

## The Re-Emergence of Whooping Cough in Sfax (Southern Tunisia)

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### Abstract

Our purpose was to assess the epidemiological situation of pertussis among infants in the region of Sfax (southern Tunisia). From January 2013 to January 2014, infants consulting for a suspected diagnosis of whooping cough were included in this study. The laboratory diagnosis was based on *Bordetella* culture and detection of *B. pertussis*-*B. parapertussis* DNA by real-time PCR. Household contacts were asked to provide pertussis diagnostic specimens for PCR and serology. One hundred thirty six infants clinically suspected of whooping cough were enrolled. Fifty two infants (38.2%) had a confirmed *B. pertussis* positive laboratory diagnosis by real-time PCR, whereas, culture was negative in all cases. For household contacts of pertussis positive cases, 8 among 20 parents tested had positive serology among them 2 mothers had also a positive nasopharyngeal *Bordetella* PCR. Our study confirms that whooping cough remains a public health problem in Tunisia affecting not only infants but also adults.

**Keywords** Pertussis; *Bordetella*; Infants; Culture; PCR; Serology

### Introduction

Whooping cough (pertussis) is a highly contagious human respiratory infection caused by *Bordetella pertussis* and *Bordetella parapertussis*. Despite the widespread use of the pertussis vaccine has resulted in a dramatic reduction in the incidence of whooping cough around the world, a resurgence of this bacterial infection noted in many countries have marked the two last decades and pertussis continues to be a public health concern even in countries with high vaccination coverage. In 2008, about 195000 children under the age of 5 died from this disease in the world which represented 2% of all deaths in this age group [1,2]. In 2010, WHO estimated the global incidence of pertussis of 50 million cases per year [2]. Waning immunity after natural infection or vaccination has been considered as one of the major reasons for the observed epidemiologic trend. In fact, according to a review of the literature, the infection-acquired immunity against pertussis disease and the vaccine-induced immunity wane after 4–20 years and 4–12 years, respectively [3].

The incidence of pertussis is still underestimated in many countries namely Tunisia due to the difficulty of clinical and laboratory diagnosis. In Tunisia, the whole-cell pertussis vaccine was introduced in 1979. The Tunisian vaccination schedule includes three primary doses at 2, 3 and 6 months and a booster at 18 months. The coverage rate for the first 3 doses is high ( $\geq 90\%$ ). According to data from the Tunisian Ministry of Health, 0 to 8 cases of pertussis have been reported annually between 2000 and 2006. However, a recent study performed at the Children's Hospital of Tunis (northern Tunisia), showed that these figures are underestimated [4]. Given the underestimation of the incidence of pertussis in our country and particularly in Sfax (southern Tunisia), we conducted this prospective study to establish a microbiological diagnosis of pertussis in Sfax, study the clinical and laboratory features of the disease and establish the possible preventive measures.

### Patients and Methods

#### Patients and samples

Between 1 January 2013 and 31 January 2014; infants clinically suspected of pertussis who were consulting or admitted in public and private healthcare facilities in the region of Sfax were enrolled. Inclusion criteria were typical pertussis with paroxysmal cough, inspiratory whoop and posttussive vomiting, prolonged and severe hypoxic access, drawing acute bronchiolitis, malignant pertussis, severe spasmodic cough lasting more than one week, apnea and deep bradycardia. Nasopharyngeal specimens obtained by swab (eSwab®) or aspirate were collected from suspected whooping cough patients for *Bordetella* culture and detection of *B. pertussis*-*B. parapertussis* DNA by real-time PCR (Polymerase Chain Reaction). Clinical data were collected from a standardized questionnaire that accompanied the specimens. Clinical and biological variables included gender and age, pertussis vaccination status, clinical course, contact with an individual with prolonged cough illness, lymphocytosis and administration of antibiotics prior to sampling. Vaccination was considered as outdated when patients hadn't received the correct number of doses corresponding to their age.

In our study, we considered a confirmed pertussis case every suspected case which was laboratory confirmed. A nasopharyngeal specimen and/or a blood sample for PCR and/or pertussis serological testing were obtained from household contacts (mother and/or father) when possible.

#### Culture

Specimens for culture were inoculated into two selective media, Bordet-Gengou agar (Difco™) supplemented with 15% defibrinated horse blood and Regan-Lowe charcoal agar (BBL™) containing 10% defibrinated horse blood. The plates were incubated at 35°C for at least 7 days in high humidity and were examined daily for colonies typical

of *B. pertussis* or *B. parapertussis*. The identification of suspicious colonies was based on the study of morphological, cultural and metabolic characteristics. The validation of culture media was done using the reference strains *B. pertussis* CIP 8132 and *B. parapertussis* CIP12822.

### Real-time PCR

DNA was extracted from a 200 µl sample using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's instructions. Each sample was eluted in 60µL of buffer. The real-time PCR assays were carried using a C1000TM thermal cycler (CFX96TM Real-Time System Bio-Rad). The detection of *B. pertussis* harboring the insertion sequence IS481 was performed by real time PCR using ARGENE kits (*BORDETELLA* R-geneTM) according to the manufacturer's recommendations. The presence of *B. parapertussis* was assessed by an in-house real-time PCR using primers and probe targeting the insertion sequence IS1001 previously described by Kusters et al, 2001 [5]. The PCR sensitivity was evaluated at the lower detection limit of ~ 1 CFU. Only samples that showed an amplification signal by the in-house real-time PCR were tested by ARGENE kits (*Bordetella parapertussis* r-geneTM) according to the manufacturer's recommendations. For all these real-time PCR assays, the result was reported as positive when a fluorescence curve appears with a threshold cycle (Ct) value lower or equal to 38 and as indeterminate when the Ct value was greater than 38 for extracted DNA tested both undiluted and diluted 1:4.

### Serology

Serological diagnosis of pertussis consisted of measurement of immunoglobulin G antibodies against pertussis toxin (anti-PT IgG) by enzyme-linked immunosorbent assay (ELISA). We used a commercial kit (EUROIMMUN) following the manufacturer's instructions. A titer greater than or equal to 100 IU/mL was considered indicative for recent pertussis infection, unless the patient has been vaccinated during the previous year.

### Statistical analysis

Statistical analysis was performed using the SPSS software version 20. Comparison of proportions was made using Chi-squared test and Fisher exact test. A difference was considered statistically significant if the p value was less than 0.05.

## Results

### Patients and samples

During the study period, a total of 136 infants with a clinical suspicion of pertussis were investigated. One hundred twenty six nasopharyngeal aspirates and 10 nasopharyngeal swabs were collected from all the patients enrolled. Of the 136 patients, 125 (91.9%) were living in Sfax, 8 (5.9%) were from others Tunisian governorates and 3 (2.2%) were foreign with two Libyan and one Algerian. One hundred seven patients (78.7%) were admitted in the pediatric wards at the university hospital, 14 patients (10.3%) were consulting other public health care facilities and 15 patients (11%) were referred by pediatricians in private sector. Laboratory diagnosis was performed for 20 household contacts of confirmed pertussis cases (17 mothers and 3 fathers). Seventeen parents were tested by serological examination and 3 mothers underwent PCR and serological testing.

### Diagnosis of pertussis-infections

*B. pertussis* infection was confirmed in 52 of the 136 patients (38.2%) by real-time PCR with a mean CT value 29.43 (range; 12.16-37.6). One sample was positive for both IS481 DNA and IS1001 DNA suggestive for *B. parapertussis* - *B. pertussis* co-infection. Culture was negative in all cases. Of the 52 patients diagnosed with pertussis, 44(84.6%) were from Sfax, 5 (9.6%) were from others Tunisian governorates and 3 (5.8%) were foreign with two Libyan and one Algerian. Among these 52 confirmed patients, 37 (71.2%) were admitted in the pediatric wards at the university hospital, 6 (11.5%) were consulting other public health care facilities and 9 (17.3%) were referred by private pediatricians. Pertussis cases were reported throughout the year with 23 cases (44.2%) occurring in the 2 months March -April 2013. The mean age of confirmed cases was 78 days (range, 18 days- 16 months) and the median age was 2 months. The male: female ratio was 1. Thirteen of the 136 suspected cases were not correctly vaccinated according to age, 10 of which were pertussis confirmed patients (76.9%). The numbers of DTP vaccination doses received by the 52 confirmed cases according to the age groups are given in Table 1.

Age	Not vaccinated Number (%)	DTP1 Number (%)	DTP1-DTP2 Number (%)	DTP1-DTP2-DTP3 Number (%)	Total Number (%)
<2 months	23 (44.2%)	-	-	-	23 (44.2%)
≥ 2 months <3 months	11 (21.2%)	1 (1.9%)	-	-	12 (23.1%)
≥ 3 months <6 months	6 (11.5%)	4 (7.7%)	4 (7.7%)	-	14 (26.9%)
≥ 6 months	-	-	-	3 (5.8%)	3 (5.8%)
<b>Total</b>	<b>40 (76.9%)</b>	<b>5 (9.6%)</b>	<b>4 (7.7%)</b>	<b>3 (5.8%)</b>	<b>52 (100%)</b>

**Table 1:** Vaccination status of pertussis confirmed cases by age group.

The most predominant clinical manifestation (51.9%) was a typical pertussis with paroxysmal cough, whooping and posttussive vomiting. Table 2 shows the clinical features in confirmed and non-confirmed pertussis cases. There were significant differences between confirmed and non-confirmed cases in typical pertussis presentation, prolonged and severe hypoxic access and cyanosis. The mean duration of symptoms at time of sampling was 9 days (range, 2- 30 days). Lymphocytosis (Lymphocytes >10 000/µl) was more common in pertussis confirmed infants (32.5% vs 1.7%; p<0.001). The infant with a *B. pertussis* - *B. parapertussis* co-infection had paroxysmal cough with whooping and cyanosis.

Clinical and biological manifestations	Non-confirmed pertussis cases (n=84): Number (%)	Confirmed pertussis cases (n=52):	p-value

Typical pertussis with paroxysmal cough, whooping and posttussive vomiting.	3 (3.6%)	27 ( 51.9%)	<0.001
Prolonged and severe hypoxic access	9 (10.7%)	13 (25%)	0.028
Drawing acute bronchiolitis	31(36.9%)	13 (25%)	0.149
Malignant pertussis	0 (0%)	1(1.9%)	0.382
Cough with one or more of the following symptoms:	56 (66.7%)	16 (30.8)	<0.001
paroxysmal cough	23 (27.4%)	11 (21.2%)	0.415
whooping	3 (3.6%)	1 (1.9%)	1
posttussive vomiting	7(8.3%)	4 (7.7%)	1
spasmodic cough	48 (57.1%)	10 (19.2%)	<0.001
cyanosis	43 (51.2%)	41 (78.8%)	0.001
Apnea	16 (19%)	7 (13.5%)	0.398
Fever	29 (34.5%)	9 (17.3%)	0.03
Lymphocytosis(Lymphocytes >10 000/ $\mu$ l)	1 (1.7%)	13 (32.5%)	<0.001

**Table 2:** Clinical characteristics of confirmed and non-confirmed pertussis cases.

There was no significant difference in clinical features according to age and vaccination status (data not shown). An antibiotic treatment prior to sampling was noted in 22 (42.3%) of positive pertussis cases; 72.7% of them were treated with macrolides. The antibiotic administration prior to specimen collection between confirmed and non-confirmed pertussis cases was not statistically significant ( $p=0.202$ ). The clinical outcome was favorable for almost all the patients except one patient who died from a malignant pertussis. This infant was aged 21 days and was unvaccinated. He was admitted to the pediatric intensive care unit at the university hospital for respiratory distress. Despite assisted ventilation and treatment with macrolide, he died 5 days after admission.

### Household contacts

Six among the 17 parents tested (4 mothers and 2 fathers) had positive serology including 5 parents who had a cough. Two among the 3 mothers tested had positive nasopharyngeal Bordetella PCR and positive serology. These 2 mothers had a cough. Anti-PT IgG antibody titers ranged from 133 IU/mL to 253 IU/mL. For household contacts of non-confirmed pertussis patients, 14 mothers and one father tested had negative serology.

### Discussion

During the study period, 52 pertussis cases were confirmed in infants. According to data from the Tunisian Ministry of Health, 0 to 8 cases of pertussis have been reported annually between 2000 and 2006. The reported cases were based exclusively on clinical diagnosis. However, a recent study performed at the Children's Hospital of Tunis (northern Tunisia) using real-time PCR showed that these numbers are underestimated. In fact, in this study extending over a period of 4 years (2007-2011), 120 pertussis cases were confirmed in infants [4].

Therefore, our study strengthens the findings from that of Tunis. These results show that the bacteria are still circulating in Tunisia. The re-emergence of pertussis in many countries could be explained by waning immunity following infection and vaccination in the absence of natural or vaccine booster ,greater awareness of pertussis, availability of better laboratory tests (PCR and serology), improved reporting via national surveillance systems and genetic changes in *B. pertussis* with the emergence of *B. pertussis* strains carrying a novel allele for the pertussis toxin promoter (ptxP3) which confers increased pertussis toxin (Ptx) production [6-9].

In our series, all confirmed cases were positive for *B. pertussis*. A coinfection *B. pertussis*-*B. parapertussis* was noted in one case. No cases have been attributed to the only presence of *B. parapertussis*. The coinfection *B. pertussis*-*B. parapertussis* has been seen in previous studies [4,10]. The *B. parapertussis* infection rate in our study is lower than those reported in others studies [4,11-13]. Pertussis cases were reported throughout the year with 23 cases (44.2%) occurring in the 2 months March –April 2013. Thus, our study did not show the seasonality summer-autumn noted in some other studies [14,15]. Pertussis mainly affects young infants none or incompletely vaccinated. In our series, 49 confirmed cases (94.2%) were aged less than 6 months. This result is consistent with that reported in other studies [4,16,17]. Regarding the vaccination status, in our study, 76.9% of pertussis confirmed patients had not received any dose of vaccination with 44.2% of pertussis cases were aged less than 2 months so younger than the age recommended for the first dose of vaccine. Similar finding were reported by the study of Tunis and a French study [4,18]. Pertussis is highly contagious. The transmission rate can reach 90% among susceptible household contacts and 50–80% in school environments [19]. Adults, particularly parents, are the most important source of infection for infants [7,20,21]. In our study, pertussis was diagnosed by serology in 4 mothers and 2 fathers and by real-time PCR and serology in 2 others mothers. All these parents had reported a cough preceding the onset of symptoms in their children except a mother who was asymptomatic. Our finding that mothers play an important role in the transmission of pertussis in the household is in agreement with other studies [22-24].

Several factors can affect the clinical manifestations of pertussis infection mainly patient age and immunization status [9,25]. Despite pertussis is known by the typical clinical presentation characterized by paroxysmal cough, whooping and posttussive vomiting, clinical features are often atypical [7,25]. In our series, typical pertussis was reported for more than half of confirmed pertussis cases (51.9%), this could be explained by the fact that we have received samples from highly suspicious infants. Because of a lack of specific symptoms particularly among patients with atypical features, pertussis is generally indistinguishable from common viral upper respiratory infections and atypical bacterial pneumonias [9,25]. Thus, both the centers for disease control (CDC) and the World Health Organization (WHO) have developed similar clinical case definitions for pertussis. It is a cough lasting at least 2 weeks with at least one of the following symptoms: paroxysms of coughing, inspiratory whooping or posttussive vomiting, without other apparent cause [26]. In our study, lymphocytosis was significantly more frequent among confirmed pertussis cases (32.5%). Indeed, lymphocytosis has been recognized as a hallmark of pertussis infection [27]. According to a review of the literature, the sensitivity and the Specificity of lymphocytosis ranged from 39% to 89% and from 57% to 75%, respectively [28].

Because accurate diagnosis of pertussis cannot be only clinical, there is a need for an efficient laboratory diagnosis. In our series, 52 cases (38.2%) were confirmed by PCR but culture was negative in all cases. Isolation of *Bordetella* is the gold standard for the diagnosis of pertussis due to its high specificity, approximately 100%. However, sensitivity of culture is relatively low; it ranges from 30% to 70% and it is influenced by the specimen collection, transport and culturing techniques, the time delay between specimen collection and inoculation, prior antimicrobial treatment, the stage of the disease (ideally within the first 2 weeks) and the immune status of the infant [9,29]. In our study, culture was negative for all samples. This could be explained by the culture media used that did not contain cephalixin therefore most cultures were contaminated by other bacteria from the nasopharyngeal flora, prior antimicrobial treatment in 42.3% of pertussis cases, 12 cases had received at least one dose of pertussis vaccine and the time delay between the onset of symptoms and the specimen collection was relatively long (mean: 9 days). Despite its limitations, culture remains important for typing of circulating strains and antimicrobial susceptibility testing [7,29,30]. PCR is revolutionizing the diagnosis of pertussis. In fact, PCR become the test of choice for pertussis diagnosis due to its high sensitivity; ranging from 70 to 99% [9,29]. Ideally, targets for PCR should be both sensitive and specific [31]. For the detection of *B. pertussis*, a multi-copy target IS481 (50-238 copies per genome) is considered as the target of choice due to its high sensitivity [31,32]. However, other *Bordetella* species may contain IS481 such *B. holmseii* (up to 10 copies per genome) and some *B. bronchiseptica* strains [31]. Thus, a single-copy target such as toxin promoter region is increasingly used as an additional target due to its better specificity [4,33]. Nevertheless, this single-copy target has low sensitivity. For the confirmation of the presence of *B. holmesii*, a specific PCR test targeting the hIS1001 (= IS1001-like) or the Rec A gene can be performed [4,31]. Some studies have used these targets but no *B. holmesii* was identified [4,34,35]. In fact, *B. holmesii* and *B. bronchiseptica* can be responsible for a pertussis like illness but *B. holmesii* affect particularly older children [31,36] and *B. bronchiseptica* affect immune-compromised patients or those in close contact with infected animals [25,31]. That's why we have not sought these two bacteria. For the detection of *B. parapertussis*, the multi-copy insertion sequence IS1001 is the most commonly used and a positive result with IS1001 is assumed to be *B. parapertussis* [31,37]. In our study, we have used as target the two insertion sequence IS481 and IS1001. Furthermore, this is a common strategy used by clinical laboratories in the United States [31]. Serology is a useful tool for the diagnosis of pertussis in patients with greater than 2 to 4 weeks of symptoms when culture and PCR testing are negative [9,31]. However, serology has no interest in neonates and young infants and it is only meaningful for older children and adults [30,31,38]. Currently, enzyme-linked immunosorbent assay (ELISA) is the most widely used method for serological diagnosis [29,31,38]. Antibodies to the pertussis toxin antigen (anti-PT antibodies) constitute the most specific serologic indicator of *B. pertussis* infection and measurement of PT-antibodies is recommended for routine diagnosis [9,29,31,38]. For dual-sample serology (acute and convalescent-phase sera), a significant increase in antibody titer ( $\geq 2$ -fold) or a decrease in antibody ( $\geq 50\%$ ) should be demonstrated [29,38]. Nevertheless, in clinical practice, a single-sample serology of anti-PT IgG is used [9,29,30,38]. Various cut-off values for IgG-anti-PT have been proposed in single serum ELISA [29,38]. The European Union reference laboratories suggest a dual cut-off between 62 and 125IU/mL as a proof of a recent infection with *B. pertussis*, provided that the patient was not vaccinated during the last 12 months [9,38]. A major problem with ELISA for pertussis

diagnosis is the lack of standardized, FDA-authorized commercially available serological tests [9,29,31]. In our study, for the diagnosis of pertussis in household contacts, we have used the EUROIMMUN kit. A titer greater than or equal to 100 IU/mL was considered indicative for recent pertussis infection, unless the patient has been vaccinated during the previous year. Given the resurgence of pertussis with a parent to infant transmission, a re-evaluation of pertussis vaccination schedules is a priority in many countries. Several potential immunization strategies have been proposed by the ACIP (Advisory Committee on Immunization Practices) and the GPI (Global Pertussis Initiative) to improve pertussis control. These strategies include: universal immunization of adolescents and adults, maternal vaccination in pregnancy, immunization of parents and possibly others in close contact with the newborn "the cocooning strategy" and vaccination of health-care workers [7,30,39]. Recently, a low dose adult-formulated diphtheria, tetanus, acellular pertussis vaccine (dTpa) has been licensed and recommended in several countries for vaccination of adolescents and adults [7]. Two formulations of Tdap booster are available: Adacel (manufactured by Sanofi Pasteur) and Boostrix (manufactured by GlaxoSmithKline) [40].

## Conclusion

Our Study confirms that whooping cough remains a public health problem in Tunisia affecting not only infants but also adults. Among vaccine-preventable diseases, pertussis is one of the most difficult to remove. So, a review of the Tunisian immunization schedule, an establishment of a national surveillance of whooping cough, a greater awareness of physicians that pertussis is not just a paediatric disease and an early and reliable biological diagnosis by improving diagnostic techniques, namely PCR assays, are required.

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