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Expression of adhesins by some *Bordetella pertussis* strains isolated in Romania in different time periods

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SUMMARY. *Bordetella pertussis* is the etiological agent of whooping cough or pertussis, a respiratory infection in humans which can be prevented by vaccination. *B. pertussis* is characterized by a set of virulence factors involved in bacterial adherence to host-cells and consecutive colonization of the respiratory epithelium and in immunity too. In this study, we compared the expression of the specific adhesins with antigenic properties in some *B. pertussis* strains, including collection strains, vaccine strains and recently-isolated strains. The specific adhesins / surface antigens of *B. pertussis* are: a surface protein, named FHA (filamentous hemagglutinin) and fimbria Fim2 and Fim3. These antigens were evidenced using an indirect ELISA method, based on specific monoclonal antibodies binding to specific epitopes. The results showed that fimbrial antigens Fim3 are expressed by all new-isolated strains, compared to the older isolates, which expressed Fim2 or both Fim2, 3.

Keywords: adherence, fimbriae, pertussis vaccine, virulence factors.

Introduction

Bordetella pertussis is a Gram-negative bacterium which infects the respiratory tract in humans and represents an important cause of worldwide deaths in children (Tsang *et al.*, 2004). It is a 3-5 years cyclically reported infection, even though whooping cough is a vaccine preventable disease (Bouchez *et al.*, 2015). Pertussis vaccination was introduced in Romania in 1961, using the whole-cell vaccine produced by Cantacuzino Institute and after that the number of infections and epidemics has drastically dropped. In 2008, the whole-cell pertussis vaccine was replaced by the acellular one and whooping cough incidence decreased from 2.8 ‰$_{000}$ in 2008 to 0.5 ‰$_{000}$ in 2017. However, the number of cases is underestimated, because there are reported only the confirmed cases, included in the surveillance programs. In our country, the vaccination coverage in 2017 was lower than 90%, the same situation being reported during the previous years (Popovici, 2017).

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B. pertussis produces a surface protein, respectively the filamentous hemagglutinin (FHA) and fimbrial proteins Fim2 and Fim3, encoded by *fim2* and *fim 3* genes (Livey *et al.*, 1987) (Willems *et al.*, 1990), which confer to the bacterial cells the capacity to adhere to host cells (Tsang *et al*., 2004). Fimbriae adhere to the ciliated epithelium and altogether with FHA ensure a prolonged bacterial colonization of the respiratory tract (Matoo *et al*., 2000) (Scheller *et al*., 2015). Fim2 and Fim3 are agglutinogens responsible for serotype determination and FHA is a non-fimbrial hemagglutinating molecule (Ashworth *et al*., 2006). *B. pertussis* binds to sulfated sugars at Fim2 receptors level, which contain 2 regions similar to heparin-binding site of fibronectin (Geuijen *et al*., 1996). These heparin-binding sites of Fim2 are parts of epitopes recognized by specific antibodies from infected patient's sera. It is thought that Fim3 has the same binding specificity and the bacteria evades host-immune responses by switching the expression of their encoding genes (Geuijen *et al*., 1998). FHA, Fim2 and Fim3 antigens were included in both whole-cell vaccines and currently used acellular pertussis vaccines, together with purified pertussis toxoid (PT) and an outer membrane protein, named pertactin (PRN).

The selective pressure observed for the circulating *B. pertussis* strains is a consequence of the introduction of pertussis vaccination, using initially the whole-cell vaccine, replaced by the acellular vaccine in 1990s (Bouchez *et al*., 2015). This pressure led to non-expression of pertactin especially in high-income countries, but the prevalence of the isolates lacking the expression of this antigen and their emergence is difficult to be interpreted (Bouchez *et al*., 2018).

The aim of this study was to estimate the expression of adhesins/antigens involved in bacterial adherence of *B. pertussis* strains and compare the expression profiles of FHA, Fim2 and Fim3 antigens of collection strains with the vaccine strains and new-isolated *B. pertussis* strains.

Materials and methods

Patients and strains. A number of 20 *Bordetella pertussis* strains were analyzed using ELISA technique in order to estimate the expression of FHA, Fim2 and Fim3 adhesins with antigenic properties: 11 *B. pertussis* collection strains, 5 new isolated strains and 4 vaccine strains, used for in house preparation of whole-cell pertussis vaccine. The tested collection strains have been isolated between 1954-1976, 3 of them being collected before the introduction of pertussis vaccine in our country (the whole-cell vaccine). The 5 new strains were isolated in 2014-2017 from unvaccinated or incompletely vaccinated children, aged from 6 months to 4 years old, 3 of them being treated with antibiotics, other than macrolides in 2 cases (a beta-lactam antibiotic and a third generation cephem) and 1 dose of azithromycin for the other patient.

Bacterial growth and identification of new-isolated strains. *B. pertussis* was isolated on selective Bordet-Gengou agar (containing 40µg/ml cephalexin) supplemented with 15% sheep blood and 1% glycerol and incubated for 3-5 days at 37^0C. Then the colonies were plated on Bordet-Gengou medium without antibiotic and molecular species identification was performed, detecting the gene encoding for pertussis toxin promoter (*ptxP*).

Expression of antigens performing indirect ELISA method. The indirect ELISA was performed using Heikkinen *et al.* adapted method (2008), modified as follows: the blocking buffer and the conjugate used in the reaction were replaced by other reagents. *B. pertussis* ATCC 9797 (American Type Culture Collection) was used as a positive control for the expression of adhesins and sterile phosphate-buffered saline-PBS (Amresco, USA-cat no E504) as negative control.

Bordetella pertussis colonies were harvested on Bordet-Gengou specific medium and incubated for 3-5 days at 37^0C. Bacterial suspensions were prepared for each tested strain and the positive control strain in PBS 1X at an optical density of 0.1. The suspensions were inactivated at 56^0C for 1h, then 96-well microtiter plates were coated with 100 µL of *B. pertussis* inactivated suspension of each tested strain and incubated over night at room temperature. On the second day, the plates were washed as described by Heikkinen *et al.* For blocking of the plates, 1% normal sheep serum (Sigma-Aldrich, USA-cat no S3772) in PBS was added for 1 h; after the washing, the microplates were incubated with a 1:1000 dilution of monoclonal antibodies specific to FHA, FIM2 and FIM3 (NIBSC, United Kingdom) antigens. Secondary antibody (goat anti-mouse IgG antibody conjugated with alkaline phosphatase-Sigma-Aldrich, cat no A3562, USA) was incubated with the microplates for 2 h, then alkaline phosphatase substrate was added (Sigma-Aldrich, USA - cat no S0942). NaOH solution (Thermo Fischer, Germany) was used to stop the reaction and the optical densities were measured at 405 nm using a Stat Fax spectrophotometer (Awareness Technology) with a reference wavelength of 630 nm.

DNA extraction. In order to identify the genes encoding for fimbrial proteins Fim2 and Fim3 by conventional PCR, genomic DNA was extracted from 200 µL of *B. pertussis* suspension using High Pure PCR Template Preparation Kit (Roche - Mannheim, Germany), according to manufacturer's instructions.

Polymerase chain reaction. PCR was performed for the identification of *fim2* and *fim3* genes, encoding for fimbriae serotype 2 and 3 (Fig.3). An in-house protocol was used to detect *fim2* gene, as follows: the reaction mixture contained 5 µL PCR buffer, 1.5mM MgCl2, 0.2 mM dNTP mix, 0.4 pmol/µL of forward and reverse primers/reaction, 1.25U Taq polymerase and 14.8 µL DNase free water. Then 1 µL of DNA was added in the reaction mixture, in a final working volume equal to 25 µL. The forward and reverse primer sequences used for PCR were designed as described by Zhang *et al.* (Table 1) and the amplification program is described in table 2.

Table 1.

Primers used to amplify *fim2* and *fim3* genes (Zhang *et al.*, 2010)

Identified gene	Forward primer	Reverse primer
fim2	ACCCATGCAAATCCCTTTCCAACGC	GGGGGTTGGCGATTTCCAGTTTCTC
fim3	ATGTCCAAGTTTTCATACCCTGCCT	TTCGTCTCCTGACGCCGCGTAGCGG

Table 2.

Thermal profile used for the amplification of *fim2* gene

Step	Temperature	Time (minutes)
Initial denaturation of DNA double strand	95^0C	2
Amplification (x 30 cycles)	Denaturation at 95^0C	0.5
	Primer annealing at 59^0C	1
	Polymerization at 72^0C	1
Final elongation of DNA double strand	72^0C	5

Results and discussion

The results indicated that the binding of specific monoclonal antibody to FHA epitope was positive for all tested strains; for Fim2, only 2 of the tested strains showed specific binding and in case of Fim3 epitope, the specific binding to the monoclonal antibody used in the reaction was positive for 50% (n=10) of the tested strains (Fig. 1).

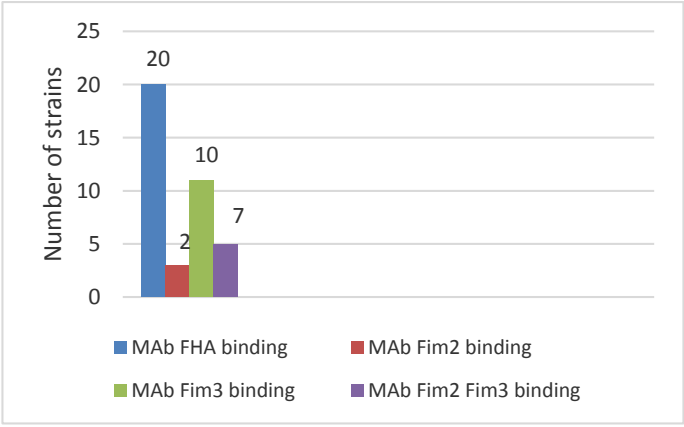

Figure 1. Monoclonal antibodies specific binding to expressed antigens

For the vaccine strains, the results showed low expression of Fim3 antigen in case of 2 strains, one of them expressed Fim2,3 and 1 strain had no expression of Fim2 or Fim3 antigen using this method.

For the older isolated strains, 2 out of 11 expressed Fim2 antigen, 4 expressed Fim3 and 5 strains expressed Fim2,3.

In case of the new-isolated strains, 4 expressed Fim3 antigen and 1 strain expressed Fim2,3 (Fig.2), indicating the same results described by other authors, that since the introduction of vaccination the main fimbriae serotype expressed in the vaccinated populations with pertussis vaccine is Fim3 (Heikkinen *et al*., 2008) (Alexander *et al*., 2012).

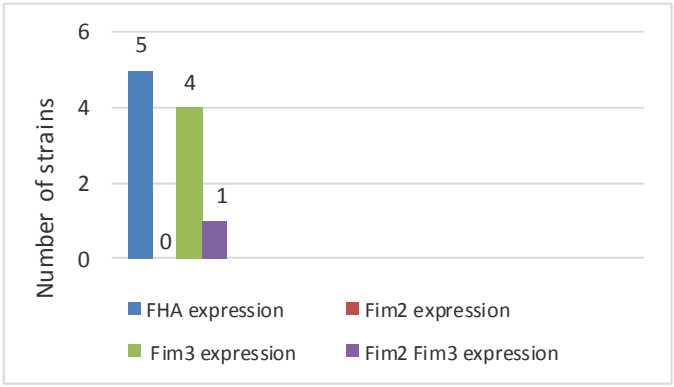

Figure 2. Antigen expression for the new-isolated strains (2014-2017)

The identification of a 700 bp amplicon indicating *fim 2* gene and of the 697 bp amplicon for *fim3* (Fig.3) gene was positive for all tested strains. Sequencing of the amplicons was not performed in this study.

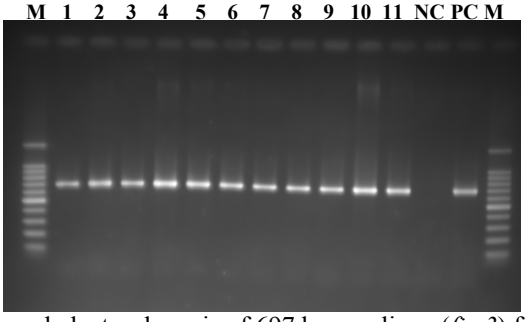

Figure 3. Agarose gel electrophoresis of 697 bp amplicon (*fim3*) for 11 *B. pertussis* collection strains; **M- molecular weight marker (100 bp DNA ladder); 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11- *B. pertussis* DNA extracted from strains; NC- negative control (DNase free water); PC- positive control (ATCC 9797).**

In our study, even though the expression of *B. pertussis* fimbriae was not tested for a large number of strains, it is important to notice that in case of all new-isolated strains, Fim3 expression was positive. This evidence is concordant with other studies in Europe, which describe the identification of Fim3 as the major fimbriae serotype, characteristic for vaccinated populations (Alexander *et al*., 2012). Fim3 prevalence is explained by different authors as a shift from Fim2 expression, as a consequence of the introduction of pertussis vaccine (Gorringe *et al*., 2014). The new strains were isolated from unvaccinated or incompletely vaccinated children and the Fim3 expression for all of them demonstrates that most of the circulating B. *pertussis* strains included in this study belong to Fim3 serotype.

This could be explained by the fact that serotype 2 antigen contained in whole-cell vaccine is more immunogenic and produces a stronger immune response than the serotype 3 antigen, as explained by other authors (Tsang *et al*., 2004; Gorringe *et al*., 2014) and the type Fim3 strains would have been selected in the population as a result of vaccine pressure. Fim3 prevalence in the population could also be a consequence of the fact that the partially immunized children are mainly infected by serotype 3 strains (Preston *et al*., 1985). Our study confirms this hypothesis, considering the fact that the tested strains were isolated from partially immunized or non-vaccinated children.

In Finland, Fim2 serotype is the most common, in spite of high vaccine coverage and Fim3 expressing strains emerge in case of epidemics (Heikkinen *et al*., 2008). In contrast to that, in other countries it was observed that for the areas with lower vaccination coverage, the prevalence of Fim2 strains increases (Gorringe *et al*., 2014). In previous studies it was revealed that in case of serotype 2 strains, *fim3* gene was not expressed and the selective pressure was not to be taken into consideration (Tsang *et al*., 2004).

For the collection strains, Fim2 and Fim2, 3 serotypes were found in case of more than half of the tested strains, compared to the recently isolated ones, with Fim3 expression positive for all the strains. It seems that Fim2 antigen contained in the whole-cell vaccine was more immunogenic and produced a better immune response than Fim3, considering the fact that in time, Fim3 type strains have not been eliminated from the circulation.

The strains included in the vaccine expressed Fim3 and Fim2,3 antigens, indicating that Fim3 serotype was not the prevalent serotype of the isolated strains, because the strains included in the vaccine expressed Fim3. In this case, the selective pressure of the vaccine could explain the prevalence of serotype 2. Other authors agreed that the methods used for serotyping may only report the predominant serotype of the infecting strain while during an infection, both antigens are expressed (Vaughan *et al*., 2014).

Expression of adhesins in *B. pertussis* circulating strains is important because the virulence of the strains is related to the antigenic profile. Whooping cough is a respiratory infectious disease, with severe symptomatology in case of non-vaccinated children. On

the other hand, the efficiency of the acellular vaccine is important to be evaluated, and the circulating strains should be isolated and tested to improve better vaccine formulas.

B. pertussis adhesins are encoded by genes regulated by the *BvgAS* operon and its expression occurs *in vivo* in the virulent phase, which is enough to produce a respiratory infection (Melvin *et al.*, 2014). The nucleotide substitutions in fimbriae genes help the bacteria to adapt to the immune pressure and fimbrial antigens are selected in the population as induced by the used vaccine or as a consequence of specific antibodies production following infection. Even though the sequencing of fim2 and fim 3 genes was not performed in this study, it would be necessary further investigation of the *B. pertussis* strains to elucidate the molecular mechanisms involved in fimbriae gene expression.

Conclusions

In our study, same as indicated by many other studies, Fim3 was found to be the main fimbrial serotype isolated from unvaccinated or incompletely vaccinated children. Further studies on adhesins expression should be performed on a larger number of strains, in order to compare the serotypes of the strains isolated before the vaccine was introduced in the population, with the recently circulating ones. This study supports the isolation and characterization of new *B. pertussis* strains, in order to estimate the social and economic impact of pertussis vaccination.

FHA and Fim2/Fim3 expression is important for the colonization and persistence of the bacterial cells in the respiratory tract and as a consequence, different mechanisms were developed by the bacteria to maintain their virulence potential.

The hypothesis related to vaccine pressure affecting the expression of pertactin, the shift from Fim2 expression to Fim3 in some countries since the introduction of the vaccine or the waning immunity to the antigens included in pertussis vaccines could explain the changes in antigen expression of *Bordetella pertussis* strains in time.

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