Research in Microbiology 174 (2023) 104058

Contents lists available at ScienceDirect

Research in Microbiology

journal homepage: www.elsevier.com/locate/resmic

Original Article

# Alterations of growth, biofilm-forming, and gene expression of *Bordetella pertussis* by antibiotics at sub-minimum inhibitory concentrations

Eda Delik [a, 1], Berfin Eroğlu [a, 1], Çiğdem Yılmaz Çolak [b], Aysun Türkanoğlu Özçelik [c], Burcu Emine Tefon Öztürk [a, *]

[a] Biology Department, Faculty of Science, Akdeniz University, 07058, Antalya, Turkiye
[b] Genetic Engineering and Biotechnology Institute, TUBITAK, 41470, Kocaeli, Turkiye
[c] Agricultural Biotechnology Department, Faculty of Agriculture, Akdeniz University, 07058, Antalya, Turkiye

ARTICLE INFO

*Article history:*
Received 10 January 2023
Accepted 3 April 2023
Available online 10 April 2023

*Keywords:*
Antimicrobials
Biofilm
Growth curve
Sub-mic
Virulence genes
Whooping cough

ABSTRACT

*Bordetella pertussis* is the primary agent of the acute respiratory disease pertussis. It has been reported that the disease has recently become more common, especially in adults and adolescents, and adaptation of the pathogen is thought to have an important influence on the recurrence of the disease. This study aims to determine the effect of erythromycin, azithromycin, and trimethoprim-sulfamethoxazole used in the treatment of pertussis on the virulence gene expressions (*prn*, *ptxS1*, *fhaB*), biofilm-forming and growth of *B. pertussis*. In this study, the minimum inhibitory concentration (MIC) values of azithromycin and erythromycin in *B. pertussis* local strain Saadet were determined to be 0.09 μg/mL and 0.3 μg/mL, respectively. However, the Tohama-I and Saadet strains were resistant to trimethoprim-sulfamethoxazole (MIC>32 μg/mL). The biofilm-forming of the Saadet strain decreased with the increase in antibiotic doses. It was observed that 1/32MIC erythromycin and 1/32MIC azithromycin upregulated the expression of *fhaB* in Tohama-I, whereas the expression of *ptxS1* and *prn* significantly decreased in sub-MICs of erythromycin. In the Saadet strain, only *ptxS1* was highly expressed at 1/16MIC azithromycin and erythromycin ($p > 0.05$). This is the first study to investigate the effect of sub-MIC antibiotics on the expression of virulence genes and biofilm-forming of *B. pertussis*.



## 1. Introduction

Antibiotics should be used in successive doses at a concentration above the minimum inhibitory concentration (MIC) to be effective against bacterial pathogens. Lower concentrations of antibiotics than the MICs are referred to as sub-minimum inhibitory concentrations (sub-MIC) and reflect the conditions of bacteria in the natural environment and in tissues [1]. These lower doses of antibiotics can select resistant bacteria, increase the rate of adaptive evolution, and affect various physiological activities of the microorganism, including virulence, biofilm formation, and gene expression [2]. Therefore, it is important to understand the effects of sub-MIC antibiotic doses on bacterial cells.

Pertussis (whooping cough) is a contagious respiratory disease with dramatic consequences for the elderly, immunocompromised persons, pregnant women, and especially infants. It is one of the infectious diseases whose severity decreases with antibiotics, especially when used in the catarrhal phase [3]. The main causative agent of this disease is *Bordetella pertussis*, which binds to the epithelial cells, especially ciliated, of the trachea and bronchi of the host's lungs [4]. This bacterium has many important virulence factors that can evade the host defences, attach to the respiratory system, and cause damage [5]. Filamentous haemagglutinin (Fha), pertussis toxin (Ptx), and pertactin (Prn) are among the important virulence factors that effectively cause the disease and are used as vaccine components. Fha is the surface and secretion protein of *B. pertussis* which is responsible for adhesion to the bronchial epithelial cells of the host and the colonisation of the entire respiratory tract [6]. Prn is one of the surface proteins that mediate

* Corresponding author. Biology Department, Faculty of Science, Akdeniz University, 07058, Antalya, Turkiye. Fax: +02423102294.
*E-mail address:* burcutefon@akdeniz.edu.tr (B.E. Tefon Öztürk).
[1] These authors contributed equally to this article.

https://doi.org/10.1016/j.resmic.2023.104058
0923-2508/

attachment to lung epithelial cells by playing a role in adhesion and colonisation [3,4]. Ptx is responsible for the irreversible activation of adenylate cyclase, which leads to excessive cAMP accumulation in the cell and prevents many cellular functions of the host [3,4]. This bacterium also forms a biofilm in the respiratory tract [4]. The biofilm is responsible for the persistence of the bacteria in the nasopharynx and for the formation of a successful infection [7].

Antibiotics are commonly used to treat the disease to reduce the symptoms of pertussis and reduce the incidence of transmission by eliminating the microorganism that causes the disease from the nasopharynx [8]. Erythromycin and azithromycin, a semisynthetic antibiotic derived from erythromycin, are bacteriostatic, which inhibit the growth of *B. pertussis* by blocking protein synthesis by reversibly binding the 50S subunit of the ribosome [9]. Azithromycin, the first azalide, has better tolerability, a broader spectrum of activity, and a better drug−interaction profile than erythromycin [10]. Trimethoprim-sulfamethoxazole (TMP-SMX) is the recommended antimetabolite that inhibits folic acid synthesis in patients who cannot tolerate macrolides, and this antibiotic has been reported to effectively clear *B. pertussis* from the nasopharynx [11]. It is known that pathogenic species of *Bordetella* spp. develop antibiotic resistance easily; however, the frequency of acquired resistance to the antimicrobial agents used in clinical has been notably low. But there are some reports which indicate the emergence of erythromycin resistance, especially in China, among *B. pertussis*. [12,13]. As the resurgence of pertussis has become an issue again [14], it is very important to study the antibiotic susceptibility and pathogenesis of the bacteria, as well as protection by vaccines and new vaccine trials. However, there are not enough studies on the antibiotic resistance of *B. pertussis* and the effect of sub-MIC doses of antibiotics on these bacterial cells. In our previous study, we reported the effects of sub-MIC doses of azithromycin and erythromycin used in the treatment of pertussis, on the growth rate and biofilm-forming capacity of *B. pertussis* Tohama-I [15]. In this study, the sub-MIC levels of these antibiotics against the local Saadet strain of *B. pertussis* were investigated, and their effects on the biofilm-forming capacity and growth rate were examined. In addition, the MIC of TMP-SMX, another antibiotic used against pertussis, was investigated for both strains. This study also examined the effects of sub-MIC levels (1⁄16, 1⁄32, and 1⁄64) of these antibiotics on *ptxS1*, *prn,* and *fhaB* gene expression in the Tohama-I and Saadet strains.

## 2. Material and method

### 2.1. Strain of B. pertussis

The *B. pertussis* strains used in this study were Tohama-1 and the local strain Saadet. The bacteria were provided by Prof. Dr. Meral Dilara Öğünç (Akdeniz University, Antalya, Turkiye). As Sato and Arai [16] stated in their study, the bacteria were grown on a modified Cohen-Wheeler (CW) agar medium, at 37 °C for 48 h, and subcultures were prepared for this study.

### 2.2. Determination of MIC levels of antibiotics

The determination of MIC values of bacteria against azithromycin, erythromycin, and TMP-SMX antibiotics was carried out using the broth dilution method [17]. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have not yet established limits for MIC values of antibiotics for the *B. pertussis* [18]. For this reason, the MIC values of antibiotics were determined according to Hoppe et al. [17]. Fresh bacterial cells were adjusted to 0.5 McFarland in 0.85% NaCl (Merck, Germany) and transferred to antibiotic-containing Morse−Bray broth (MB). Antibiotic-free bacterial cultures were established as positive controls. In the first step, cultures of antibiotics in a wide concentration range (0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 μg/mL) were established. The prepared cultures were incubated at 150 rpm and 37 °C for 96 h. Then, the lowest antibiotic concentration without bacterial growth and the highest concentration with growth was determined as the MIC range. For the TMP-SMX, additional higher antibiotic concentrations (10, 20, 30, 40, 50, 100 μg/mL) were tried. The bacterial cultures with the antibiotics were reconstituted according to the determined MIC intervals, and the lowest antibiotic concentration without bacterial growth was determined as the MIC. The experiments were performed in 3 replicates.

To determine the MIC levels of TMP-SMX against bacterial strains, the E-test method was used [19]. Freshly grown bacterial cultures were adjusted to 0.5 McFarland in 0.85% NaCl and spread on CW agar medium. Commercially produced E-test strips (0.002−32 μg/mL) (Bioanalyze, Ankara, Turkiye) were then placed on the medium according to the manufacturer's instructions. After incubating the prepared media at 37 °C for 96 h, the inhibition zones were analysed. *Staphylococcus aureus* (ATCC 29213) strain was used as the MIC quality control [20]. The experiments were performed in 3 replicates.

### 2.3. Effect of sub-MIC doses on the growth rate of bacteria

Similar to the studies by Nikbin et al. [21], the growth curve of bacteria was generated at sub-MICs of antibiotics. The concentrations of fresh bacterial cells were adjusted to an initial concentration of 0.05 at 600 nm wavelength in MB broth. Antibiotic-containing bacterial cultures were established at sub-MICs of 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 of the determined MIC levels. Antibiotic-free bacterial cultures were set up as a control group. The cultures were incubated at 150 rpm, and 37 °C, and the $OD_{600}$ values were measured and recorded every 12 h for 120 h. The experiments were performed in 3 replicates.

### 2.4. Effect of sub-MICs on biofilm production

The biofilm-forming capacity of bacteria was observed using the slightly modified method of Conover et al. [22]. Freshly grown *B. pertussis* cells were adjusted to a concentration of 0.1 cells at $OD_{600}$ in the MB medium. Antibiotic bacterial suspensions were then prepared to obtain the desired sub-MICs at the final concentration, and 200 μL were added to a 96-cell culture plate. The blank MB was used as a negative control and the antibiotic-free bacterial suspension was used as the positive control. The prepared samples were incubated at 37 °C for 96 h under static conditions. After incubation, the plates were washed with sterile water and dried for 25 min at 37 °C. Then, 200 μL of a freshly prepared 0.1% crystal violet solution (Merck, Germany) was added to each well and stained for 30 min at room temperature. After that, the plates were washed thoroughly three times with distilled water. Then 200 μL of ethanol (95%) was added to the wells to dissolve the dye and measure the absorbance values of the samples at a wavelength of 590 nm. The experiments were performed in 3 replicates.

### 2.5. Total RNA extraction and cDNA synthesis

Bacterial cells were incubated in MB broth containing 1/16, 1/32, and 1/64 sub-MICs of antibiotics for 48 h at 37 °C at 150 rpm. Total RNA was isolated using a total RNA isolation kit (Zymo Research, USA) according to the manufacturer's recommendations. The purity and quantity of total RNA obtained were determined using a NanoDrop spectrophotometer (Thermo Scientific, USA). In addition,

1.5% agarose gel electrophoresis was performed to visualize the isolated RNA. Subsequently, cDNA synthesis was performed from the isolated RNA, following the instructions of the commercially available cDNA synthesis kit (Jena Bioscience, Germany). The synthesised cDNAs were stored at −20 °C for later use.

### 2.6. Quantitative PCR (qPCR) assays

qPCR reactions were performed to analyse the expression levels of *ptxS1*, *prn,* and *fhaB*, which confer important virulence characteristics to *B. pertussis*. The *rpoB* was used as a housekeeping gene, and the synthesised primers of the relevant gene regions are listed in Table 1.

The qPCR analyses were performed using the QuantiNova SYBR Green PCR Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The 20 μL PCR mixture contained 2X SYBR Green PCR master mix, 10 μM forward and reverse primers and 0.1 ng template DNA. All reactions were prepared as triplicate and analysed with RotorGene Q 5plex HRM (Qiagen, Hilden, Germany). PCR initial activation step of 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s was applied. Standard curves were formed by four logs dilution series and E values were generated. Reaction tubes without cDNA were prepared to control for nucleic acid contamination (non-template control). Melting curve analysis was carried out to analyse the accuracy of the experiments. qPCR analyses of each gene region were performed in triplicate. The virulence gene expression levels were normalised to the levels of *rpoB* gene transcripts. The relative expression levels of cultures with antibiotics were compared with cultures without antibiotics, and the data were analysed using the $2^{-\Delta\Delta CT}$ method.

### 2.7. Statistical analysis

Each test was performed in triplicate; results were expressed as the mean of 3 independent experiments. Statistical analysis was performed with one-way analysis of variance (ANOVA), and Tukey's test for multiple comparisons (IBM SPSS 22 software was used (SPSS, USA)) [26]. Values were considered statistically significant when $p < 0.05$.

## 3. Results

### 3.1. Determination of MIC levels of antibiotics

In this study, the MICs of the antibiotics erythromycin and azithromycin against the *B. pertussis* Saadet strain were determined (Table 2). Since the MIC of TMP-SMX for both *B. pertussis* strains could not be determined by the broth dilution method and E-test, the remaining experiments were performed with erythromycin and azithromycin. The *S. aureus* strain used as a control for the E-test was able to form an inhibition zone and the results were in accordance with the CLSI standard (Fig. S1) [27].

**Table 2**
MIC levels of antibiotics against *B. pertussis*.

| Strains | Azithromycin (μg/mL) | Erythromycin (μg/mL) | TMP-SMX (μg/mL) |
|---|---|---|---|
| Tohama-I | 0.08[a] | 0.2[a] | >32 |
| Saadet | 0.09 | 0.3 | >32 |

[a] MIC data of our previous study [15].

### 3.2. Effect of sub-MIC doses on the growth rate of bacteria

The data on the effects of antibiotics on the growth rate of *B. pertussis* at sub-MICs is shown in Fig. 1. Similar to a previous study with Tohama-I [15], it was observed in Saadet that the growth of the bacteria was significantly slowed at sub-MIC antibiotic doses, and growth was strongly inhibited in the presence of 1⁄2, 1⁄4, and 1⁄8MICs of antibiotics ($p < 0.05$). It was found that the growth rate of Saadet was close to control at MICs of 1⁄32 and 1⁄64 of azithromycin and MIC of 1/64 of erythromycin compared with other MICs of antibiotics. However, the bacterial growth of the samples at this MIC values was statistically significantly lower compared to the bacterial growth of the control group ($p < 0.05$).

### 3.3. Effect of sub-MICs on biofilm production

In this study, the ability of the Saadet strain to form a biofilm was investigated using the crystal violet assay at different sub-MIC levels of erythromycin and azithromycin (Fig. 2). Similar to our previous study with Tohama-I [15], the highest biofilm-forming capacity was observed in antibiotic-free cultures and was statistically different from all antibiotic-containing cultures ($p < 0.05$).

### 3.4. Effects of sub-MICs on gene expressions

To investigate the effect of erythromycin and azithromycin on the expression of *prn, ptxS1,* and *fhaB*, mRNAs were isolated in the mid-log phase, and it was found that sub-MICs of azithromycin and erythromycin had different effects on the expression of virulence genes (Fig. 3). In Tohama-I, the expression of *ptxS1* was statistically significantly decreased in all sub-MICs of erythromycin ($p < 0.05$). In Saadet, *ptxS1* was overexpressed at 1/16MICs of azithromycin and erythromycin; the fold change was 1.7 and 1.2, respectively ($p > 0.05$) (Table S1). In both isolates, the expression of *prn* was downregulated by erythromycin. However, the expression was significantly downregulated by azithromycin only in Saadet ($p < 0.05$). In contrast, in Tohama-I, the expression of *prn* was slightly increased by azithromycin ($p > 0.05$). In Tohama-I, *fhaB* expression was significantly upregulated by azithromycin and erythromycin in 1/32MICs ($p < 0.05$). In Saadet, it was significantly downregulated by azithromycin and 1/16MIC of erythromycin ($p < 0.05$).

**Table 1**
Primers used in qPCR analysis and their amplification product sizes.

| Gene | Primers | Amplicon size (bp) | References |
|---|---|---|---|
| *ptxS1* | Forward primer: 5′-TTCCAGAACGGATTCACGGC -3′<br>Reverse primer: 5′-TGCTGCTGGTGGAGACGAA -3′ | 112 | [23] |
| *prn* | Forward primer: 5′- TGCCGACTGGAACAACCA-3′<br>Reverse primer: 5′- GTCGGAGCCCTGGATATGG-3′ | 73 | [24] |
| *fhaB* | Forward primer: 5′-TGTCCGCCATGGAGTATTTCA- 3′<br>Reverse primer: 5′-CCCAAATGTACTCGTAGCGATTC-3′ | 153 | [25] |
| *rpoB* | Forward primer: 5′- GCTGGGACCCGAGGAAATCA-3′<br>Reverse primer: 5′- GCGCCAATGTAGACGATGC-3′ | 93 | [23] |

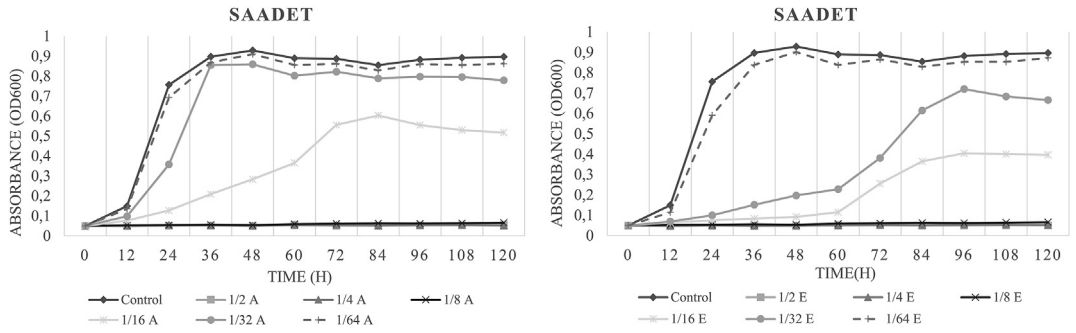


**Fig. 1.** The effects of sub-MICs on the growth rate (Control: *B. pertussis* grown in an antibiotic-free environment, A: Azithromycin, E: Erythromycin).

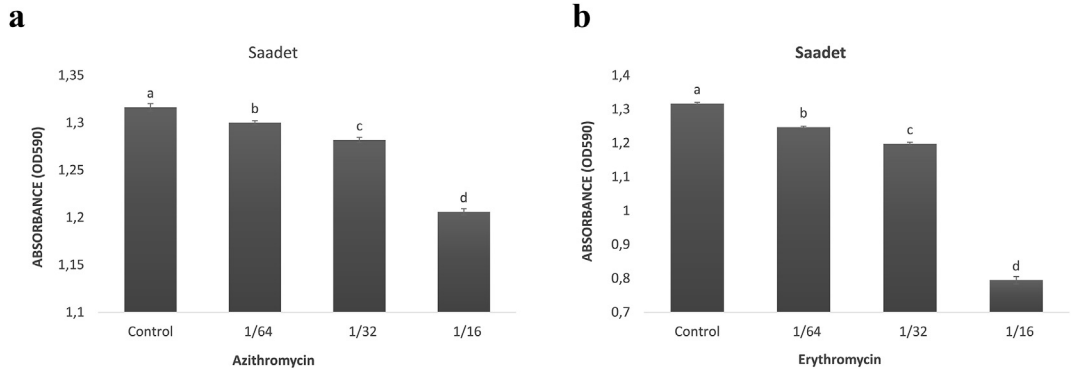


**Fig. 2.** The effect of sub-MICs on the biofilm (Control: *B. pertussis* incubated in an antibiotic-free environment, different letters in superscripts indicate a statistical difference ($p < 0.05$)).

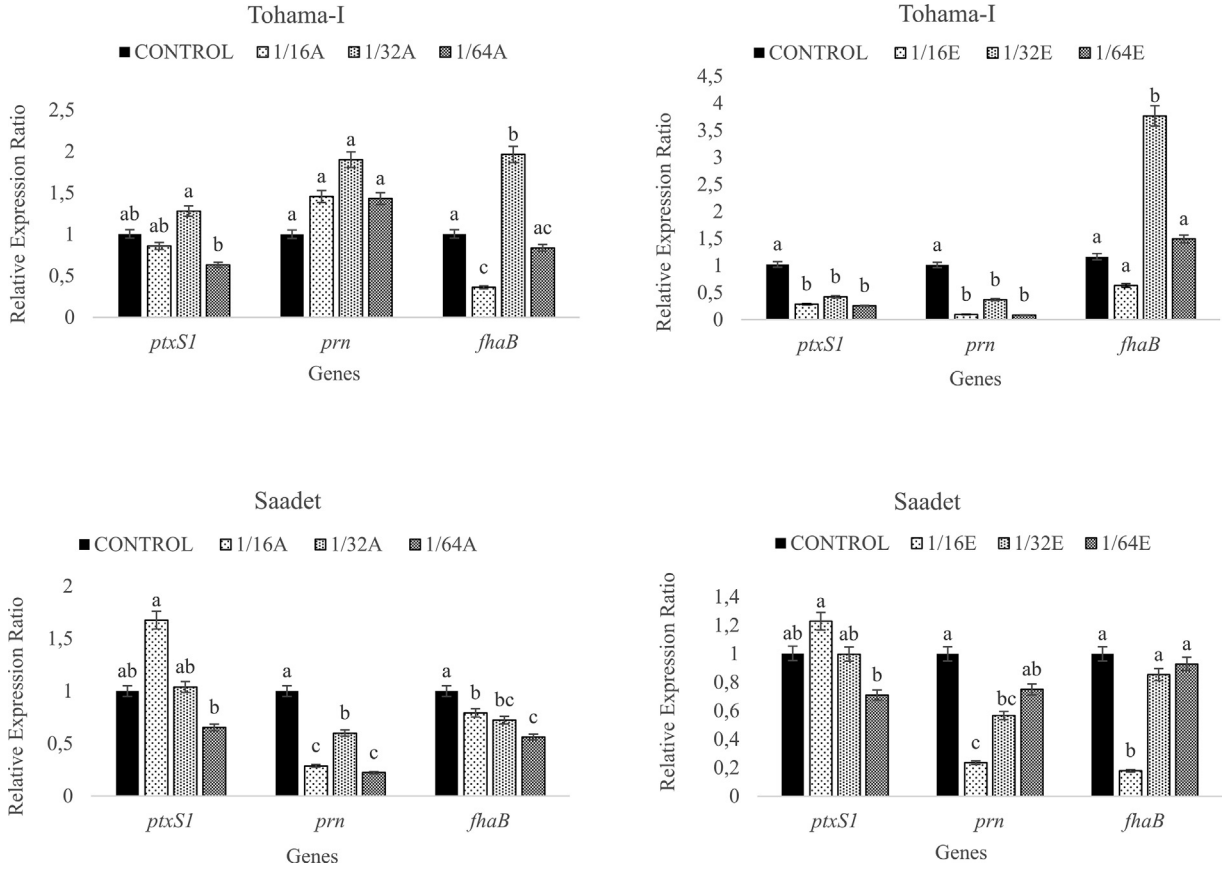


**Fig. 3.** Relative gene expression of *B. pertussis* in response to sub-MIC antimicrobial agents (Control: *B. pertussis* incubated in an antibiotic-free environment, A: Azithromycin, E: Erythromycin, *ptxS1*: Pertussis toxin, *prn*: Pertactin, *fhaB*: Filamentous haemagglutinin, different letters in superscripts indicate a statistical difference ($p < 0.05$)).

## 4. Discussion

TMP-SMX is still used for treating pertussis and is generally recommended for the treatment of patients 2 months of age and older who cannot tolerate macrolides or are infected with a *B. pertussis* strain resistants to macrolides [8,28,29]. Interestingly, according to the results, both Tohama-I and Saadet were highly resistant to this antibiotic. Similarly, Dorji et al. [30] investigated the antimicrobial susceptibility of 21 clinical *B. pertussis* isolates in Western Australia and reported that Tohama-I and all other isolates were resistant to TMP-SMX. However, Lönnqvist et al. [31] studied the prevalence and antimicrobial susceptibility of *B. pertussis* strains in Finland and reported that all the strains they used were susceptible to TMP-SMX. TMP-SMX was introduced in the 1970s, and there are few studies supporting the use of this antibiotic in the treatment of pertussis [8]. Henry et al. [32] found that bacteria could be re-isolated in patients after ten days of the 7-day treatment regime of TMP-SMX. The results of this study and the interpretation of other studies in the literature raise several questions regarding the effectiveness of TMP-SMX in treating pertussis.

In this study, Saadet was more sensitive to azithromycin than to erythromycin (Table 2). In previous studies, researchers also speculated that Saadet was superior to Tohama-I in terms of reproduction, toxigenicity, and antigenicity [33,34]. Lönnqvist et al. [31], in their study in Finland to determine the antimicrobial susceptibility of *B. pertussis* strains, found that the MIC levels of isolates ranged from 0.016 to 0.19 μg/mL for azithromycin and 0.016–0.25 μg/mL for erythromycin, and identified no isolates resistant to azithromycin and erythromycin. However, Mi et al. [13], in their study found that 78 of the 125 *B. pertussis* isolates they used were resistant to erythromycin and azithromycin (MIC>256 mg/L). Hua et al. [12] reported that 95 of 126 *B. pertussis* strains they used in their study were resistant to erythromycin, azithromycin, and clindamycin (MIC>256 mg/L). They also reported that a macrolide-resistant *B. pertussis* isolate was detected in four patients who did not receive antibiotic treatment.

There is not enough up-to-date study in the literature on the effects of sub-MIC doses of antibiotics on the growth rate of *B. pertussis* cells. Hewlett et al. [35] investigated the inhibitory and lethal effects of chlorpromazine for *Bordetella* species. They reported that 1/2 and 1/4MICs of this antibiotic inhibited *B. pertussis* growth. There are studies conducted with many different bacteria that have investigated the effects of sub-MIC doses of antibiotics on bacterial growth. Reeks et al. [36] stated that 1/8 and 1/16MICs of chlortetracycline and chlortetracycline-sulfamethazine significantly impaired the growth of *Mannheimia haemolytica* and *Haemophilus somnus*. Feng et al. [37] studied the effects of sub-MICs of ceftazidime on the pathogenicity of *Escherichia coli.* They stated that bacterial growth at 1⁄4 and 1⁄8MICs did not differ with the control group; therefore, they used 1⁄4MIC antibiotic doses in their other studies. Similarly, in this study, *B. pertussis* cells were able to tolerate 1⁄16, 1⁄32, and 1⁄64MICs. For this reason, the effects of these determined sub-MIC doses of antibiotics on the biofilm-forming capacity of *B. pertussis* were investigated.

Biofilms can play a role in bacterial resistance. Bacteria in a biofilm are generally 100–1000 times more resistant to important substances than corresponding populations of planktonic bacteria [38]. In the Saadet strain grown in an antibiotic-containing medium the amount of biofilm decreased in parallel with the increase in the antibiotics' doses ($p < 0.05$), similar to our previous study with Tohama-I [15]. In their study, Dorji et al. [30] classified the *B. pertussis* strains with absorbance values above 1.204 as strongly biofilm-forming strains. Accordingly, we also classified the control group in this study as a strong biofilm-forming strain because the absorbance was higher than 1.204. However, Saadet showed moderate biofilm formation at 1/16 and 1/32MICs of erythromycin (absorption value < 1.204). Cattelan et al. [39], showed in their study of *B. pertussis* strains in the USA and Argentina that the amount of biofilm produced by circulating strains was significantly higher than that of the Tohama-I. However, we have seen that the biofilm formation of Tohama-I and Saadet is similar in comparison.

The literature search shows that there has been no study on the effects of sub-MIC doses of antibiotics on the capability of *B. pertussis* cells to form biofilms. However, there are studies that have been conducted with many different bacterial species. For example, in their study on the effects of sub-MIC doses of azithromycin, curcumin, and gentamicin on *Pseudomonas aeruginosa* cells, Bahari et al. [40] reported that 1/4 and 1/16MICs significantly decreased the bacteria's ability to form biofilms and reported that biofilm formation decreased in parallel with the increase in sub-MIC used. Some studies have reported that sub-MICs of antibiotics induce biofilm formation in some bacterial species. Liu et al. [41] studied the effect of a sub-MIC dose of norfloxacin on biofilm-forming capacity and virulence gene expression in *Streptococcus suis*. They reported that bacteria formed more biofilms at a 1⁄4MIC of norfloxacin and that biofilm contained more live bacteria at this dose. Yousefpour et al. [42], in their study investigating the biofilm formation of *P. aeruginosa* at sub-MICs of gentamicin, reported that 31.3% of isolates at 1/2 and 1/4MICs of gentamicin showed a significant increase in biofilm formation ability. In this study, sub-MICs of azithromycin and erythromycin reduced the biofilm formation ability of *B. pertussis* cells.

Different types of antibiotics' low concentrations can act like chemical molecules and affect various processes, including pathogenicity. Liu et al. [43] mentioned that this interaction could affect strains with different resistance traits differently. Ptx, Prn, and Fha are important virulence factors of *B. pertussis*, and they are present in all pertussis vaccines. The literature review shows that there is no study on the effects of sub-MIC antibiotic doses on the gene expression of *B. pertussis* cells. Examination of gene expressions showed that azithromycin differentially affected the expression of the virulence genes of *B. pertussis* Tohama-I. While the *prn* gene was upregulated at all sub-MIC concentrations of azithromycin, the expressions of the *ptxS1* and *fhaB* genes were found to change with the concentration of the antibiotic. The expression of the *fhaB* gene was found to increase statistically significantly at a 1/32 concentration of azithromycin. The expression of the *ptxS1* and *prn* genes was also downregulated at the sub-MIC levels of erythromycin in the Tohama-I strain ($p < 0.05$). Expression of the *fhaB* gene showed a statistically significant increase at the 1/32 MIC level of erythromycin, just as with azithromycin. In the Saadet strain, gene expression was generally decreased at sub-MIC concentrations of azithromycin and erythromycin; only *ptxS1* gene expression was increased at 1/16 sub-MIC azithromycin and 1/16 sub-MIC erythromycin concentrations ($p > 0.05$). Atshan et al. [44] investigated the effects of daptomycin and tigecycline sub-MICs on the expression of genes involved in *S. aureus* adhesion and biofilm. They reported that sub-MICs caused an increase in some strains and a decrease in some strains in the expression levels of the genes they specifically studied. As with the results of this study, the sub-MIC dose of an antibiotic can increase the expression of one gene, while the same dose can decrease the expression level of another gene. Similarly, Sadredinamin et al. [45], in their study investigating the effects of sub-MIC azithromycin and ciprofloxacin doses on different virulence factors in *Shigella* serogroups, reported that in the presence of sub-MIC azithromycin in serotype 4a, the *icsA* gene was upregulated, while other genes in the *virF* pathway were downregulated. Navidifar et al. [46] investigated at the expression levels of genes involved in *Acinetobacter baumannii* biofilm formation in sub-MICs of meropenem and tigecycline. They founded

that the expression levels of genes in AB55 and AB13 strains did not regularly increase or decrease with the increase of tigecycline dose. Liu et al. [41], in their study investigating the effect of a sub-MIC dose of norfloxacin on biofilm formation and virulence gene expression of *S. suis*, reported that the expression of the *sly*, *ef*, *cps*, *gapdh,* and *gdh* genes increased in 1/4MIC, while the expression of the *mrp* gene decreased. In addition, gene expression did not increase or decrease in parallel with the increase in sub-MICs in this study.

In conclusion, the overuse and indiscriminate use of antibiotics have led to an increase in resistant bacteria around the world, which is a public health concern given the lack of effective antimicrobials, especially for children. Our findings indicated that although TMP-SMX is one of the antibiotics used in treating pertussis in some children, the Tohama-I and Saadet strains in this study were resistant to this antibiotic. In the present study, it is also reported the modulatory effects of sub-MIC doses of azithromycin and erythromycin on biofilm formation, growth rate, and virulence gene expression. It was observed that sub-MICs of antibiotics resulted in a decreased ability for biofilm formation and growth rate of *B. pertussis*. Furthermore, the effects of these doses on the expression of *ptxS1*, *prn*, and *fhaB* were quite variable, which can be explained by the different transcriptional changes that occur in the presence of each antibiotic at low concentrations. This bacteria may have different regulatory pathways in response to environmental stresses. More work is needed to determine the existence and regulation mechanisms of these pathways. Data in this study indicate that it is very important to study the vital activities and pathogenicity of *B. pertussis* exposed to sub-MICs of antibiotics. To obtain sufficient information on the contribution of sub-MIC doses of antibiotics to the treatment process of pertussis, the results obtained in this study should be supported by large-scale studies with more isolates and molecular experiments.

## Funding

This work was supported partly by The Scientific and Technical Research Council of Turkiye (TUBITAK 121Z656) and Akdeniz University Scientific Research Project Coordination Unit (FYL-2021-5606 and FYL-2021-5610).

## Declaration of Competing Interest

The authors have no conflict of interest to declare.

## Acknowledgements

The authors thank M.Sc. Mesut Cihan Aydemir for his support and advice.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2023.104058.

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