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Whooping cough in South-East Romania: a 1-year study

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ABSTRACT

The incidence of whooping cough in Romania is substantially underestimated, and, as noted by the health authorities, this is mostly due to the lack of both awareness and biological diagnosis. We conducted a 1-year study in Bucharest in order to assess the circulation of *Bordetella pertussis*, the main etiological agent of whooping cough. Fifty-one subjects suspected of whooping cough were enrolled. Culture, real-time PCR, and enzyme-linked immunosorbent assay were used for laboratory diagnosis. Whooping cough patients (63%) were distributed among all age groups, and most were unvaccinated, incompletely vaccinated, or had been vaccinated more than 5 years previously. *Bordetella holmesii* DNA was detected in 22% of the bordetellosis cases; these patients included adults; teenagers; and, surprisingly, young children. *B. pertussis* isolates were similar to the clinical isolates currently circulating elsewhere in Europe. One isolate does not express pertactin, an antigen included in some acellular pertussis vaccines.

1. Introduction

Bordetella pertussis and *Bordetella parapertussis* are the etiological agents of whooping cough, a highly contagious human respiratory disease. After the introduction of mass immunization in infants in the 1950s, with whole-cell pertussis vaccine (wP), the incidence of whooping cough decreased substantially in the child population (Edwards and Decker, 2008; Mattoo and Cherry, 2005; Zepp et al., 2011).

Mass immunization against pertussis was introduced in Romania in 1961 using a local diphtheria-tetanus–whole cell pertussis vaccine (DTwP). The primary immunization schedule included 3 vaccine doses (at 2, 4, and 6 months of age) followed by 2 boosters, at 12 and 30–35 months of age (Lutsar et al., 2009). In 2008, DTwP was replaced by acellular vaccines and 2 other changes in the vaccination program followed in 2010 and 2012. According to the current national program (http://www.ms.gov.ro/?pag=133), the children are vaccinated with acellular vaccine when 2, 4, 6, and 12 months old and 1 booster is administered at 6 years of age. Official reports indicate that the vaccination coverage is under the target of 95% with a significant decrease (88.2%) registered for the cohort of July 2011 (Popovici, 2013a). The data collected by the Romanian Ministry of Health for the last 14 years describe a median incidence of whooping cough of around 0.4 per 100,000 inhabitants. Peak values in pertussis incidence were recorded in 2000, 2004, and 2008. In 2012, the year we conducted this study, the estimated incidence of whooping cough was the same as the median value for the last 14 years (Popovici, 2013b). As Romanian health authorities state, pertussis incidence values have been greatly underestimated. The relevant epidemiological analyses were biased due to under-reporting of cases, assessing only the clinical diagnosis, disregarding adults as potential whooping cough patients, and the use of laboratory diagnosis methods with poor sensitivity and specificity.

To improve the description of the circulation of *B. pertussis* in Romania, we performed a 1-year pilot surveillance study involving the "Dr Victor Babeş" Clinical Hospital for Infectious and Tropical Diseases in Bucharest, 3 general practitioners, the "Cantacuzino" National Institute of Research-Development for Microbiology and Immunology (National Reference Centre for Pertussis, and Molecular Epidemiology Laboratory), and Institut Pasteur in Paris. The project also aimed to improve biological diagnosis performed by the Romanian National Reference Centre.

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2. Materials and methods

2.1. Patients and samples

Suspected patients were addressing to "Dr Victor Babeş" Clinical Hospital for Infectious and Tropical Diseases or to a general practitioner facility both in Bucharest.

The criteria for enrolment were cough lasting for more than 1 week and at least 1 of the following symptoms: paroxysmal cough, nocturnal cough, post-tussive emesis, fever, apnea, or facial cyanosis. A single asymptomatic individual was enrolled because she was a contact of a possible whooping cough case. Clinical data were collected from the questionnaire that accompanied the specimens. Clinical variables recorded included gender and age, the clinical symptoms listed above, contact with a laboratory-confirmed case, administration of antibiotics prior to sampling, and immunization history. Nasopharyngeal swabs (NPS) and sera were collected from suspected whooping cough patients. Physicians were trained to perform NPS sampling correctly (http://youtu.be/d6d-y7SX_dY), and recommended collectors were used (Copan, 482CE; Brescia, Italy).

The patients/legal guardians were informed about the study; they signed a consent form, and the study was carried out in an anonymous way.

2.2. Culture

NPSs were plated on Bordet Gengou agar (Difco, Le Pont-de-Claix, France) supplemented with 1% glycerol (Calbiochem, Darmstadt, Germany) and 15% sheep blood and incubated at 37 °C. All the samples were tested in duplicate on media containing and not containing 1% cephalexin. The plates were visually inspected every day for 1 week.

2.3. Characterization of the isolates

Isolates were identified on the basis of colony aspect, morphological characteristics, and biochemical properties. Susceptibility to macrolides (azitromycin, clarithromycin, erythromycin) and sulfamethoxazole/trimethoprim was tested by a disc diffusion method.

DNA pulsed-field gel electrophoresis (PFGE) fingerprinting was performed as previously described (Caro et al., 2005).

DNA was extracted from the isolates with DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany). The repeated regions I and II of the *prn* gene encoding pertactin (PRN), the region encoding the S1 subunit of pertussis toxin (PT), and the *ptxA* promoter (*ptx*A-Pr) were used for genotyping (Hegerle et al., 2012). The PRN open reading frame was amplified and sequenced using primers described previously (Hegerle et al., 2012).

The production of each PT, adenylate cyclase-hemolysin (AC-Hly), the other major toxin produced by *B. pertussis*, PRN, and filamentous hemagglutinin (FHA) were assessed by Western blotting with specific murine polyclonal sera (Weber et al., 2001). Monoclonal antibodies were used to detect fimbria 2 (FIM2) and fimbria 3 (FIM3) proteins (Guiso et al., 2001).

2.4. Real-time PCR

DNA was extracted from 300 μL aliquots of Amies transport medium provided with the NPS collector, after collection of samples, using High Pure PCR Template Kits (Roche, Mannheim, Germany) following the instructions supplied with the kit.

The presence of *Bordetella* strains harboring IS*481* and IS*1001* was assessed by real-time PCR using Argene kits (catalog no. 69-0011B and 71-012; Argene, Verniolle, France) according to the manufacturer's instructions. *B. pertussis* DNA was then retrospectively identified using primers and probes targeting the *ptx*A-Pr as previously described (Njamkepo et al., 2011). *Bordetella holmesii* DNA was detected using primers and probes targeting the H-IS*1001*–like insertion sequence (Tatti et al., 2011). The limits of detection by real-time PCR in our conditions were 30 CFU for the in-house *ptx*A-Pr–based PCR and 1 CFU for the in-house H-IS*1001*–based PCR (Njamkepo et al., 2011).

2.5. Serology: enzyme-linked immunosorbent assay (ELISA)

Anti-pertussis toxin IgG antibodies were detected and quantified using the in-house reference ELISA method (Simondon et al., 1998). The purified PT was kindly provided by Sanofi Pasteur, and the reference serum was purchased from NIBSC as recommended (Guiso et al., 2011). The criteria used to confirm the disease were those proposed previously (Riffelmann et al., 2010). Briefly, a value of anti-PT IgG below 40 IU/mL indicates no recent contact with *B. pertussis* strains. A second serum sample collected after an interval of 3 weeks is required if the value is between 40 and 100 IU/mL. A value greater than 100 IU/mL confirms a recent infection, unless the individual has been vaccinated during the previous year.

We also purchased and used a new commercial kit (cat. no. 1231-01, SeroPertussis Toxin IgG; Savyon® Diagnostics Ltd., Ashdod, Israel) designed to detect anti-PT IgG antibodies. The objective was to compare this kit with the in-house reference ELISA in terms of specificity, sensitivity, and rapidity; the performance of this kit had not been independently and rigorously tested. This ELISA was performed manually according to the instructions given in the package insert. Three different investigators tested 15 sera in duplicate (6 positive sera with values between 52 and >400 IU/mL and 9 negative sera) and 2 batches of the kit. Intra-assay agreement was good, and the differences never exceeded 17%. Sixty-five sera from patients confirmed to have *B. pertussis* infections by culture or PCR or non-infected were tested.

3. Results

3.1. Patients and samples

Fifty-one patients were enrolled in the study between February 2012 and January 2013. Forty-six individuals were enrolled by the hospital, and 5 were recruited by the general practitioners. Most of the individuals were living in Bucharest (29), and the others lived in counties in the South-East of Romania: Călăraşi (8), Ilfov (6), Buzău (5), Constanţa (1), Dâmboviţa (1), and Ialomiţa (1). NPSs were collected from all the 51 patients enrolled. Blood was obtained from 35 patients at the same time as NPS sampling. Seventeen of the 35 individuals returned after approximately 21 days to provide a second blood sample.

3.1.1. Diagnosis of B. pertussis infections

B. pertussis infection was confirmed in 32 (63%) of the 51 patients by culture, specific real-time PCR, and testing for anti-PT antibodies. Four *B. pertussis* infections were diagnosed by culture, specific real-time PCR, and IgG anti-PT detection; 6, by specific real-time PCR and IgG anti-PT detection; 4, by specific real-time PCR only; and 18, by detection of anti-PT antibodies only (12/18 cases based on single-sample serology).

The age of the patients ranged from 3 months to 75 years old (Table 1). All the patients, except a contact of a possible whooping cough case, were symptomatic. Paroxysmal cough was reported for 94% of the patients, nocturnal cough for 81%, post-tussive emesis for 53%, cyanosis for 25%, apnea for 25%, and fever for 12.5%. All the symptoms, except fever, were less frequent among non-confirmed cases: paroxysmal cough (84%), nocturnal cough (58%), post-tussive emesis (42%), apnea (11%), and fever (16%).