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ORIGINAL PAPER

RESURGENCE OF PERTUSSIS IN NORTHEASTERN BULGARIA DURING THE 2024-2025 EPIDEMIC: A MOLECULAR STUDY ON INCIDENCE AND MACROLIDE SUSCEPTIBILITY

Denis Niyazi[1,2(A,D,E,F)], **Marina Miroshnikova**[1,2(B,C)], **Milena Bozhkova**[1,2(B,C)], **Temenuga Stoeva**[1,2(A,D,E)]

[1]Clinical Microbiology Laboratory, University Hospital "St. Marina", Varna, Bulgaria
[2]Department of Microbiology and Virology, Faculty of Medicine, Medical University of Varna, Varna, Bulgaria

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Address for correspondence: Denis Niyazi, Department of Microbiology and Virology, Faculty of Medicine, Medical University of Varna, 55 Marin Drinov Str., 9002 Varna, Bulgaria, e-mail: denis.niyazi@gmail.com, phone: +359 52 677 050
ORCID: Denis Niyazi https://orcid.org/0000-0003-3666-3802, Marina Miroshnikova https://orcid.org/0009-0007-6495-0098, Milena Bozhkova https://orcid.org/0000-0002-9843-379X, Temenuga Stoeva https://orcid.org/0000-0001-6202-4532

Summary

Background. Despite being vaccine-preventable, pertussis is often detected, resulting in outbreaks, which is the case in many European countries, including Bulgaria, in 2024.

Material and methods. Nasopharyngeal swabs from symptomatic individuals from 6 regions (R1-R6) were tested by PCR. The DNA was extracted using the SaMag DNA Extraction Kit. Quantitative PCR was used to detect the macrolide resistance determinant and was further confirmed with melt curve analysis**.**

Results. 149 samples were tested at the Clinical Microbiology laboratory of the University Hospital "St. Marina", Varna, Bulgaria, between May 2024 and May 2025. Of them, 23 (15.4%) were positive for *Bordetella* DNA, and in 22, *Bordetella pertussis* infection was confirmed. Most of the positive cases were from R1 and were detected in the warmer months (n=18; 78.2%). Positive cases were prevalent in females (n=15, 65.2%), and the median age of positive individuals was 9 years (IQR: 1-11). The PCR and following melt curve analysis did not detect any macrolide-resistant cases.

Conclusions. This study demonstrates a rise in cases of pertussis (15.4%) in Northeastern Bulgaria, which is consistent with the national and European epidemiological reports. The most common mutation, associated with macrolide resistance in *B. pertussis* was not detected. Our results also confirm the seasonality of the disease and identified individuals between 9-10 years of age as the main risk group for contracting and developing this symptomatic disease.

Keywords: macrolide susceptibility, melt curve analysis, Northeastern Bulgaria, PCR, epidemic

Introduction

The *Bordetella* species (*Bordetella* spp.) is a Gram-negative, aerobic coccobacilli belonging to the genus *Bordetella*, a part of *Alcaligenaceae* family [1,2]. Several species are known to cause respiratory infections in humans (*Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica* and *Bordetella holmesii*), among which *Bordetella pertussis* (*B. pertussis*) is the most commonly isolated representative of the genus. The disease caused by *B. pertussis* is pertussis, also known as "whooping cough" for its characteristic cough. The other Bordetellae are associated with usually milder, pertussis-like infections [1]. The wide variety of virulence factors found in *B. pertussis* (fimbriae, filamentous hemagglutinin, pertactin, pertussis toxin, adenylate cyclase toxin, dermatonecrotic toxin,

tracheal cytotoxin, type 3 secretion system, lipopolysaccharides) aid in the colonization of the respiratory tract, confer resistance to immune cells and cause damage to the host organism [1,3].

Pertussis is a vaccine-preventable, highly contagious anthroponotic respiratory infection. In non-immune individuals, after an incubation period of 7-10 days on average, it progresses through three distinct stages [4]. The initial catarrhal stage (1-2 weeks), characterized by a runny nose, sore throat and conjunctivitis, is followed by the paroxysmal stage (2-6 weeks), which is marked by prolonged, recurrent episodes of distinctive coughing. The last convalescent stage is long and can take months to fully recover [2]. The infection typically causes epidemics every couple of years, despite the use and mandatory nature of vaccines in many countries [5]. In some cases, infection-related complications (pneumonia, encephalopathy) can occur, and the estimated global death rate is more than 160,000 cases annually in children below the age of 5 [6].

Prevention of the disease is of paramount importance. In Bulgaria, the vaccination against pertussis is mandatory and is given as a combined shot starting at two months of age and is followed by revaccination at three, four, and sixteen months and then at six and twelve years of age [7]. Two types of vaccines against whooping cough are available. The whole-cell vaccine, developed and introduced in 1940, led to a significant decline in the spread of whooping cough in many countries [1]. In Bulgaria, the vaccine was successfully introduced in 1957. Before the implementation of the vaccine in the immunization schedule, in the 1930s, 1940s and 1950s, there were between 4,000 and 12,000 cases of pertussis in Bulgaria per year [8]. Due to the association of the vaccine with rare but severe adverse reactions, its use was discontinued, and a new acellular vaccine containing different components of the bacterium was developed [9]. This vaccine was introduced in Bulgaria in 2010 [8]. Despite the better acceptance of this type of vaccine and fewer side effects, it has drawbacks related to rapid immune depletion and the need for revaccination [1].

Since the 1970s, erythromycin has been the recommended antimicrobial agent to treat pertussis [10]. Following the development of new macrolides (clarithromycin and azithromycin), erythromycin is no longer recommended due to its more frequent side effects [11].

In addition to virulence factors, *B. pertussis* can also exhibit resistance to antibiotics. The most problematic of these is resistance to macrolides [12]. Resistance to macrolides may be due to three mechanisms. The first and most common is associated with point A2047G mutation in the 23S rRNA gene [13], which prevents macrolides from binding to the large ribosomal subunit and inhibit protein synthesis [12]. Another mechanism is the acquisition of

the *erm* gene, which is associated with methylation of 23S rRNA and the inability of erythromycin to bind to the ribosome [14]. The expression of the MexAB-OprM efflux pump, which helps the bacteria to expel macrolides from the cell, is the third identified mechanism [15].

In May 2024, the European Centre for Disease Prevention and Control (ECDC) reported a significant increase in the incidence of pertussis between January and March 2024 in the EU (more than 32,000 cases) compared to 2023, when 25,000 cases were diagnosed [16].

In line with this, proven cases in Bulgaria also began to increase in early 2024, leading to the declaration of an epidemic situation from May 9 to July 31, 2024 [17], which was later extended to December 31 by the Ministry of Health [18].

Aim of the work

The aim of this study was to assess the dissemination of pertussis in Northeastern Bulgaria during the period May 2024 – May 2025 and to detect the presence of macrolide resistance using a molecular genetic method.

Material and methods

Nasopharyngeal swabs (NPSs) from individuals with clinical symptoms suspected of pertussis were tested in the Clinical Microbiology laboratory of the University Hospital "St. Marina", Varna, Bulgaria. The laboratory was chosen by the Bulgarian Ministry of Health to perform the testing for pertussis or to confirm positive cases of pertussis in Northeastern Bulgaria, an area with a population exceeding 1 million. Samples of hospitalized individuals and outpatients from Varna (R1) and another 5 regions (R2 – Dobrich; R3 – Shumen; R4 – Targovishte; R5 – Silistra) were examined. All NPSs were accompanied by informed consent from the patient or a parent/legal guardian if the individual was under 18 years of age.

The seasonality of pertussis was based on the positive samples for *Bordetella* sp. throughout the spring/summer period (March to August) and autumn/winter period (September to February).

Polymerase chain reaction (PCR) was used to diagnose pertussis. Nucleic acid from the samples was extracted by the automated nucleic acid extraction system SaMag-12 (Sacace, Italy), using the SaMag Bacterial DNA Extraction Kit (Sacace, Italy). A qPCR kit for *B. pertussis*, *B. parapertussis,* and *B. bronchispetica* detection (B84-100FRT, Sacace, Italy) was

used for DNA amplification. All tests were performed according to the manufacturer's recommendations.

The positive DNA extracts for *Bordetella* spp. were subjected to a second PCR for macrolide resistance determinant using Wang's protocol with some modifications [19]. FP (GTGATGGGGTGCAAGCTCTT), RP (TCTGGCGACTCGAGTTCTGC), and MP (ATCTACCCGCGGCTAGACAGG) oligonucleotides were used for detection of point A2047G mutation in the 23S rRNA gene. The PCR reactions were performed in 20 µl. The PCR mix included 10 µl of NZYSupreme qPCR Green Master Mix (2x) (NZYtech, Portugal), 10 µmol of each oligonucleotide (Metabion, Germany), 2 µl DNA template, and 5 µl purified DNase-free water (Thermo Fisher, USA). After the amplification protocol, melt curve analysis was performed to determine the wild-type or mutant variant of the alleles. The melt curves were generated by heating the amplicons at 0.5°C/s increments (from 65°C to 95°C). Samples producing only one product were considered carriers of wild-type allele. A Ct value of 16.4 was accepted as positive for DNA amplification. SaCycler-96 (Sacace, Italy) was used for all PCR tests.

Results

Between May 2024 and May 2025, a total of 149 non-repetitive samples were obtained from patients with clinical symptoms suspected of pertussis. 89 samples (59.7%) were collected in the spring/summer period and 60 (40.3%) in the autumn/winter period. Of these, 23 (15.4%) tested positive with qPCR (20 in 2024 and 3 in 2025). *B. pertussis* was confirmed in 22 cases. One patient was positive only for the *ptxA* gene, but based on the epidemiological data, the patient was considered having pertussis. No cases associated with *B. parapertussis* and *B. bronchiseptica* were detected.

The highest number of positive samples was from the Varna region (n=13, 56.5%), with females being the more dominant (n=15, 65.2%). The median age of positive individuals was 9 years (IQR: 1-11). The highest number of positive cases was in the spring/summer period of 2024 – 18 (78.2%).

21 out of all those positive for *Bordetella* spp. DNA extracts (21/23) were subjected to a second qPCR to detect macrolide resistance related to point A2047G mutation. Two samples were not tested due to poor storage. Wild allele was detected in 19/21 cases, and in 2, no amplification was observed. Melt curve analysis confirmed wild allele in all 19 isolates (Tm 84.2-84.6° C) (Figure 1, Table 1).

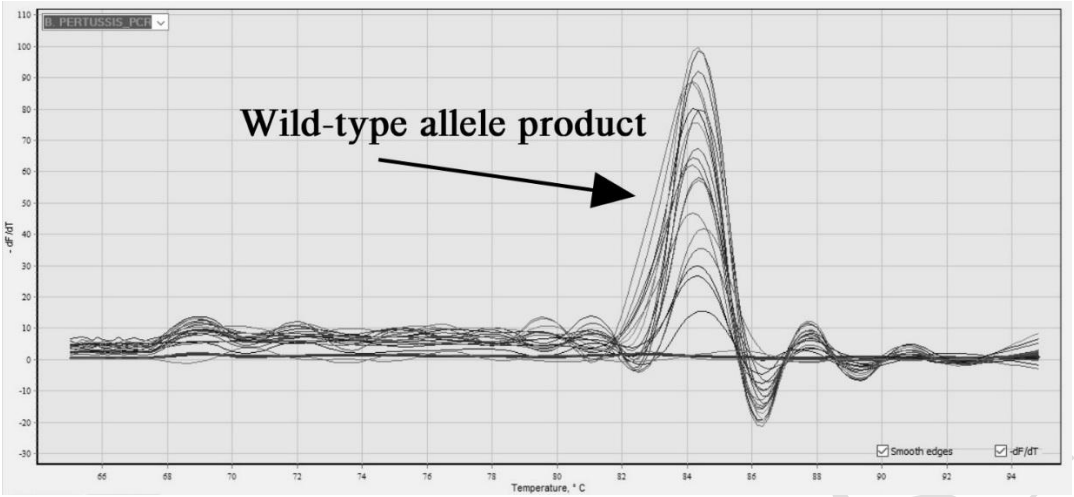

Figure 1. Melt curve analysis of PCR for detection of macrolide resistance in *Bordetella pertussis*

Table 1. Epidemiological and PCR data of the positive cases of pertussis

ID	Gender	Age (years)	Region	Month	qPCR (Ct)		PCR_{MR} + melt curve
					ptxA	*B. pertussis* DNA	
BP4_2024	M	11	R1	May	24.9	26.2	WT
BP8_2024	F	9	R1	May	27.1	27.8	NA
BP22_2024	F	9 m	R1	June	13.6	13.9	WT
BP33_2024	F	15	R1	July	19.4	19.5	WT
BP39_2024	M	15	R1	July	24.2	23.6	WT
BP42_2024	F	1	R1	July	21	20.2	WT
BP47_2024	M	3	R1	July	18.9	18.7	WT
BP48_2024	M	1	R1	July	18.2	17.4	WT
BP52_2024	F	6	R1	July	13.4	13.1	WT
BP100_2024	F	1	R1	October	20.8	20.6	WT
BP3_2025	F	4 m	R1	January	22.3	22.5	WT
BP6_2025	F	11	R1	January	19.7	21.7	WT
BP8_2025	M	10	R1	February	30.5	ND	NA
BP21_2024	M	12	R2	June	12.2	13.5	WT
BP23_2024	F	68	R2	June	24.1	24.6	WT
BP2_2024	F	4	R3	May	19.2	18.4	WT
BP3_2024	M	12	R4	May	19.2	18.1	WT
BP62_2024	F	6	R4	August	24.5	24.8	*
BP63_2024	F	6	R4	August	28.8	28.5	*
BP71_2024	M	8 m	R4	September	14	14.8	WT
BP6_2024	F	10	R5	May	21	21.9	WT
BP7_2024	F	10	R5	May	23.9	23.7	WT
BP18_2024	F	10	R5	June	24.7	24.4	WT

Notes: F – female, M – male, m – months, NA – not amplified, ND – not detected, WT – wild-type, * – PCR not performed, Ct – cycle threshold, *ptxA* – pertussis toxin gene, PCR_{MR} – PCR to detect macrolide resistance, R1 – Varna, R2 – Dobrich, R3 – Shumen, R4 – Targovishte, R5 – Silistra.

Discussion

Although whooping cough is a vaccine-preventable infection, the pathogen often circulates, and epidemics occur every 3-5 years [5]. This is the case in many European countries, including Bulgaria, with over 2,700 Bulgarian cases reported in 2024. According to the National Centre of Infectious and Parasitic Diseases (NCIPD), the incidence of pertussis during the 2015-2023 period in Bulgaria ranged between 3 and 116 cases per year, while the cases reported in 2024 amounted to 2,721. Of these categorized as proven were 2,588 cases, with 72 probable and 61 reported as possible cases [20]. With the dramatic increase in cases in the country, healthcare authorities declared an epidemic situation and began mass testing. Their recommendations included testing of nasopharyngeal secretions from suspected individuals using quantitative PCR. The rate of positive samples, confirmed in our laboratory by qPCR, from 6 regions in Northeastern Bulgaria was 15.4%. Our results significantly exceed the rate of positive cases in a similar Italian study (5.7%) [21] but are in concordance with the report of Hitz et al. for pertussis cases in Germany (15%) [22]. Similar to these studies, our positive cases were more frequent during the warmer months (78.2%), which is also consistent with national data [20]. Before the introduction of the pertussis vaccine, the disease followed a seasonal pattern, with most cases detected in spring/summer months [23]. After implementation of the immunization practice, the seasonality almost completely disappeared [24], only to reappear decades later [25]. Some authors speculate that the spring/summer increase of cases might be associated with the age group of infected individuals, waning immunity, and the less common occurrence of other respiratory infections in this period or better surveillance and diagnostic practices [22]. In our study, female individuals predominated, which is also in parallel with the report of NCIPD, while in the Italian study, positive cases were evenly distributed according to the gender of the individuals.

The median age of positive cases confirmed in our study was 9 years (IQR: 1-11). This is similar to the data reported by Hitz et al. (8 years; IQR: 3-12) [22] and to the findings reported by the Bulgarian National Center for Public Health and Analysis (10-14 age group) [20]. It is well-known that with the newer pertussis vaccines, the immunity wanes quickly after the initial vaccine doses, which could be the reason for the higher rate of positive individuals in this age

range detected in our study. Thus, European (ECDC) and American (CDC) healthcare authorities recommend additional boosters with Tdap (low dose pertussis vaccine) at the age of 11-12 [26,27]. In our study, information on vaccination status was not disclosed by those who sent the samples, but given the age of the individuals, we can only assume that they were not vaccinated or were partially vaccinated.

In our study, we used qPCR with melt curve analysis to determine macrolide resistance associated with the most common mechanism – point A2047G mutation in the 23S rRNA gene. No isolates containing the mutation were detected. Two of the isolates did not show amplification. A similar result was observed by Wang et al. [19]. The authors speculate that the lack of amplification may be due to the low amount of DNA in the sample. In our study, the cycle threshold (Ct) for the two samples in the first PCR was 27.1 and 30.0, respectively, which is the most likely reason.

The first case of a macrolide-resistant isolate was reported in 1994 in the United States in a sample of a 2-month-old child [28]. Cases associated with similar isolates have also been reported in China, the first being in 2011 [29]. Fu et al. conducted research on the molecular evolution and macrolide resistance of *B. pertussis* isolates (n=283) collected in Shanghai, China between 2016 and 2022 [30]. The study revealed a sharp rise in macrolide resistance from 36.4% in 2016 to 97.2% in 2022, driven by the A2047G mutation in the 23S rRNA gene. The authors used various methods such as PCR, multilocus variable-number tandem-repeat analysis (MLVA), genotyping, and DNA sequencing to uncover the relation and the evolution of the circulating isolates. Initially, macrolide-resistant strains (MRBP) were mainly *ptxP1* allele-positive and belonged to the MT195 type, but after 2020, *ptxP3*-MRBP of the MT28 type emerged rapidly and became dominant. The transition from *ptxP1*- to *ptxP3*-positive strains is worrisome as the latter is associated with increased pertussis toxin production. This genetic shift was accompanied by a change in patient age distribution – from mostly infants before 2020 to more older children afterward (similar to our study). The authors hypothesize that the change is likely due to vaccine-driven selection and heavy macrolide use [30]. Relying on the reports of MRBP in China, current guidelines no longer recommend using macrolides as a first treatment option, with alternative antimicrobials (trimethoprim/sulfamethoxazole, SXT) being preferred instead [2,31]. In their study, Fu et al. report preserved SXT susceptibility of all tested *B. pertussis* isolates [30]. Macrolide-resistant isolates have also been documented in Europe [32]. Almost all of the cases were exclusively associated with the A2047G mutation. The other two mechanisms (*erm* gene and MexAB-OprM efflux pump) have not been detected in *Bordetella pertussis* at present [12].

Conclusions

This study demonstrates a rise in cases of pertussis (15.4%) in Northeastern Bulgaria, which is consistent with national and European epidemiological reports. The most common mutation, associated with macrolide resistance in *B. pertussis*, was not detected, a finding confirming that macrolides are still the drugs of first choice in our region. Our results also confirm the seasonality of the disease and identified individuals between 9-10 years of age as the main risk group for contracting and developing this symptomatic disease.

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All the tests have been performed in accordance with the principles stated in the Declaration of Helsinki and informed consent was obtained from all tested individuals or their families.

Artificial intelligence (AI) was not used in the creation of the manuscript.

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