ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2020; 25(4): 1696-1670
doi: 10.25083/rbl/25.4/1696.1670

Received for publication, September, 1, 2018
Accepted, October, 2, 2018

Original paper

Methods used for direct detection of Bordetella pertussis infections in Romania

GEORGETA CRISTINA OPREA[1,2], SORIN DINU[1], MARIA DAMIAN[1], VERONICA LAZĂR[2]

[1]Cantacuzino National Institute for Medico-Military Research – Development, Bacterial Respiratory Infections Laboratory, Bucharest, Romania
[2]University of Bucharest, Faculty of Biology, Bucharest, Romania

Abstract Vaccination against whooping cough was introduced in Romania in 1961, using the whole-cell vaccine, until 2008, when it was replaced by the acellular pertussis vaccine. The number of infections decreased continously since the introduction of the vaccine, but the number of cases identified in non-vaccinated individuals has been increasing during the last decade. During all this period, the diagnostic method used was mainly based on specific antibodies detection , but the number of cases is considered underreported or misinterpreted. It is known that this indirect method cannot be used for diagnosing vaccinated persons or, whenever a rapid identification is necessary. These methods are also not useful in all cases, due to the antigenic variation in bacterial strains, if the population was vaccinated with the acellular vaccine, or when it is less effective in immunodeficient individuals. The purpose of this study was to comparatively evaluate the results of different molecular methods for direct identification of *Bordetella pertussis*, such as conventional PCR targeting the *ptx S1* region and *ptxP* (pertussis toxin promoter), real-time PCR and, on the other hand, the results of the conventional methods, namely the direct method, based on pathogen isolation and cultivation on specific medium.

Keywords Whooping cough, real-time PCR, pertussis toxin, pertussis vaccination.

To cite this article: OPREA GC, DINU S, DAMIAN M, LAZĂR V. Methods used for direct detection of *Bordetella pertussis* infections in Romania. *Rom Biotechnol Lett*. 2020; 25(4): 1696-1700. DOI: 10.25083/rbl/25.4/1696.1700

*Corresponding author: GEORGETA CRISTINA OPREA, Cantacuzino National Institute for Medico-Military Research – Development, Bacterial Respiratory Infections Laboratory, Bucharest, Romania
E-mail: crisstina_bio@yahoo.com

Introduction

Pertussis or whooping cough is a highly contagious respiratory infection, caused by the Gram-negative, aerobic coccobacillus *Bordetella pertussis*, an infection considered the most prevalent of all vaccine preventable diseases (HEGERLE & GUISO [1]). This disease affects especially unvaccinated children and is associated with severe symptoms in infants aged less than 1 year, but there are also pertussis reported cases in adolescents and adults (HIGGS & al [2]); BARKOFF & al [3]); CHLIBEK & al [4]). Immunoprophylaxy by vaccination has been implemented worldwide, using the whole-cell vaccine or the acellular vaccine, containing inactivated pertussis toxin or detoxified pertussis toxin (PT), using different chemical agents (hydrogen peroxide, formaldehyde or glutaraldehyde) or genetically detoxified toxin, which does not alter the functional and immunological properties of PT (SEUBERT & al [5]). The acellular vaccine may also contain other components obtained from *B. pertussis* virulence factors, in fact bacterial adhesins which confer to the pathogen the ability to adhere and colonize the epithelium of the respiratory tract: filamentous hemagglutinin (FHA), pertactin (PRN) and fimbrial proteins Fim 2 and Fim 3 (AUSIELLO & al [6]). Other important virulence factors are represented by tracheal colonization factor (TcfA), the adenylate cyclase toxin (ACT), tracheal citotoxin (TCT) and dermonecrotic toxin (DNT). The FHA binds to the galactose residues of the sulfatides from the surface of the ciliated cells, the fimbrial proteins mediate the adherence to the sensitive substrate and pertactin binds to the epithelial cells through the RGD (Arg-Gly-Asp) motifs. The main toxins involved in *B. pertussis* pathogenesis are: adenylate cyclase which enters the mammalian cells and reduces the phagocytic activity, and pertussis toxin which is important in the initial colonization and toxemic stages of the disease (TODAR [7]).

In Romania, the vaccination against whooping cough using the whole-cell vaccine was introduced in 1961 and the doses were administered at 2, 4, 6 months of age and boosters at 12 and 30-35 months (LUTSAR & al [8]). Since 2008, the whole-cell vaccine was replaced by the acellular vaccine with the same vaccination schedule until 2010, when the second booster was introduced at the age of 4 years old. Starting with 2015, another change in the vaccination program occured, with doses administered at 2, 4, 11 months of age and a booster at 6 years old. Vaccination coverage in our country during the last decade and the incidence of pertussis have been reported in an epidemiologic study describing the situation af whooping cough in Central and Eastern European countries. The study revealed a lower vaccination coverage in Romania (<90%) than in other Eastern European countries (HEININGER & al [9]). Although DTaP vaccine is included in the national surveillance programs, the highest incidence of pertussis (5,1‰) confirmed for 0-4 year-old children has been reported in 2016 and a percentage of 79% of the confirmed cases were non-vaccinated children for the same year (POPOVICI [10]).

The diagnosis of *B. pertussis* infection in our country is performed by conventional methods, based on pathogen isolation and cultivation and serological methods, based on specific antibodies detection. More recently was introduced the molecular method, namely real-time PCR, as demonstrated by a previous study regarding the molecular identification of *B. pertussis* in Romania (DINU & al [11]). The conventional methods have some inconveniences: the bacterial cells grow slowly on Bordet-Gengou agar, requiring 3 to 6 days for culture (HEGERLE & GUISO [1]). At the same time, the eventual treatment with antibiotics already initiated may influence the bacterial growth (CRAIG-MYLIUS & WEISS [12]), leading to false negative results. Molecular identification of *Bordetella* species using real-time PCR is recommended for rapid identification of the pathogen, targeting genes encoding for insertion elements: IS*481* for *B. pertussis*, *B. Bronchiseptica* and *B. holmesii*, IS*1001* for *B. parapertussis* / *B. bronchiseptica* or *recA* for *B. holmesii* (MARTINI & al [13]). In case of *Bordetella sp.* infections, IS*481* can be identified by real-time PCR targeting a 181 bp region of this insertion sequence (WHO [14]). The molecular identification of pertussis toxin, which is specific for *B. pertussis* can be performed either for structural subunit S1 (also known as subunit A) or pertussis toxin promoter (*ptxP*) using conventional PCR. This promoter regulates the expression of *ptl* (pertussis toxin liberation) genes located downstream of the *ptx* genes (RAMBOW-LARSEN & WEISS [15]).

The aim of this study was to correlate the conventional methods used for identification of *Bordetella sp.* infections with the molecular methods, in order to optimize the direct diagnostic of the whooping cough and to isolate new *B. pertussis* strains.

Materials and Methods

Patients and samples

Nasopharyngeal swabs were collected from 20 patients with prolonged coughing and other associated symptoms, suspected of *Bordetella pertussis* infection, who presented from August 2013 to February 2015 to a clinical hospital in Bucharest. Most of the patients were children aged 2 months to 15 years old, seven patients were adults from 20 to 33 years old and one patient was 58 years old. Antimicrobial treatment was recommended and already initiated for 15 of the studied patients, nine of them receiving macrolides (Table 3).

Culture

The nasopharyngeal nylon flocked swabs (Copan, Italy) were sent to Cantacuzino Institute (at the National Reference Center for *Bordetella*) and then were plated on Bordet-Gengou agar selective medium (Liofilchem, Italy)

supplemented with 1% glycerol, 15% sheep blood and 1% cephalexin (Liofilchem, Italy) and used for primary isolation of *Bordetella pertussis*. The samples were also plated on Bordet-Gengou agar without antibiotic and incubated at 37°C for 3-6 days, with daily examination. Biochemical testing for the isolated strains included oxidase test using oxidase strips (Oxoid, UK), catalase test using hydrogen peroxyde and urease test using urea broth (Calbiochem, Germany).

DNA extraction

DNA was extracted using High Pure PCR Template Preparation Kit (Roche – Mannheim, Germany), from 300 µL of Amies medium provided with the swab. We added 7,5 µL of 10 mg/mL lysozyme and 60 µL proteinase K solution contained in the extraction kit for each sample. DNA was eluted in 200 µL elution buffer. For the extraction of presumed *B. pertussis* DNA, we used 10 µL of Internal Control (IC2) contained in the real-time PCR kit (cat. no. 69-0011B Argene, Verniolle, France).

The conventional PCR was performed on DNA extracted from *B. pertussis* strains using the same kit as above.

Real-time PCR

Real-time PCR was performed using the kit afo-rementioned, according to the manufacturer`s instructions, with the primers contained in the reaction mix and TaqMan probes 6-carboxyfluorescein labeled (FAM) for *Bordetella sp*. DNA and cyanine-3 (CY3) labeled for the internal control.

End-point PCR

The detection of IS*481* cannot confirm the existence of *B. pertussis* infection, because this sequence is not species-specific. In order to identify specific targets from *B. pertussis* DNA, molecular identification using con-ventional PCR was performed for the S1 subunit of pertussis toxin (*ptx-S1*) and also for the promoter region of pertussis toxin gene (*ptx-P*) (Table 1).

Table 1. Primer sequences used in conventional PCR reactions (Schouls & al [16])

Identified gene	Forward primer	Reverse primer
ptxP (5' to 3')	AATCGTCCTGCTCAACCGCC	GGTATACGGTGGCGGGAGGA
ptx-S1 subunit (5' to 3')	CCCCTGCCATGGTGTGATC	AGAGCGTCTTGCGGTCGATC

For the PCR reactions, in-house protocols were implemented, using Promega kit (Madison, USA); for *ptxP*, the reaction mixture contained: 5 µL PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 pmol/µL of each forward and reverse primer/reaction, 1.25U Taq polymerase and 14.8 µL DN-ase free water. Then 1 µL of extracted DNA was added in the reaction mixture for each sample in a final working volume of 25 µL. The amplification protocol was set as described in table 2.

Bordetella pertussis reference strain ATCC 9797 was used as a positive control for the reaction and nuclease-free water as a negative control.

Table 2. Thermal profile used for the amplification of *ptxP* promoter region

Step	Temperature	Time (minutes)
Initial denaturation of DNA double strand	95°C	2
Amplification (x 30 cycles)	Denaturation at 95°C	0.5
	Primer annealing at 57°C	1
	Polymerization at 72°C	1
Final elongation of DNA double strand	72°C	5

The PCR amplification was performed on a Corbett Research thermocycler. The amplification of S1 subunit of *pertussis toxin* gene was performed using the same protocol as for *ptxP*, except that the annealing of specific primers indicated in table 2 was set at 59°C.

Results and Discussion

Real-time PCR was positive for IS*481* in case of 11 of the tested patients, demonstrating the infection with *Bordetella spp*. for 5 children and 6 adults, four of them being correctly treated with macrolides. Among the children clinically suspected of having whooping cough, seven were not confirmed as harbouring *Bordetella* DNA using any of the methods used (Table 3).

The other 9 patients which were positive for IS*481*, were only identified as *Bordetella sp.* infection, because the conventional PCR performed on DNA extracted from the nasopharyngeal swab could not give relevant results that

could be interpreted as negative for *ptxP*, and thus for *B. pertussis* infection.

Bordetella pertussis was isolated on Bordet-Gengou agar supplemented with 15% sheep blood and 1% glycerol, from two patient samples, the results for IS*481* identified by real-time PCR, *ptxP* and S1 subunit of pertussis toxin identified by conventional PCR being also positive (Fig. 1). The two patients with positive culture were children aged 2 and respectively 4 years old, treated with other antibiotics, than macrolides. This indicates that only by clinical examination, with no laboratory confirmation, the diagnosis of *B. pertussis* infection could be sometimes confused with other respiratory infections (WIRSING von KONIG & al [17]). The results also indicate that the culture is not positive for all the IS*481* positive patients, only 2 out of the 11 patients with *Bordetella sp*. infection, being confirmed as *B. pertussis* positive, using culture and conventional PCR. This could be due to the fact that the antibiotic received by the infected patients did not make possible the isolation of *Bordetella sp*. on Bordet-Gengou agar or the sampling of the nasopharyngeal swab was done at more than one month after the cough onset (LEE & al [18]).

Oxidase and catalase tests were positive in case of both isolated strains and urease test was negative, confirming *B. pertussis* identification.

Table 3. Data regarding the patients' distribution, anamnesis and results of the specific molecular test for *Bordetella sp.* infection

Grouping criteria of investigated patients	**Different groups of patients**	**Patients positive for IS *481***	**Patients with other respiratory infections (non-*Bordetella*)**
Age	Children	5	7
	Adults	6	2
Gender	Male patients	4	2
	Female patients	7	7
Vaccination history	Vaccinated children	1	2
	Incompletely vaccinated children	1	2
	Unvaccinated children	3	3
	Adults vaccinated in childhood	#NA	1
Clinical symptoms	Prolonged coughing	7	3
	Paroxysmal coughing	6	7
	Nocturnal coughing	5	5
	Cyanosis	0	0
	Post-tussive vomiting	2	2
	Patients with apnea	1	0
	Patients with fever	1	3
Treatment	Patients treated with macrolides	4	5
	Patients treated with other antibiotics	2	4

#NA=not-available

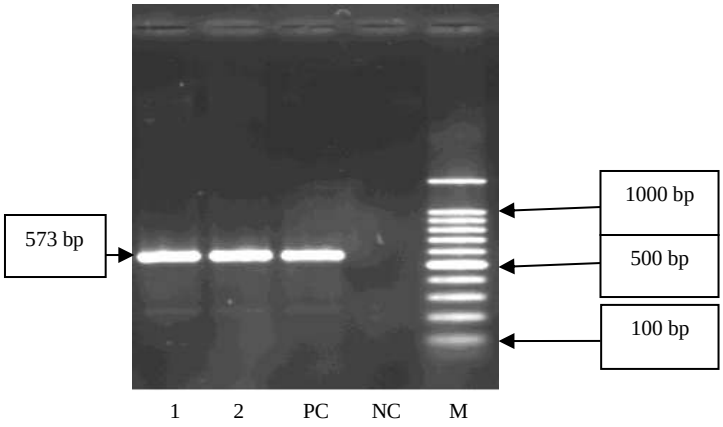

Figure 1. Agarose gel electrophoresis of 573 bp amplicon representing *ptxP* gene identified for *Bordetella pertussis* strains.

1, 2- *Bordetella pertussis* strains isolated in this study
PC- positive control (ATCC 9797)
NC- negative control
M- molecular weight marker (100 bp)

IS*481* has been used as a target to identify *Bordetella spp*. infections (WILLIAMS & al [19]; MARTINI & al [13]), but the lack of specificity of this element has also been reported by other authors (FARRELL & al [20]; PROBERT & al [21]) and imposes the necessity of confirmation of a specific target for *B. pertussis* infections, such as *ptxP*, also confirmed in our study. However, the conventional identification as *Bordetella pertussis* using *ptxP* target was highly specific, but performed on DNA extracted from culture. Molecular identification of *Bordetella pertussis* DNA extracted from the clinical specimen, requires the use of an additional real-time PCR, targeting a specific gene.

Conclusions

Real-time PCR is a test widely used for the identification of *Bordetella spp*. infections directly from the pathological product due to its high sensitivity and it is recommended especially for rapid identification of these infections in children, mostly for those who are less than 1 year old and require a rapid and adequate treatment. In these cases, and also for vaccinated children, adolescents and adults, PCR is the indicated test to be performed, instead of serology, which can provide results indicating an antibody titer that could sometimes be a response to the vaccination and also represents a retrospective method for whooping cough diagnosis. Serology based on detection of specific antibodies should be the chosen method for the correct interpretation of a result, only if the last dose of the vaccine or booster has been administered at least one year before the sampling of the serum.

References

1. HEGERLE N. and GUISO N. Epidemiology of whooping cough and typing of *Bordetella pertussis*. *Future Microbiology* 8(11), 1391-1403 (2013).
2. HIGGS R., HIGGINS S.C., ROSS P.J., MILLS K.H.G. Immunity to the respiratory pathogen *Bordetella pertussis*. *Mucosal Immunology* 5, 485-500 (2012).
3. BARKOFF A.M., GRONDAHL-YLI-HANNUKSELA K., HE Q. Seroprevalence studies of pertussis: what have we learned from different immunized populations, *Pathog Dis* 73(7) doi: 10.1093 (2015).
4. CHLIBEK R., SMETANA J., SOSOVICKOVA R., FABIANOVA K., ZAVADILOVA J., DITE P., GAL P., NAPLAVA P., LZICAROVA D. Seroepidemiology of whooping cough in the Czech Republic: estimates of incidence of infection in adults. *Public Health* 2017 Sep; 150:77-83. doi: 10.1016 Epub (2017).
5. SEUBERT A., D'ORO U., SCARSELLI M., PIZZA M. Genetically detoxified pertussis toxin (PT-9K/129 G): implications for immunization and vaccines. *Expert Rev Vaccines* 13(10): 1191-204 (2014).
6. AUSIELLO C.M. and CASSONE A. Acellular pertussis vaccines and pertussis resurgence: revise or replace? *mBio* 5(3):e01339-14. doi:10.1128/mBio.01339-14 (2014).
7. TODAR K. www.textbookofbacteriology.net (2008)
8. LUTSAR I., ANCA I., BAKIR M., USONIS V., PRYMULA R., SALMAN N. Epidemiological characteristics of pertussis in Estonia, Lithuania, Romania, the Czech Republic, Poland and Turkey–1945 to 2005. *Eur J Pediatr* 168:407-15 (2009).
9. HEININGER U., ANDRE P., CHLIBEK R., KRISTUFKOVA Z., KUTSAR K., MANGAROV A. MESZNER Z., NITSCH-OSUCH A., PETROVIC V., PRYMULA R., USONIS V., ZAVADSKA D. Comparative epidemiologic characteristics of Pertussis in 10 Central and Eastern European countries 2000-2013. *PLOS ONE* 11(6): e0155949 (2016).
10. POPOVICI O. Analiza epidemiologică a cazurilor de tuse convulsivă intrate în sistemul de supraveghere în anul 2016. www.cnscbt.gov.ro (2016).
11. DINU S., GUILLOT S, DRAGOMIRESCU C.C., BRUN D., LAZAR S., VANCEA G., IONESCU B.M., GHERMAN M.F., BJERKESTRAND A.F.D., UNGUREANU V., GUISO N., DAMIAN M. Whooping cough in South-East Romania: A 1- year study. *Diagnostic microbiology and infectious disease* 78(3) (2013).
12. CRAIG – MYLIUS K.A. and WEISS A.A. Antibacterial agents and release of periplasmic pertussis toxin from *Bordetella pertussis. Antimicrobial Agents and Chemotherapy*, 44(5): 1383-1386 (2000).
13. MARTINI H., DETEMMERMAN L., SOETENS O., YUSUF E., PIERARD D. Improving specificity of *Bordetella pertussis* detection using a four target real-time PCR. *PLOS ONE*; 12(4): 1-11 (2017).
14. WORLD HEALTH ORGANISATION. Laboratory manual for the diagnosis of whooping cough caused by *Bordetella pertussis – Bordetella parapertussis*. Immunization, Vaccines and Biologicals WHO/IVB/14.03 (2014)
15. RAMBOW – LARSEN A. A. and WEISS A. A. Temporal expression of pertussis toxin and Ptl secretion proteins by *Bordetella pertussis*. *Journal of Bacteriology*, 186(1): 43-50 (2004).
16. SCHOULS L.M., van der HEIDE H.G., VAUTERIN L., VAUTERIN P., MOOI F.R. Multiple locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. *Journal Bacteriol*. 186(16): 5496-505 (2004).
17. WIRSING von KONIG C.H., ROTT H., BOGAERTS H., SCHMITT H.J. A serologic study of organisms possibly associated with pertussis-like coughing. *Pediatr Infect Dis J* 17(7): 645-9 (1998).
18. LEE A.D., CASSIDAY P.K., PAWLOSKI L.C., TATTI K.M., MARTIN M.D., BRIERE E.C., TONDELLA M.L., MARTIN S.W. Clinical evaluation and validation of laboratory methods for the diagnosis of *Bordetella pertussis* infection: culture, polymerase chain reaction (PCR) and anti-oertussis toxin IgG serology (IgG- PT). *PLOS ONE* e0195979 (2018).
19. WILLIAMS M.M., TAYLOR T.H. Jr., WARSHAUER D.M., MARTIN M.D., VALLEY A.M., TONDELLA M.L. Harmonization of *Bordetella pertussis* real-time PCR in the US, 2012. *Journal of Clinical Microbiology* 53(1): 118-23 (2015).
20. FARRELL D.J., McKEON M., DAGGARD G., LOEFFELHOLZ M.J., THOMPSON C.J., MUKKUR T.K.S. J. *Clin. Microbiol.* 38(12): 4499-4502 (2014).
21. PROBERT W.S., ELY J., SCHRADER K., ATWELL J., NOSSOFF A., KWAN S. Identification and evaluation of new target sequences for specific detection of *Bordetella pertussis* by real-time PCR. *Journal of Clinical Microbiology* 46(10): 3228-31 (2008).