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Pertussis diagnosis in Belgium: results of the National Reference Centre for *Bordetella anno* 2015

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SUMMARY

In 2015, the Belgian National Reference Centre for *Bordetella* analyzed 4110 respiratory samples by qPCR and 4877 serum samples by serology. Whereas about 50% of respiratory samples were from infants and children below the age of five, serum samples were distributed among all age categories. A total of **394** (9·6%) cases was diagnosed as positive for *Bordetella pertussis* by qPCR and **844** (17·3%) cases were diagnosed as acute infection by serology (anti-pertussis toxin (PT) IgG > 125 IU/ml). Another **1042** (21·4%) sera had anti-PT IgG between 55 and 125 IU/ml reflecting a vaccination or pertussis infection during the last 1–2 years. Seventy per cent of the pertussis cases diagnosed by qPRC were in infants and children younger than 14 years old, whereas the highest number of sera with anti-PT levels >125 IU/ml was in the age group of 10–14 years old. Based on the limited data of the last vaccination (reported for only 15% of the samples), recent booster vaccination in the teenager group may have contributed only minimally to these elevated anti-PT levels. The highest number of sera with anti-PT titers between 55 and 125 IU/ml was found in the age category 50–59 years old. It is clear that pertussis continues to be a problem in Belgium and that other vaccination strategies (maternal vaccination, cocoon vaccination) and ultimately better vaccines will be needed to control this highly infectious respiratory disease.

Key word: *Bordetella pertussis*.

INTRODUCTION

Pertussis, caused by *Bordetella pertussis*, is a highly contagious respiratory illness. It is potentially life-threatening to infants and young children and a major cause of childhood morbidity and mortality worldwide. Although disease incidence declined dramatically after the introduction of whole-cell-based vaccines in the late 1950s, the number of reported cases across all age groups has increased again during the past decade in many industrialized countries with high vaccination coverage [1]. Furthermore, severe morbidity and mortality still occurs primarily in young infants who are not (or not fully) vaccinated [2].

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The underlying mechanisms of this evolution are probably multifactorial: waning immunity in adults (related to reduced exposure), switch from wP (whole-cell pertussis) to less reactogenic aP (acellular pertussis) vaccines, increased awareness of physicians and the general public coupled to an easier and earlier diagnosis by PCR and possibly changes in virulence of the circulating pertussis strains [3, 4].

PCR on nasopharyngeal swabs or aspirates is the method of choice during the first 2–3 weeks of disease and always for young children <1 year old. Serology has expanded the time-interval for diagnosis and although it has limited value during the first 2–3 weeks of clinical symptoms (or after a recent vaccination), serodiagnosis is the best diagnostic method at later time points, particularly in older adolescents and adults. Since 2011, the Belgian National Reference Centre (NRC) for *Bordetella* (financed by the Health Insurance System RIZIV-INAMI) works as a consortium of two partners: University Hospital Brussels performing microbiological diagnosis by qPCR and culture on respiratory samples and Scientific Institute of Public Health (WIV-ISP) performing the serodiagnosis using a validated anti-pertussis toxin (PT) IgG antibody ELISA. Serodiagnosis of pertussis has a limited role for the antibiotic treatment of the disease, as macrolides are only effective during the first 1–2 weeks of *Bordetella* infection. However, besides being an important tool in diagnosis and surveillance of the disease, it can also play a role in differential diagnosis of respiratory diseases, as we reported for a 47-year-old patient with persistent cough for whom, after a painstaking series of complementary investigations (gastroscopy, thorax radiography and scan, functional respiratory tests, etc.), serology finally identified whooping cough after 2 months of symptoms [5].

PT is present in the acellular pertussis vaccines administered in Belgium (besides the two other pertussis antigens filamentous hemagglutinin (FHA) and pertactin (Prn)), and therefore anti-PT antibodies are also induced by vaccination. However, vaccine-induced anti-PT levels decrease after 1–2 years to values below a cut-off level that can be used for the diagnosis of acute infection [6]. Information on the date of the last vaccination is essential for correct interpretation of samples with high anti-PT titers and this information was asked for on a routine basis, but only reported for a minority of samples.

We previously reported on the pertussis serodiagnosis for 2013 by the NRC for *Bordetella* at WIV-ISP [7]. Of 2129 samples tested, **521** (24·5%) had antibody levels indicative of an acute infection and **261** (12·3%) samples were diagnosed as positive (indicative of a pertussis infection during the last 1–2 years). Combined with the PCR/culture data, the NRC *Bordetella* reported a total of **827** cases in 2013, 521 cases detected by serology, 299 pertussis cases diagnosed by PCR and seven cases by both methods.

In order to increase the efficiency of the serological analyses and in order to cope with an increasing number of demands linked to a peak in pertussis incidence in 2014, from that year onwards it was decided to analyse only sera for which the duration of cough at time of blood sampling was known. Furthermore, as of 2014, reporting was changed and instead of the Virotech ELISA Units used previously, results were now expressed in international units (IU)/ml.

The number of annual reported cases of pertussis is known to fluctuate, with peak incidences reported every 5–7 years. As just mentioned, in Belgium the last peak occurred in 2014, with the number of pertussis cases reported by the Belgian NRC consortium increasing dramatically to a total of **1395**, with 930 cases detected by serology, 458 by PCR and seven cases by both methods.

In 2015, the total number of cases of pertussis reported by both NRC labs was still high, although at the end of the year incidence showed a tendency to decrease. In total **1246** cases of pertussis were diagnosed by the Belgian NRC consortium: 844 cases by serology, 394 cases by PCR and 8 cases by both methods. The NRC also reported 28 cases of *Bordetella parapertussis* and nine cases of *Bordetella holmesii*. Here we report in more detail on the results of the 2015 pertussis diagnosis by the Belgian NRC consortium.

METHODS

The Belgian NRC for *Bordetella* performs the laboratory diagnosis of pertussis using two complementary methods: real-time PCR on clinical respiratory samples and culture of PCR-positive samples and detection of anti-PT IgG levels in serum by ELISA.

Real-time PCR was used for the detection of *B. pertussis* directly on clinical respiratory samples. Nasopharyngeal swabs and aspirates were preferred, but other samples, such as sputa, were accepted as well. Four targets were used to ensure both sensitive and specific detection of *B. pertussis* as opposed to other *Bordetella* spp. such as *B. parapertussis* and

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B. holmesii. The targets used were IS*481*, IS*1001*, *recA*, and IS*1002*. Detection of IS*481* in a clinical sample indicates the presence of *B. pertussis*, which can be confirmed by the presence of IS*1002*. Other combinations may indicate the presence of *B. parapertussis* or *B. holmesii*.

DNA extraction was performed on 100 µl of the clinical sample, using the NucliSENS easyMAG (bioMérieux, Grenoble, France). Viscous clinical samples were treated with Sputasol (Oxoid Ltd., Basingstoke, England) prior to DNA extraction. Likewise before DNA extraction, phocine herpesvirus (PhHV) was added as an internal control [8].

Two multiplex real-time PCR's were performed, one for the detection of IS*481*, IS*1001*, and PhHV, and one for the detection of *recA* and IS*1002*. Primers and probes were based on Roorda *et al*. [9], Guthrie *et al*. [10], and van Doornum *et al*. [11]. The reactions were performed in a 50 µl mixture containing 0·3 µM of each of the primers, 0·2 µM of each of the probes, iQ Multiplex Powermix (Bio-Rad Laboratories, Temse, Belgium), and 5·0 µl extracted DNA. Amplification was done on the LightCycler 480 II PCR system (Roche Diagnostics, Mannheim, Germany), using the following profile: 3 min at 95 °C; 45 cycles of 15 s at 95 °C and 1 min at 60 °C; 30 s at 40 °C. Analysis was done based on Cp values and the 'second derivative maximum' method in the Roche LightCycler 480 Software, version 1.5. Samples were considered negative for *B. pertussis* if the Cp value for IS*481* equalled or surpassed 38·5. For *B. parapertussis* this cut-off was 40·0 for IS*1001*.

All qPCR-positive samples were **also cultured** by inoculation on laboratory-prepared Regan–Lowe agar and incubation at 35 °C in a humidified aerobic atmosphere. Plates were examined for suspect colonies for up to 12 days. Identification was done with the Microflex LT MALDI-TOF platform and MALDI Biotyper 3·0 software (Bruker Daltonics GmbH, Leipzig, Germany) [12, 13], as well as based on biochemical characteristics (growth on charcoal agar, Haemophilus agar, MacConkey agar, presence of oxidase and presence of urease).

Pertussis **serodiagnosis** was performed using a one-point serology based on the detection of IgG antibodies against PT. This is the gold standard for pertussis serology [14]. Only serum from patients with clinical symptoms and for whom the duration of cough is documented are analyzed free of charge in the frame of the NRC. Antibodies against PT are specific for the species *B. pertussis* [15]. The validated test used by the NRC in 2015 was the PT IgG ELISA of Virion–Serion which expresses its results in International Units/ml. The Virion–Serion kit recommends cut-off values of 40 IU/ml for a negative sample, 40 < × <100 for an equivocal and >100 IU/ml for a positive sample. However, based on previous data from literature [16, 17], slightly different thresholds were used for interpretation of the results: <50 IU/ml: negative, 50–55 IU/ml: doubtful (borderline between negative and positive), 55 < × <125 IU/ml: positive (recent vaccination or past infection) and >125 IU/ml: acute or very recent infection.

Anti-PT antibodies increase over time and generally become maximal only 3–4 weeks after onset of the symptoms. Therefore a serum sample collected during the first 3 weeks of cough may give a false-negative value below 125 IU/ml. In this case a second serum sample is required to make a definitive diagnosis. Unfortunately, for the majority of patients a second serum sample was requested but not received (see details in the report).

RESULTS

qPCR and culture

In 2015, 4110 samples were tested by qPCR of which 394 (9·6%) were evaluated as positive for *B. pertussis*. The number of samples sent was highest during the first 3 months of 2015 (reflecting the end of the 2014 peak), whereas the percentage of positive cases per month was highest in August (21·8%). The percentage of PCR-positive samples from which *B. pertussis* could be cultured was stable around 30% throughout the whole year (Table 1). However, a strong correlation was seen between the Cp value for IS*481* and the success rate of cultivation (Table 2). Lower Cp values correlated with a higher recovery rate.

A total of 1949 out of 4110 (47%) of the sent respiratory samples were from children younger than 5 years old. In this age group 31% of the total number of positive cases were detected. The age group 5–14 years represented 15·6% of the sent samples and 39% of the total number of positive cases were detected in this age group. Hence 70% of the pertussis cases diagnosed by qPRC were in infants and children (Table 3). Since 2004, one dose of aP vaccine is recommended at 5–7 years of age and since 2009, the Belgian Superior Health Council recommends an additional booster vaccination for adolescents at the age

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Table 1. *Monthly distribution of number and percentage of pertussis cases detected by qPCR*

Month	Number of samples on which PCR was performed	Number of samples positive for *B. pertussis* (%)	*B. pertussis*-positive culture (%)
January	588	44 (7·5)	12 (27·3)
February	493	31 (6·3)	9 (29·0)
March	442	37 (8·4)	8 (21·6)
April	352	39 (11·1)	11 (28·2)
May	298	37 (12·4)	13 (35·1)
June	301	48 (15·9)	12 (25·0)
July	232	33 (14·2)	12 (36·4)
August	165	36 (21·8)	11 (30·6)
September	246	33 (13·4)	12 (36·4)
October	300	21 (7·0)	3 (14·3)
November	343	23 (6·7)	8 (34·8)
December	350	12 (3·4)	4 (33·3)
Total	4110	394 (9·6)	115 (29·2)

Table 2. *Association between Cp value and recovery in culture, for samples positive for B. pertussis*

Cp value IS481	PCR positive	Culture positive (*B. pertussis*)	Recovery culture/PCR (%)
Cp $\leqslant$ 20	51	42	82·4
20 < Cp $\leqslant$ 25	50	32	64·0
25 < Cp $\leqslant$ 30	64	26	40·6
Cp > 30	229	15	6·6
Total	394	115	29·2

of 14–16 years (HGR 8532). Unfortunately, the limited access of the NRC to vaccination data makes it very difficult to know to what extent these 70% was an indication of vaccine failure or of insufficient vaccine coverage.

Serodiagnosis

In 2015, a total of 4877 sera were received for pertussis serodiagnosis. During the first quarter of 2015 an average number of 600 sera was received monthly, reflecting the end of the 2014 peak, and this number decreased to about 300 sera monthly for the rest of the year. The absolute number of cases with anti-PT titers >125 IU/ml was also highest at the beginning of 2015, and it gradually decreased to about 30 cases at the end of the year. Interestingly, and confirming the PCR results, the percentage of monthly cases with this elevated anti-PT IgG titer was highest in the summer months August (34·5%) and September (30·7%), as compared with the average 17·3% detected on annual basis (Table 4).

The anti-PT levels measured in the different age categories are shown in Table 5 and Figure 1. In total **844** (17·3%) serum samples had anti-PT IgG levels >125 IU/ml, indicative of an acute infection. In 2015, the highest number of sera with anti-PT levels >125 IU/ml was in the age group of 10–14 years old. This age group contributed to 21·8% of the total number of cases detected by qPCR and 13% of the total number of cases evaluated by serology. Furthermore, the percentage of sent samples that scored positive was also the highest (28·7% and 43·8% in qPCR and serology, respectively) in this age category. As vaccination can also induce PT-specific antibodies, we checked for the vaccination status in this age group when possible. Of the 251 children tested in this age group, the date of the last vaccine dose was only reported to the NRC for 38 (15·1%). Among these children, 14 (37·8%) had anti-PT levels higher than 125 IU/ml indicative of an acute pertussis, ranging from 170 IU/ml to >500 IU/ml. One child (462 IU/ml) had reportedly received a booster vaccination during the last year, whereas the 37 other children had received their last dose at the age of 4–6 years. This was the case for 13 children with anti-PT levels >125 IU/ml, eight children with anti-PT levels between 55 and 125 IU/ml and for 16 children with anti-PT levels <55 IU/ml. Based on these (limited) figures, we estimated that <3% of the

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Table 3. *Age distribution of pertussis cases detected by qPCR and culture*

Age (year)	Number of samples on which PCR was performed	Number of samples positive for *B. pertussis* (% per age group)	*B. pertussis* positive culture (%)
<1	1220	**64** (5·2)	29 (45·3)
1–4	729	**59** (8·1)	16 (27·1)
5–9	340	68 (20·0)	16 (23·5)
10–14	300	86 (**28·7**)	19 (22·1)
15–19	111	10 (9·0)	6 (60·0)
20–24	68	7 (10·3)	2 (28·6)
25–29	79	7 (8·9)	2 (28·6)
30–34	102	7 (6·9)	3 (42·9)
35–39	136	8 (5·9)	2 (25·0)
40–44	138	16 (11·6)	3 (18·8)
45–49	147	9 (6·1)	3 (33·3)
50–54	139	11 (7·9)	3 (27·3)
55–59	143	9 (6·3)	3 (33·3)
60–64	112	6 (5·4)	2 (33·3)
65–69	75	7 (9·3)	3 (42·9)
70–74	65	8 (12·3)	0 (0·0)
75–79	32	4 (12·5)	0 (0·0)
80–84	25	2 (8·0)	0 (0·0)
>85	19	0 (0·0)	0 (/)

Age group with highest percentage of positive samples is indicated in bold.

high-titered serum samples in the teenager group may have reflected a recent vaccination.

Also the age groups 40–44 and 45–49 year each accounted for more than 10% of the total number of reported pertussis cases, confirming the notion that pertussis is no longer exclusively a childhood disease.

Another **1042** (21·4%) serum samples had anti-PT titers between 55 and 125 IU/ml, indicative of a pertussis infection during the last 1–2 years. The percentage of these samples was about 20% in all age categories, except for the age group of young adolescents 15–19 years old, in which 31·8% of the samples had this intermediate titer. Although vaccination status was not recorded for most patients, the high percentage in this age category probably reflected the booster vaccination recommended for adolescents at the age of 14–16 years by the Superior Health Council (HGR 8532).

Pertussis cases detected both by qPCR and serology

Eight cases of pertussis were detected both by qPCR and anti-PT IgG serology. Details are shown in Table 6. One of the cases was also positive in culture.

Table 4. *Monthly distribution of number and percentage of cases diagnosed as acute infection*

Month	Number of sera analyzed	Number of sera with anti-PT > 125 IU/ml	Monthly percentage with anti-PT > 125 IU/ml
January	701	102	14·5
February	643	79	12·0
March	571	87	15·0
April	503	75	14·9
May	341	63	18·5
June	342	78	22·8
July	253	59	23·3
August	197	68	**34·5**
September	306	94	**30·7**
October	371	65	17·5
November	325	40	12·3
December	324	34	10·5
Total	**4877**	**844**	**17·3**

Months with highest percentages of sera having anti-PT levels >125 IU/ml are indicated in bold.

Six of the cases diagnosed by both methods had been showing symptoms for 3 weeks or more before the date of diagnosis. The other two cases were diagnosed 1 week after onset of symptoms. Among the eight cases diagnosed by both methods, there were four children under the age of 10, as well as one adolescent of 14 years old, and three adults. The oldest patient, 41 years old, was hospitalized. The date of the last vaccine was known in only three cases, all children under the age of 10, whereas the vaccination status was unclear in the four adult cases.

DISCUSSION

Notification of pertussis to the Health Inspection is mandatory in Belgium. Both sentinel laboratories and the NRC for *Bordetella* report the cases of acute infection to the authorities. In the peak incidence year 2014, 1056 cases were reported in Flanders, 830 cases in Wallonia and 117 cases in Brussels Capital region [18]. Using a log-linear capture–recapture method, the total number of cases of pertussis in Belgium in 2014 (all ages) was estimated to range from 24·2 to 30·8/100 000 [19]. As reported in detail in this paper, a total **1246** cases of pertussis were diagnosed by the Belgian NRC consortium in 2015: 844 cases by serology, 394 cases by PCR and eight cases by both methods. Furthermore, adult pertussis can even be asymptomatic, as indicated by our

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Table 5. *Age distribution of number and percentage of sera with anti-PT titers indicative of acute infection (>125 IU/ml) or recent vaccination/pertussis infection during the last 1–2 years (55–125 IU/ml)*

Age (year)	Total number of sera tested	Number of sera with anti-PT > 125 IU/ml (% of total number, $n = 844$)	Percentage of acute infections/age group	Number of sera with anti-PT of 55–125 IU/ml (% of total number, $n = 1042$)	Percentage of positive sera/age group
<1	33	2 (0·02)	6	2 (0·02)	6
1–4	98	28 (3·3)	28·5	14 (1·3)	14
5–9	164	49 (5·8)	29·9	28 (2·6)	17
10–14	251	**110 (13)***	**43·8**	39 (3·7)	15·5
15–19	214	26 (3·1)	12·1	68 (6·5)	**31·8**
20–24	178	34 (4·0)	19·1	37 (3·5)	20·8
25–29	228	30 (3·5)	13·1	55 (5·3)	24·1
30–34	299	28 (3·3)	9·4	68 (6·5)	22·7
35–39	398	79 (9·3)	19·8	89 (8·5)	22·4
40–44	480	91 (10·8)	18·9	84 (8·1)	17·5
45–49	455	90 (10·7)	19·8	93 (8·9)	20·4
50–54	478	65 (7·6)	13·6	**115 (11·0)**	24·1
55–59	500	77 (9·1)	15·4	**111 (10·6)**	22·2
60–64	381	50 (5·9)	13·1	81 (7·8)	21·3
65–69	273	33 (3·9)	12·1	72 (6·9)	26·4
70–74	174	28 (3·3)	16·1	36 (3·4)	20·7
75–79	92	16 (1·9)	17·4	19 (1·8)	20·6
80–84	76	3 (0·3)	**3·9**	23 (2·2)	**30·3**
>85	40	7 (0·8)	17·5	8 (0·8)	20·0

The highest numbers and percentages for both categories are highlighted in bold.
* Based on the data of the last vaccination (reported for 15% of the samples), recent booster vaccination in the teenager group (10–14 year) may have contributed for <3% of these high titered samples.

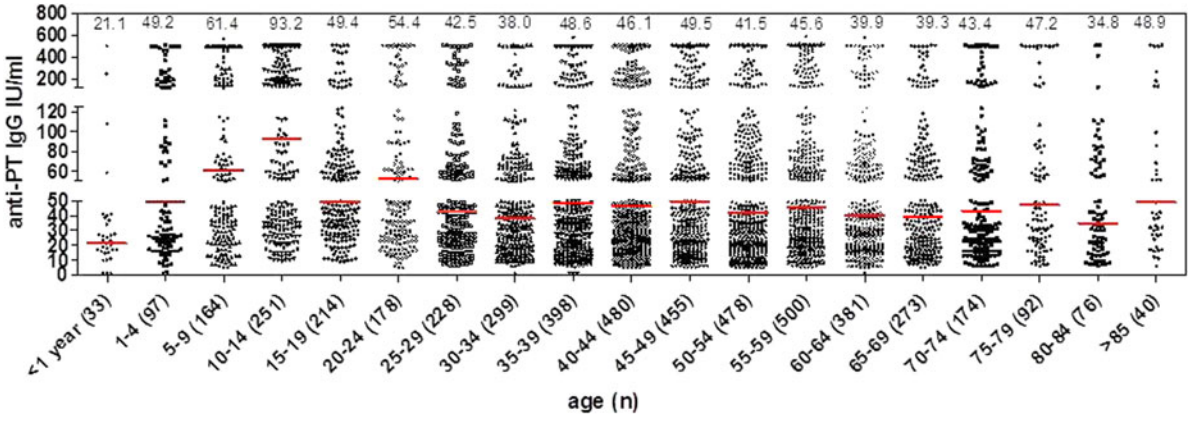

Fig. 1. Overview of the NRC results of 2015 pertussis serodiagnosis for the different age categories (the number of samples tested is given in parentheses). The red line and figure on top of each age category indicate the median concentration calculated for each age group.

Belgian 2012 serosurveillance study on a group of 1500 'healthy' persons of 20–39 years old, among whom 4% had anti-PT antibody levels indicative of a very recent infection [16]. Although the whole Belgian territory was not covered in that serosurveillance study, the results were strongly suggestive of an asymptomatic *B. pertussis* reservoir in the adult Belgian population, confirming reports from other European countries [20].

The number of reported pertussis cases across all age groups has increased during the past two decades in many industrialized countries despite high vaccination coverage, first with a whole-cell-based vaccine and since the end of the 1990s with an acellular

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Table 6. *Patient characteristics of pertussis cases detected both by qPCR and serology*

Patient	Age	Sex	Duration cough (weeks)	Hospitalisation	Vaccination	PCR	Culture	Anti-PT IU/ml
1	4·7	F	4	N	4	pos	neg	>500
2	6·6	F	3	N	4	pos	neg	301
3	6·9	M	5	N	4	pos	neg	>500
4	9·5	F	1	N	4	pos	neg	464
5	14·8	M	3	N	UNK	pos	neg	>500
6	21·6	M	3	N	UNK	pos	pos	427
7	22·2	F	3	N	UNK	pos	neg	223
8	41·4	M	1	Y	UNK	pos	neg	>500

F, female; M, male; N, no; Y, yes; UNK, unknown.

vaccine, composed of 1–5 purified protein antigens in alum adjuvant [1]. Also in Belgium, the number of reported cases has increased in recent years and in a 20-year follow-up study, we have previously reported that the age distribution of cases (diagnosed by serology) shifted from children <5 years old in 1990 to teenagers and adults in 2009 [21].

Neither vaccination nor infection induce a lifelong protection against pertussis and waning is thought to occur 4–12 years after the last booster dose and 7–20 years after an episode of illness [22]. In Belgium, acellular pertussis vaccine composed of PT, FHA, and Prn is administered to infants in a combined vaccine targeting also poliomyelitis, diphtheria, tetanus, hepatitis B, and *Haemophilus influenzae* type B, in three doses at 8–12–16 weeks, followed by a fourth dose at 15 months. Since 2004, one dose of aP vaccine is recommended at 4–6 years of age and since 2009, the Superior Health Council recommends an additional booster vaccination for adolescents at the age of 14–16 years (HGR 8532). Also since 2009, a so-called cocoon vaccination of one dose of Tdap vaccine is recommended for all adults in frequent contact with not or not fully vaccinated babies, <1 year old (future parents and grandparents, staff in nurseries and health care personnel). As pertussis is particularly serious and can be life-threatening for these young babies, the Superior Health Council promotes since 2013 the vaccination of all pregnant women between week 24 and 32 of their pregnancy (HGR 8547) [23]. A quantitative multicenter survey study has been performed in Flanders between October 2014 and May 2015 in postpartum women ($N = 823$, response rate = 89·2%) to assess the coverage of pertussis (and influenza) vaccination during pregnancy. Overall coverage of pertussis vaccination during pregnancy was 64·0%, most often in the third trimester (74·0%) of pregnancy [24]. In the context of this maternal pertussis vaccination in Flanders, we have reported on a follow-up study of vaccine responses in infants until 1 month after the fourth infant pertussis vaccination at 15 months of age. A good humoral immune response was elicited against pertussis, diphtheria, and tetanus antigens in children born from mothers vaccinated during pregnancy and control mothers. However, pregnancy vaccination was associated with a minor blunting effect on anti-PT IgG antibody level and antibody avidity that persisted 1 month after the fourth vaccine dose [25, 26].

In summary, the results of our NRC consortium for *B. pertussis* have shown that optimal pertussis diagnosis requires the combination of both microbiological and immunological methods. It is clear that pertussis continues to be a problem in Belgium and that other vaccination strategies (maternal vaccination, cocoon vaccination, adult boosters?) and ultimately better vaccines will be needed to control this highly infectious respiratory disease [27, 28].

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