pertussis. Vaccination is the most effective method used to prevent pertussis and help limit transmission, although a study with baboons indicated that vaccines are not able to prevent transmission of the pathogen (1, 2). However, antibiotics are needed for the treatment of patients with diagnosed pertussis, especially in infants. Treatment is effective only during the first weeks of symptoms in microbial clearance, but do not often alter the clinical symptoms (3). Nevertheless, clearance of the pathogen is needed to prevent further transmission of the disease and early antibiotic treatment in young infants seems to be beneficial (4). Macrolides, such as erythromycin and azithromycin, are the first line drugs of choice for treatment of *B. pertussis* infections (5). *B. pertussis* isolates resistant to macrolides have been sporadically identified in the USA, France, Brazil, Iran, and China since 1994 (6-12) . Recently macrolide resistant *B. pertussis* isolates have been increasingly reported in China (7, 8, 13). One Chinese study reported that out of 16 isolates collected between 2012 and 2013 in Xi'an, 87.5 % were resistant to erythromycin (8). A point mutation, changing nucleotide A to a G at position 2047, in the domain V of the 23S rRNA gene of *B. pertussis* has been associated with macrolide resistance (6-12). So far no other mechanisms causing macrolide resistance in *B. pertussis* have been identified. However, as it is not known whether other mutations also cause resistance to macrolides in *B. pertussis*, culture based antimicrobial susceptibility testing is needed for determination of resistance. Quinolones are an alternate option for treatment of pertussis in cases where macrolides cannot be used e.g. due to hypersensitivity or allergy. However, *B. pertussis* strains resistant to nalidixic acid (NAL) have been reported in Japan (14). These strains all possessed a mutation of A260G in the *gyrA* gene. In addition, trimethoprim/sulfamethoxazole (TMP/SMX) is also used for treatment of pertussis. There are no reports of TMP/SMX resistant *B. pertussis* isolates nor any specific mutation identified in

the *B. pertussis* genome to cause TMP/SMX resistance. Studies on antimicrobial susceptibility testing of *B. pertussis* isolates in Europe are quite limited (10, 15). In Finland, antibiotic susceptibility of *B. pertussis* isolates has not previously been determined.

The primary aim of this study was to develop a rapid assay for surveillance of antimicrobial resistance of Finnish *B. pertussis* isolates. The secondary aim was to determine the MIC of two macrolides, erythromycin and azithromycin, and to screen for the presence of quinolone (NAL) and trimethoprim/sulfamethoxazole (TMP/SMX) resistant strains using antimicrobial susceptibility testing (AST).

2. Materials and methods

2.1 Strains and culture conditions

The study comprised of 148 *B. pertussis* isolates collected in the National Reference Laboratory for Pertussis and Diphtheria at National Institute for Health and Welfare (THL) and recently at the University of Turku (transferred since 2015) during a period of 2006-2017. All isolates collected at the laboratory were included in the study. Last epidemic pertussis season in Finland was during 2003-2004. However, a few minor local epidemics occurred after this season. Therefore, many isolates were collected from Rautavaara in 2010. Number of isolates collected each year with available epidemiological and patient data is shown in table 1. Vaccination coverage for primary vaccination in Finland has been more than 95% after 1990s (16) . However, we do not have individual vaccination data of the patients included in table 1. The isolates were cultured on Regan-Lowe charcoal agar (EN ISO/IEC 17025 FINAS accredited, Reagent preparation laboratory, Turku University Hospital, Turku, Finland) without cephalexin (RL-C) and incubated at 35^0C for 48-72 hours as recommended by earlier studies (6, 17).

2.2 PCR assay conditions and targets

Heated bacterial suspensions were used as PCR templates, which were prepared by inoculation of a 1 µl loop full of bacteria in 200 µl of sterile nuclease-free (SNF) water (E476-100ML, VWR, USA). From this suspension 50 µl were diluted with 350 µl of SNF water and heated at 95^0C for 30 minutes on a dry heat block.

In one study, two PCR assays were developed for specific identification of the macrolide resistance associated A2047G mutation in 23S rRNA gene of *B. pertussis*. These

assays follow the same setup, but different set of primers were designed to detect either the wild-type or the mutated gene . In the wild-type assay, the primers have been designed to only amplify a single PCR product of 286 bp if the sequence corresponding to macrolide resistance is present in the *B. pertussis* genomic DNA. However, if the sequence corresponding to macrolide sensitivity is present in the *B. pertussis* genome, PCR amplification of an additional product of 121 bp would also be present. All samples were screened with the wild-type (no-mutation) PCR assay (7, 8). *B. pertussis* strains ATCC 9797T and NAP-12-30 (a Chinese strain, kindly provided by Z. Wang, not publicly available) (7) were included in every assay as sensitive and resistant controls, respectively.

For detection of the quinolone resistance associated mutation A260G, the region of the *gyrA* gene containing the mutation was first amplified by PCR using forward primer gyrA1 and reverse primer gyrA8 as published by Ohtsuka et al. (14). Successful amplification of the *gyrA* product was confirmed by gel electrophoresis on a 1.5% agarose gel and visualized using GelDoc XR+ Imaging System (BioRad, California, USA). The *gyrA* gene products were sequenced using only the forward primer gyrA1, at the Institute of Molecular Medicine Finland (FIMM). Reverse sequencing was not performed and no reference strain was publicly available. The sequences were analysed using Seq Scanner 2 (Applied Biosystems, the USA).

2.3 Antimicrobial agents and testing

Four antimicrobial agents, azithromycin (E-test, ref. 501618, bioMerieux, France), erythromycin (E-test, ref. 510518, bioMerieux, France), NAL (30µg disk, CT0031B, OXOID, United Kingdom) and TMP/SMX (25 µg disk, CT0052B, OXOID, United Kingdom) were tested in this study. Antimicrobial susceptibility testing was performed on every third *B. pertussis* isolate (N=50). E-tests were used to determine the MIC of erythromycin and azithromycin. Resistance to NAL and TMP/SMX was determined by single disk diffusion. Mueller-Hinton (MH), Mueller-Hinton (MHB) supplemented with blood, and RL-C agars were evaluated for susceptibility testing of *B. pertussis*. Plates were inoculated with a bacterial suspension density equivalent to 0.5 McFarland standard, and incubated at 35^0C for 72 hours. The MIC and the inhibition zone diameter were recorded after 48 and 72 hours of incubation. Macrolide resistant Chinese *B. pertussis* strains NAP-12-30 (with A2047G mutation), and macrolide sensitive *B. pertussis* strain ATCC 9797T (no mutation) were included as controls in each macrolide MIC test. No resistant control strains for NAL or TMP/SMX were available.

Sensitive *Escherichia coli* strain ATCC 25922 was used for quality control of the TMP/SMX disk, according to EUCAST guidelines (inhibition zone range 23-29 mm)(18).

3. Results

3.1 Allele-specific PCR for macrolide and quinolone resistance

The working principle of this allele-specific PCR has been described earlier (7). Allele-specific PCR for detection of macrolide resistance showed that all 148 isolates carried the wild type alleles (sensitive) with the sizes of 281 bp and 121 bp (Figure 1). No mutation in the 23S rRNA gene was detected in any of the isolates. For quinolone susceptibility, the sequenced *gyrA* gene showed that all 148 isolates possessed the wild type A at the position of 260. No A260G mutation in the *gyrA* gene was identified among the isolates.

3.2 MIC and disk susceptibility tests

For optimization of the culture plate for MIC and disk tests, when cultured with 0.5 McFarland standard *B. pertussis* grew in a semi-confluent layer on RL-C agar. However, no growth could be seen on MH or MHB agars. However, MHB was further tested with inoculation suspensions equivalent to 4.0 and 8.0 McFarland standards. Only the suspension equivalent to 8.0 McFarland standard rendered a semi-confluent layer of growth. Therefore, antimicrobial susceptibility testing of *B. pertussis* strains was performed on RL-C charcoal agar using an inoculation suspension with a density equivalent to 0.5 McFarland standards.

The MIC of azithromycin and erythromycin were determined for 50 *B. pertussis* isolates. For azithromycin, the MIC ranged between 0.016 and 0.19 µg/ml. For erythromycin, the MIC ranged between 0.016 and 0.25 µg/ml. Isolates resistant to azithromycin and/or erythromycin were not identified. The susceptibility for NAL and TMP/SMX was determined for the same 50 *B. pertussis* isolates that were tested for macrolide susceptibility. The size of the inhibition zone for NAL surrounding the disk ranged between 22 and 27 mm in diameter. None of the isolates expressed resistance towards NAL. The size of the inhibition zone surrounding the TMP/SMX disk ranged between 24 and 37 mm in diameter. No resistant *B. pertussis* isolates were identified. All results are presented in Table 2 and the representative pictures of plates are presented in Figure 2.

4. Discussion

Macrolide resistant *B. pertussis* strains have emerged in Europe, America, and Asia (8-11). Therefore, in this study we aimed to determine the prevalence of macrolide, quinolone and TMP/SMX resistant *B. pertussis* strains in Finland. We did not detect any resistant *B. pertussis* isolates. It should be kept in mind that the 148 tested isolates were all isolates collected at the National Reference Laboratory of this country during 2006-2017. Our findings indicate those *B. pertussis* currently circulating in Finland are sensitive to macrolide, quinolone and TMP/SMX.

So far, there has been no standardised method for antimicrobial susceptibility testing of *B. pertussis*. Further clinical breakpoints for determination of resistance or sensitivity are not available at the EUCAST web-pages. Previous studies have found that *B. pertussis* isolates that are resistant to erythromycin and azithromycin have, in most cases, a MIC > 256 µg/ml (6-8, 11). In the USA, two isolates with erythromycin MIC of 32 µg/ml and 64 µg/ml have been isolated, and were also considered as resistant (9, 19). In the UK, the MIC of erythromycin and azithromycin in macrolide sensitive isolates have been recorded as ⩽0.064 µg/ml in isolates from 2001-2008 (15). In the USA, the MIC of erythromycin for sensitive isolates has been determined as ⩽0.094 µg/ml, and the MIC for azithromycin has been determined as ⩽0.125 µg/ml (6). The MIC of the Finnish *B. pertussis* strains for both erythromycin and azithromycin was less than 0.25 µg/ml and 0.19 µg/ml, respectively, which corresponds to the previous studies. In Japan, *B. pertussis* isolates resistant to NAL have been identified by E-test (MIC ⩾ 256 µg/ml) and disk diffusion test (30 µg disk, no inhibition zone) (14). We used disk diffusion test to detect NAL resistant isolates among the Finnish *B. pertussis* isolates. The inhibition zone was recorded as ⩾22 mm, indicating that the strains are sensitive. However, we did not possess a quinolone resistant *B. pertussis* control strain and were therefore unable to compare and confirm the inhibition zone breakpoint. Disk diffusion test was also used to detect TMP/SXM resistance. The inhibition zone recorded was ⩾24. We did not possess a control strain of TMP/SXM resistant *B. pertussis*.

It is possible that the lack of macrolide resistant strains in Finland is because our vaccination policy with several boosters has been successful so far and the number of pertussis cases has been low (Finnish infectious diseases register, https://www.thl.fi/ttr/gen/rpt/tilastot.html), which has limited the use of macrolides. Furthermore, low rate of macrolide usage may have less selective pressure for the strains to

develop resistance. An increase in the number of pertussis cases would cause more patients to be treated with antibiotics, mainly macrolides, which could theoretically lead to an increase in the number of resistant strains. As resistance levels increase, new effective antibiotics for treatment of *B. pertussis* are required. One potential drug is the fourth-generation macrolide solithromycin, which has been found to be more effective than azithromycin and trimethoprim-sulfamethoxazole on *B. pertussis* (20).

We acknowledge that there are some limitations to this study. The number of strains tested is limited as these are the only *B. pertussis* isolates collected in Finland during 2006-2017. In addition, we did not possess any quinolone or TMP/SMX resistant control strains. We propose that standardised methods for antimicrobial susceptibility testing of *B. pertussis*, with corresponding clinical breakpoints for determining resistance, should be established in Europe and included in the EUCAST guidelines. Aggregating comparable data from antimicrobial susceptibility testing of *B. pertussis* is essential for monitoring possible emergent resistant strains in Europe and to ensure effective treatment and patient health.

5. Conclusions

In the present study, the results showed that *B. pertussis* strains currently circulating in Finland are sensitive to macrolide, quinolone and TMP/SMX. However, routine surveillance of *B. pertussis* resistance should be considered as resistant strains have been emerging. Culture of *B. pertussis* isolates is less used at routine microbiology laboratories performing pertussis diagnosis at present. Therefore, the use of bacterial DNA, isolated from nasopharyngeal swabs collected for routine PCR diagnostics, could be considered for allele specific PCR testing described in this present study.

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Ethical approval: Not required

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Year	Number of isolates	Origin of isolates	Gender of patients (Male %)	Age of patients (range in years)
2006	14	Different regions of Finland	41	0.17-51.0
2007	9	Different regions of Finland	45	0.17-42.3
2008	8	Mainly from southwest Finland	63	0.08-34.5
2009	6	Mainly Helsinki area	33	0.17-42.3
2010	13	Mainly Rautavaara (local epidemy)	54	0.08-13.0
2011	26	Different regions of Finland	58	0.08-10.7
2012	22	Different regions of Finland	32	0.08-62.3
2013	5	Mainly Helsinki area	20	0.08-13.5
2014	12	Mainly Southern Finland	42	0.08-26.6
2015	5	Mainly Southern Finland	20	0.08-8.9
2016	26	Different regions of Finland	46	0.08-71.3
2017	2	Lahti and Oulu	0	0.58-0.67

Table 1. Yearly number of isolates collected with available epidemiological and patient data

*Clinical data of patients is not available. However, diagnostics are always based on clinical symptoms and then laboratory confirmation of the pathogen.

E-test (µg/ml)			Disk diffusion (mm)		
Antibiotic	MIC Geometric mean	MIC range	**Antibiotic**	Inhibition zone Geometric mean	Inhibition zone range
Erithromycin	0.049	0.016-0.25	**Nalidixic acid (30 µg)**	24	22-27
Azithromycin	0.023	0.016-0.19	**Trimethoprim-sulphametoxazole (25 µg)**	31	24-37

Table 2: Antibiotic susceptibility testing of 50 Finnish *B. pertussis* isolates.

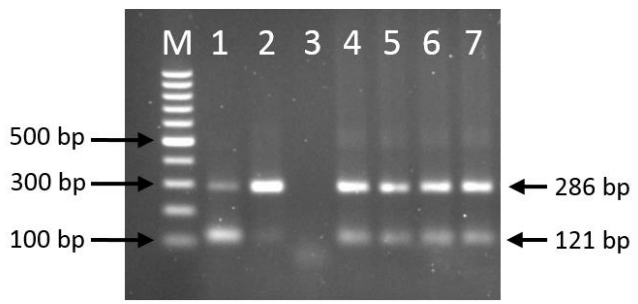

Figure 1: Allele specific PCR assay. M) GeneRuler 100 bp DNA Ladder (Thermo Scientific) 1) ATCC 9797T does not contain a mutation which gives two bands 2) NAP-12-30 contains mutation which allows detection of only one band 3) No template control 4-7) Finnish strains without mutation yields two bands.

Figure 2: Antimicrobial susceptibility testing of *B. pertussis* on Regan-Lowe charcoal agar with inoculation density equivalent of 0.5 McFarland standard. To the top left: erythromycin E-test, top right: azithromycin E-test. Bottom left: nalidixic acid 30 µg, bottom right: trimethoprim-sulfamethoxazole 25 µg.