CVI Accepts, published online ahead of print on 4 December 2013
Clin. Vaccine Immunol. doi:10.1128/CVI.00641-13

Antibody responses in Sweden to individual *Bordetella pertussis* fimbriae serotype 2 or 3 following immunization with whole cell or two- or five-component acellular pertussis vaccines and following pertussis disease and in children in 1997 and 2007.

Hans Hallander[1], Abdolreza Advani[1], Frances Alexander[2], Lennart Gustafsson[1], Margaretha Ljungman[1], Catherine Pratt[2], Ian Hall[2] and Andrew R. Gorringe[2#]

[1]Swedish Institute for Communicable Disease Control, Stockholm, Sweden. [2]Public Health England, Porton Down, Salisbury, UK

Running title: *Bordetella pertussis* anti-Fim2 and anti-Fim3 IgG responses

Corresponding Author: Andrew Gorringe, andrew.gorringe@phe.gov.uk

ABSTRACT

Bordetella pertussis fimbriae (Fim2 and Fim3) are components of a five component acellular pertussis vaccine (DTaP5) and antibody responses to fimbriae have been associated with protection. We have analysed the IgG responses to individual Fim2 and Fim3 in sera remaining from a Swedish placebo-controlled efficacy trial that compared a whole cell (DTwP), a two-component acellular pertussis vaccine (DTaP2) or DTaP5. One month following three doses of the Fim-containing vaccines (DTwP or DTaP5), anti-Fim2 geometric mean IgG concentrations were higher than anti-Fim3, with a greater anti-Fim2: anti-Fim3 IgG ratio elicited by DTaP5. We have also determined responses in vaccinated children following an episode of pertussis. For those who received DTaP5, there was a large rise in anti-Fim2 IgG, reflecting the predominant Fim2 serotype at the time. In contrast, those who received DTwP showed an equal rise in anti-Fim2 and anti-Fim3 IgG, indicating DTwP may prime more efficiently for a Fim3 response following contact with *B. pertussis*. Anti-Fim2 and anti-Fim3 IgG concentrations were also determined in samples from two seroprevalence studies conducted in Sweden in 1997, when no pertussis vaccine was used and Fim2 isolates predominated, and in 2007 when either DTaP2 or DTaP3 vaccines without fimbriae were used and Fim3 isolates predominated. Very similar distributions of anti-Fim2 or anti-Fim3 IgG in 1997 and 2007 were obtained, except that anti-Fim3 concentrations in 1997 were lower. This observation, together with the number of individuals with both anti-Fim2 and Fim3 IgG strongly suggests that *B. pertussis* expresses both Fim2 and Fim3 during infection.

250 words

45 **INTRODUCTION**

46 *Bordetella pertussis* causes whooping cough which despite high vaccine coverage continues to
47 be a public health concern. Indeed, rates of pertussis have increased in many countries in recent
48 years with a concern that immunity provided by acellular vaccines may be waning more quickly
49 than that provided by whole cell pertussis vaccines (1, 2, 3). Thus it is important to understand
50 the antibody responses induced by the components of acellular pertussis vaccines, particularly
51 those associated with protection.

52 Fimbriae are important antigens in the pathogenesis of pertussis disease, functioning as
53 adhesins (4). They are built up by subunits to make long filamentous structures on the surface of
54 the bacteria (5). Two different serologically distinct fimbriae are expressed which are composed
55 of either Fim2 or Fim3 major subunits, 22.5 and 22.0 kDa respectively (6). Subtypes of Fim2 (*fim*
56 *2-1* and *fim 2-2*) and Fim3 (*fim3-1*,*-fim3-2*, and *fim3-3*) have also been described (7, 8). Fimbriae
57 are present in whole cell pertussis vaccines and in 1979 the WHO, supported by epidemiological
58 data, suggested that both Fim2 and Fim3-expressing strains should be included in whole cell
59 pertussis vaccines (9, 10, 11). Fim2 and Fim3 are also included in a 5-component acellular
60 pertussis vaccine (DTaP5). The fimbriae are co-purified during the vaccine manufacturing
61 process and the specific content of Fim2 and Fim3 has not been defined (12).

62

63 In Sweden the population was not vaccinated against pertussis between 1979 and 1996. During
64 this period a number of vaccine trials were therefore performed. In Sweden trial I (13) the
65 efficacy of acellular vaccines composed of two components (DTaP2 containing pertussis toxin,
66 Ptx and filamentous haemagglutinin, FHA), or five components (DTaP5 containing Ptx; FHA;

pertactin, Prn; and co-purified Fim2+Fim3, 5µg per dose) and a whole cell vaccine (DTwP) was compared. Efficacy of 85.2% was demonstrated for DTaP5, when compared to a placebo DT in a 2-, 4-, 6-months schedule using the WHO-defined criteria that included culture positive as well as paired serology-positive cases, with 58.9% and 48.3% efficacy reported for DTaP2 and DTwP respectively.

In Sweden trial II (14), a trial without a placebo control, the same two component (DTaP2), a three component (DTaP3 containing Ptx, FHA and Prn) and a five component DTaP5 acellular vaccine and another whole cell DTwP vaccine were compared. It was shown that when mild pertussis disease was included in the case definition there was significantly lower relative effectiveness of the three-component acellular pertussis vaccine not containing fimbriae (DTaP3) compared to the DTaP5 vaccine.

An interesting observation arose from the long term follow-up of children in trial II. The relative effectiveness of DTaP5 has diminished slightly (15) compared to both the DTwP and the DTaP3 vaccine over the same time period. One hypothetical explanation is that the expression of fimbriae in circulating *B. pertussis* changed from predominantly Fim2 during the period when trials were performed to predominantly Fim3 around 1998-1999 and later. The DTaP5 vaccine might have been more effective in an environment dominated by Fim2 strains due to a stronger immune response produced by the Fim2 antigen in the vaccinated subjects than by the Fim3 antigen. This hypothesis is supported by serotype data from about 500 culture positive children in trial I. There was a statistically significant lower rate of Fim2 isolates among pertussis cases

89 vaccinated with DTaP5 compared with the rate of Fim2 isolates in the non-fimbriae group (16),
90 indicating that the Fim2 antigen in the DTaP5 vaccine was more protective than the Fim3
91 antigen.
92
93 Over the years there has been much interest concerning the correlation between antibodies to
94 *B. pertussis* virulence factors and protection. In the early days of pertussis vaccination
95 agglutinins were used as markers for protection (17). It is now thought that the agglutinins are
96 directed to fimbriae, Prn and lipopolysaccharide (18). Meade et al. showed that appropriate
97 anti-fimbriae2+3 ELISA assays (19) gave results similar to those provided by agglutinin assays in
98 the evaluation of acellular vaccine immunogenicity. Correlation between levels of antibodies to
99 acellular vaccine components and protection against pertussis disease after household exposure
100 to *B. pertussis* was also studied in the Sweden vaccine trial I. The correlation between levels of
101 anti-pertactin antibodies and antibodies against co-purified Fim2+3 antigen and disease was
102 clear and statistically significant. A weak protective relationship was revealed for anti-Ptx
103 antibodies (20). Similar results were reported by Cherry et al. in a German household study (18).
104
105 Recently, we have described the antibody response to individual Fim2 or Fim3 following
106 immunization with DTaP5 (containing 5µg per dose Fim2+3) or pertussis disease (21). It was
107 found that all individuals showed increases in anti-Fim2 and anti-Fim3 IgG concentrations
108 following vaccination with DTaP5, with 3-fold greater anti-Fim2 than anti-Fim3 IgG
109 concentrations seen in 15-month-old and 4- to 6-year-old children. It was also shown that
110 individuals with evidence of recent pertussis disease (confirmed by Ptx serology) had greater

anti-Fim3 than Fim2 IgG concentrations, consistent with the predominant serotype of *B. pertussis* isolates during the sampling period.

In a previous report we characterized anti-Fim antibodies after immunization at 2, 4 and 6 months of age and after a later episode of pertussis (verified by paired anti-Ptx serology) in 370 sera from 96 participants of Sweden trial I using co-purified anti-Fim2+3 antigen (22). The separate Fim2 and Fim3 antigens have now made it possible to go back and characterize the concentrations of anti-Fim2 and anti-Fim3 IgG separately in the subset of those children with sera still available. In addition, sera from two seroepidemiology collections were also re-analysed for anti-Fim2 and anti-Fim3 IgG. The first set of serum samples were collected in 1997 in Sweden just after vaccination against pertussis was reintroduced after a hiatus of 17 years. The children included in this serosurvey were all non-vaccinated and at this time Fim2 strains were predominant. The other set of serum samples was collected in 2007 after 10 years use of either of the non-fim vaccines DTaP2 (Pentavac, SanofiPasteurMSD)® or DTaP3 (Infanrix®, SmithKline Beecham) when Fim3 strains predominated (22) .

The aims of this study were to determine the specific anti-Fim2 and anti-Fim3 IgG responses after vaccination with either DTwP or DTaP5 vaccines, as well as specific anti-Fim IgG profiles before and after disease in primed and non-primed children. An additional aim was to analyze separate anti-Fim2 and anti-Fim3 IgG responses in seroprevalence samples obtained in Sweden in 1997 and 2007, and positive with the combined anti-Fim2/3 antigen, to determine the effect of the prevailing *B. pertussis* serotype.

MATERIALS AND METHODS

Vaccines and sera used in Sweden trial I (1992-1995):

Vaccines used in Sweden trial I

1. The experimental two-component acellular DTaP2 vaccine (SmithKline Beecham, Rixensart, Belgium; now GlaxoSmithKline).

2. The experimental five-component acellular DTaP5 vaccine (Connaught Laboratories, Toronto; now Sanofi Pasteur).

3. The whole cell DTwP vaccine licensed in the United States (Connaught Laboratories Swiftwater, Pa).

4. The DT vaccine (SBL-vaccin AB, Stockholm) used as placebo contained diphtheria and tetanus toxoids.

All vaccines were adsorbed to aluminium salts.

Collection of serum samples in Sweden trial I.

Sweden trial I was a placebo-controlled randomized vaccine efficacy trial that enrolled 9829 children born in 1992 who were immunized at 2, 4, and 6 months of age in one of four vaccine arms as described above (13). Serum samples in this study were obtained from subsets of the following:

1. Pre-planned serum samples were collected from almost all of the children in trial I at the pre-determined age of 1 year, and later at either 2, 2.5 or 3 years of age.

2. Pre-scheduled serum samples for immunogenicity studies were also collected systematically at one study site, Linköping. All children recruited in Linköping during trial I were routinely bled

155 at 2 months of age just before the first trial dose and thereafter at 7, 13 and 29 months of age,
156 i.e. 1, 7 and 23 months after the third vaccine dose.
157 3. Acute and convalescent serum samples, obtained during episodes of suspected pertussis, and
158 analyzed for diagnostic purposes during the pertussis trial I.
159
160 *Serum samples in Sweden trial I used in this study*
161 1. All serum samples obtained one month post the third dose of either DTaP5 (182 children) or
162 DTwP (146 children), at one of the fourteen study sites, were analysed for anti-Fim2 and anti-
163 Fim3 IgG concentrations.
164 2. In addition, serum samples from infected young children from all four vaccine arms in the
165 Sweden trial I were used to study anti-Fim2 and anti-Fim3 IgG concentrations (amplitude, decay
166 and persistence) before, during and after a pertussis infection.
167 For this study we identified a subset of 91 children who received three trial vaccine doses and
168 with a pertussis infection confirmed by anti-Ptx IgG ELISA after the third trial dose according to
169 the criteria in trial I (13), and with one or more serum samples obtained, before, during and
170 after the end of the pertussis episode. A total of 334 serum samples still available, three to four
171 per child, were obtained in trial I from the 91 culture-negative but anti-Ptx positive children.
172 These samples were re-analysed for both anti-Fim2 and anti-Fim3 IgG concentrations. Typically,
173 for each child, a 1-year sample was taken before the positive episode, an acute and a
174 convalescent sample taken during the episode, and a 2-, 2.5- or a 3-year blood sample taken
175 after the end of the pertussis episode. A pre-episode sample for re-analysis existed for 79
176 children. Sera (n=255) after onset of pertussis disease were still available from 91 children.

There were 13 children available given DTaP5, 16 given DTwP, 46 given DTaP2 and 16 given DT with pertussis infection during the trial and with blood samples taken after onset of the pertussis episode. Children in the DTP groups may be regarded as vaccine failures, while the DT group served as a placebo control.

Vaccines in the universal vaccination programme in Sweden after 1996. During the 10-year period after reintroduction of pertussis vaccination the vaccines used in infancy differed in time and by geographic regions. During 1996 and 1997 a trivalent three-component DTaP3 containing Ptx, FHA and Prn (Infanrix®, SmithKlineBeecham) was used in the whole country, except in Gothenburg area where a one-component DTaP1, containing Ptx only, was used. From the end of 1998 Infanrix® was replaced in a number of counties by a pentavalent two-component DTaP2-IPV-Hib with Ptx and FHA (Pentavac®, Sanofi Pasteur MSD).

Seroepidemiology serum samples 1997 and 2007

In 1997 a seroepidemiology study was conducted in Sweden (22). None of these children had received pertussis vaccine. A similar seroepidemiology study was conducted in 2007 (22). Children sampled in 2007 had not been immunized with pertussis vaccines containing fimbriae. In the age group 2-15 years altogether 184 and 95 sera respectively which had measurable (≥2 EU/ml) anti-Fim2+3 IgG were available for analysis with separate Fim2 and Fim3 antigens.

ELISA for anti-Fim2 and anti-Fim3 IgG.

An indirect ELISA method was used to measure the concentrations of IgG antibodies to Fim2

and Fim3 separately. The general outlines were described in previous publications (23, 24). Fim2 and Fim3 antigens were prepared as described by Alexander et al. (21) and were both treated with 4M urea before use in the assays. The antigens were diluted to 1.0 mg/L in PBS pH 7.4 and were used for coating plates overnight at room temperature. A NIBSC reference human antiserum, 89/530, previously determined to contain 74.1 EU/ml of IgG anti-Fim2 and 14.8 EU/ml of IgG anti-Fim3 was used as primary calibrator (21). A pool of fractioned human plasma (Ig42) was used as in-house secondary calibrator. Following parallel assays in duplicate on three different days, Ig42 was assigned 2300 EU/ml for anti-Fim2 and 2200 EU/ml for anti-Fim3 and diluted 1/100 when used in the assay. The minimum level of detection (MLD) was 2 EU/ml for anti-Fim2 and 1 EU/ml for anti-Fim3. For the analyses of antibody concentrations 5 EU/ml was used as cut-off as it was previously used in a study addressing correlates of protection for anti-Fim 2+3 (20)

Statistical methods. Geometric mean antibody concentrations with 95% confidence intervals were calculated. Reverse cumulative distribution curves were plotted as described by Reed et al. (25). Comparison of groups was performed using the Mann Whitney test and Minitab version 16.2.2.

RESULTS

Quantification of anti-Fim2 and anti-Fim3 IgG concentrations in sera from Sweden trial I at 7 months of age following vaccination at 2, 4, and 6 months of age. Sera from 182 children who received three doses of DTaP5 and 146 children who received DTwP were analysed (Table 1).

Geometric mean anti-Fim2 IgG concentrations were significantly higher in children who received DTaP5 compared to those that received DTwP ($P<0.01$). The anti-Fim2 IgG concentrations were greater than anti-Fim3 following both vaccines, although the ratio of anti-Fim2:3 was lower in individuals who received the DTwP vaccine (ratio = 1.6) compared with those that received DTaP5 (ratio = 6.6, $P<0.05$). The distribution of anti-Fim2 and anti-Fim3 IgG concentration values obtained is shown in the reverse cumulative distribution curves in Figure 1. The greater proportion of individuals with higher anti-Fim2 IgG concentrations can be seen for both vaccines, with over half of those who received DTaP having anti-Fim2 IgG concentrations greater than 100 EU/ml. In addition, the gradient of the anti-Fim3 curve for the subjects who received DTwP is steeper than for the subjects who received DTaP, indicating a more uniform response with the DTwP vaccine.

Quantification of anti-Fim2 and anti-Fim3 IgG concentrations in sera from Sweden trial I at 12 months of age following vaccination at 2, 4 and 6 months of age

To determine the anti-Fim2 and anti-Fim3 IgG concentrations in vaccinated individuals before the onset of pertussis disease we identified 79 pre-disease serum samples, 74 of which were taken at 1-year of age, i.e. at about 6 months after the third vaccine dose. The other 5 were early acute samples collected when the "sample trigger" for a study child was present which was either pertussis or suspected pertussis in another household member. These samples were taken between 17 and 595 days prior to onset of cough. These sera were analysed for anti-Fim2- and anti-Fim3-specific IgG and the geometric mean concentrations of sera from each vaccine group are shown in Table 2.

There were 13 samples from children vaccinated with DTaP5 vaccine and 12 vaccinated with DTwP vaccine. All but one of these samples was taken at 1 year of age. The geometric mean IgG antibody concentrations seen for Fim2 were higher than those for Fim3 following either DTaP5 or DTwP although this difference was not significant (P>0.1). However, there was a greater anti-Fim2: anti-Fim3 ratio following DTaP5 vaccination than following DTwP (P=0.03). Only one of the samples in each of the DTaP5 and DTwP groups had a ratio below 1.

One child (8%) who received DTaP5 was below the 5 EU/ml cutoff for anti-Fim2 IgG and so were two children (17%) who received DTwP. For anti-Fim3 IgG, 69% (n=9) and 33% (n=4) of samples were below this cutoff for the DTaP5 and DTwP groups respectively. Only one sample was below the MLD for both anti-Fim2 and anti-Fim3 IgG following immunization with a Fim-containing vaccine.

In individuals vaccinated with DT or DTaP2, 52 of 54 samples (96%) were previously found to have anti-Fim2+3 IgG below the protective cutoff of 5-EU/ml (20). Of these, 49 were below the MLD for anti-Fim2 and 29 were below the MLD for anti-Fim3.

Peak anti-Fim2 and anti-Fim3 IgG concentrations determined after vaccination and onset of pertussis disease.

Two hundred and fifty five samples from 91 children from Sweden trial I following vaccination with either DTaP5, DTaP2, DTwP or DT were available for quantification of anti-Fim2 and anti-Fim3-specific IgG after onset of pertussis disease. There were 29 children with 80 samples in the two Fim-containing vaccination groups and 62 children with 175 samples in the two Fim-naïve groups. The geometric mean concentrations of the "peak" values obtained for each child are shown in Table 3. The trends are also visualized by reverse cumulative distribution curves of the peak IgG concentrations as shown in Figure 2, although based on few cases (n=13 for DTaP5, n=16 for DTwP). It can be seen that the Fim-containing vaccines provide a clear priming effect for both Fim2 and Fim3 with large increases in IgG concentration to both antigens following disease. In children who received DTaP5, the post disease peak geometric mean IgG concentration is 11.1-fold greater for anti-Fim2 and 4.2-fold greater for anti-Fim3 than was observed at 6 months following vaccination. Those who received DTwP appeared equally primed for anti-Fim2 and anti-Fim3 IgG responses with rises of 9.4 and 8.5-fold respectively (Table 3 compared to Table 2). Significantly lower anti-Fim2 and anti-Fim3 IgG geometric mean concentrations were seen in children who received the vaccines without fimbriae (DTaP2 and DT, $P<0.05$, Table 3) compared to the Fim-vaccine group.

276

277 The reverse cumulative distribution curves of the post vaccination, post disease samples
278 analysed for anti-Fim2 and anti-Fim3 IgG (Figure 2) clearly show the higher concentrations of
279 anti-Fim2 IgG in those who previously received DTaP5 or DTwP than for the Fim-naïve group
280 with a steeper gradient of responses evident for DTaP5 recipients, indicating a more uniform
281 response to this antigen with the acellular vaccine. The curves for anti-Fim3 IgG concentrations
282 reinforce the observation that DTwP provides effective priming for the anti-Fim3 response
283 following disease and that the Fim3 component of DTaP5 is inferior in this respect, with a
284 similar profile of post disease anti-Fim3 IgG concentrations in those who received DT, DTaP2 or
285 DTaP5. Among those not primed with fimbriae, 80% of samples in the DTaP2 group were below
286 5 EU/ml for anti-Fim2 IgG and 64% for anti-Fim3 IgG following the pertussis episode. In the DT
287 group the corresponding figures were 60% and 56% of samples respectively. The anti-Fim2 and
288 anti-Fim3 IgG peak responses were also lower for those vaccinated with DTaP2 compared to
289 those who received DT, although these differences did not reach significance DTaP2 vs DT, Fim2
290 *P*=0.19; Fim3 *P*=0.14).

291

292 **Kinetics of the anti-Fim2 and anti-Fim3 IgG responses in young children with disease which**
293 **occurred after the third DTaP5 or DTwP vaccine dose.** For analysis of the kinetics of anti-Fim2
294 and anti-Fim3 IgG before and after infection we examined 105 sera (80 post onset) from 29
295 children who were positive for pertussis by anti-Ptx IgG ELISA (3-4 sera per child). The first day
296 of cough was defined as the onset of an episode and the samples were from a period from 595
297 days before onset of symptoms until 700 days after the first day of cough. Samples were

298 grouped into one of twelve time intervals before or after the onset of cough. The kinetics of the
299 anti-Fim2 and anti-Fim3 IgG responses are shown in Figure 3. The increase of anti-Fim2 IgG
300 commenced within the first two to three weeks of cough, confirming a booster response in
301 these DTaP5 or DTwP-vaccinated children. Maximum values for anti-Fim2 (approximately 150
302 EU/ml) were reached within 2 months after onset of cough. The increase in anti-Fim3 antibodies
303 started later, at about 5 weeks after onset, and the median peak value at two months post
304 onset of cough was just over 25 EU/ml. The increase from pre-to maximum post onset was
305 about 10-fold for anti-Fim2 IgG and about 4-fold (and from a lower pre-onset level) for Fim3.
306 The decay of the curve indicates there was a change from a rapid to a slower decay between 3-5
307 months after the first day of cough. Anti-Fim2 IgG returned to pre-disease levels at about 18
308 months after the onset of disease.
309
310 **Anti-Fim2 and Fim3 IgG concentrations in seroepidemiology samples obtained in Sweden in**
311 **1997 and 2007.** Seroepidemiology samples were obtained in Sweden in 1997 from children who
312 had not received pertussis vaccination and when Fim2 was the predominant *B. pertussis*
313 serotype. Similar samples were also obtained in 2007 from children who had received acellular
314 pertussis vaccines that did not contain fimbriae and when the predominant serotype was Fim3.
315 All children used in this study, aged 2-15 years, were selected as they had measurable
316 antibodies against Fim2+3 (22). There is no reason to believe that samples negative with the
317 combined antigen should be positive with the separate Fim2 and Fim3 antigens. There were 184
318 sera included from 1997 and 95 sera from 2007. Reverse cumulative distribution curves of anti-
319 Fim2 and antiFim3 IgG concentrations obtained in samples from these two years is shown in

Figure 4. It can be seen that the curves obtained for anti-Fim2 and anti-Fim3 in 2007 and for anti-Fim2 in 1997 are aligned with no significant difference between these values (P>0.05). However, lower anti-Fim3 IgG concentrations were obtained in samples from 1997 (P < 0.001). These data are also presented in Table 4 which shows the number and per cent of individuals with anti-Fim2, anti-Fim3 IgG in the ranges 4 - <14, 14 - <100 or >100. This analysis highlights that there are the same percentage of observations in each range for anti-Fim2 or anti-Fim3 in 2007. It also highlights the greater percentage of individuals in 1997 with anti-Fim2 IgG concentrations between 14-100 EU/ml (58%) than anti-Fim3 (40%, P<0.05).

DISCUSSION

B. pertussis fimbriae have been shown to be important components of both whole cell and acellular vaccines and antibodies to these proteins have been associated with protection in early trials of whole cell vaccines (MRC) and in household exposure studies (18, 20). IgG responses to co-purified Fim2+3 have been measured in the clinical trials of acellular pertussis vaccines (13, 26) and many subsequent studies. The predominant serotype expressed by *B. pertussis* isolates has changed in a number of countries during different time periods (27, 28, 29) and the reasons for this are not understood. *B. pertussis* isolates possess *fim2* and *fim3* genes and expression is dependent on a run of C residues in the promoter regions of these genes (12). Spontaneous changes in the poly C tract length allow or stop expression but despite this genetic flexibility the predominant Fim type remains stable over time until a switch in the population occurs. It is not known if this is driven by immune evasion following a rise in anti-Fim2 or anti-Fim3 IgG levels in those infected.

342

343 To understand the role of antibodies to Fim2 and Fim3, we have purified individual Fim2 and
344 Fim3 from *B. pertussis* and used these to quantify anti-Fim2- and anti-Fim3-specific IgG
345 following vaccination with DTaP5 and in individuals with clear evidence of on-going and recent
346 pertussis (21). The ELISA IgG concentration values assigned to standard serum 89/530 in the
347 previous study (21) were determined by aligning the ELISA dose response curve for a 1:1
348 mixture of Fim2 and Fm3 with the dose response curves obtained with this serum and individual
349 Fim2 or Fim3 coated at an equal protein concentration. Thus the anti-Fim2 and anti-Fim3 IgG
350 concentration values assigned to each serum allow comparison of IgG antibody responses to
351 these antigens, although the comparison should be treated with caution as the functional
352 activity of the antibodies is not known. These separate Fim2 and Fim3 preparations have now
353 been used to quantify anti-Fim2 and and anti-Fim3 IgG responses in sera from Sweden Trial I
354 obtained between 1992 and 1995 (13) and from seroepidemiology samples obtained in Sweden
355 in 1997 and 2007 (22).

356

357 A unique serum collection from a well-controlled group of small children, vaccinated with three
358 doses of either DTaP5, DTaP2, DTwP or placebo DT vaccine and assessed previously for anti-
359 Fim2+3 IgG by ELISA (13), has allowed anti-Fim2 and anti-Fim3 IgG responses to be determined.
360 Anti-Fim2 and anti-Fim3 IgG were determined in 7-month-old children for the DTaP5 and DTwP
361 groups only. The anti-Fim2 IgG concentrations were greater than anti-Fim3 for both vaccines. It
362 is interesting to note that the anti-Fim2 and anti-Fim3 IgG responses following three doses of
363 vaccine were 7 and 14-fold lower than those previously determined in sera from 15-month-old

children following a fourth dose of DTaP5 (21). This previous study showed that the ratio of anti-Fim2:anti-Fim3 IgG concentration decreased with additional doses and this study confirms this trend continues to post dose three and that the anti-Fim2:anti-Fim3 ratio is greatest in young children. Anti-Fim2 and anti-Fim3 IgG responses following DTwP have not been reported previously and show that this particular whole cell vaccine elicited lower anti-Fim2 IgG than following DTaP5 (P <0.05) but higher anti-Fim3 IgG geometric mean titres (Table 1) with a lower ratio of anti-Fim2:anti-Fim3 values (1.6) than DTaP5 (6.6)

This serum collection also included children with anti-Ptx IgG-confirmed pertussis infection after the primo-vaccination and this allowed both the anti-Fim2 and anti-Fim3 IgG responses to be determined following disease so that the priming effect of these vaccines for anti-Fim2 and anti-Fim3 responses could be determined. The 6-month post vaccination and pre-disease anti-Fim2 and anti-Fim3 IgG geometric mean concentrations shown in Table 2 were low for the control (DT) and the DTaP2 vaccine without fimbriae. With both DTaP5 and DTwP, anti-Fim2 IgG values were higher than those obtained for anti-Fim3 continuing the trend observed at 7-months of age. These higher anti-Fim2 antibody levels were probably not influenced by earlier episodes of subclinical pertussis as the anti-Fim2+3 concentrations seen in the DTaP5- and DTwP subgroups in an earlier study did not differ from what was seen in a larger data-set of one-year samples after vaccination (22). The values reported previously for the 15-month-old children in samples obtained before the fourth dose of DTaP5 are comparable to those reported in this study from children 6 months following three doses of DTaP5 (21).

386 The anti-Fim2 and anti-Fim3 IgG peak levels found in these children in a current episode of
387 pertussis (Table 3 and Figure 3) show the clear priming effect of prior vaccination with a Fim-
388 containing vaccine. Children who received DTaP5 or DTwP vaccines showed much greater post
389 disease anti-Fim2 and anti-Fim3 IgG levels than those who previously received DT or DTaP2. The
390 responses to Fim2 were greater than to Fim3, corresponding with the predominant Fim2
391 serotype of isolates at this time. However, for interpretation of these data it must be kept in
392 mind that the distinction between serotypes of isolates (2, 3 or 2/3) is based on an insensitive
393 agglutination technique. It might well be that the technique does not detect small amounts of
394 either Fim3 or Fim2 in vitro. Also, both antigens may be expressed in vivo as suggested by
395 Heikkinen et al (30).
396
397 There is also the possibility that the anti-Fim2 response is more efficiently primed, particularly
398 by DTaP5 possibly due to the greater Fim2 than Fim3 content of this vaccine. It is interesting to
399 observe that the ratio of pre- to peak post-disease anti-Fim2 IgG levels following DTaP5 was
400 greater than that for anti-Fim3 (Table 2 and 3), whereas the pre to post disease anti-Fim2 and
401 anti-Fim3 IgG levels were similar in those that had received DTwP. Thus it appears that DTwP is
402 a more effective priming vaccine for a anti-Fim3 response. This clearly shown in Figure 2B,
403 where the reverse cumulative distribution curves for anti-Fim3 IgG levels is clearly shifted to the
404 right for those who have received DTwP. This is perhaps surprising as most isolates during this
405 time were serotyped as Fim2 but may reflect a proportion of strains expressing Fim3. However,
406 some caution is recommended in drawing conclusions from these data as peak anti-Fim2 and
407 anti-Fim3 IgG responses were only determined with sera from a small number of individuals.
408 The observation that anti-Fim2 IgG responses were greater than those for anti-Fim3 following

409 DTaP5 vaccination may tie in with a study also performed in Sweden in 1992-1995 that
410 suggested that DTaP5 may be less effective against Fim3 strains as described in Introduction.
411
412 It is clear that the anti-Fim2 and anti-Fim3 responses seen following disease were greatly
413 influenced by previous vaccination, with greater anti-Fim2 and anti-Fim3 responses seen in
414 those who received DTaP5 or DTwP compared to those vaccinated with a non-fim vaccine. It has
415 been shown that previous vaccination can also blunt responses to antigens not included in the
416 vaccine (31). It can be seen in Figure 2 that the distribution of anti-Fim2 and anti-Fim3
417 responses is lower in those who received DTaP2 compared to those that received the control DT
418 vaccine although this was not significant with the sample size used. Cherry et al. (31) proposed
419 that this was due to linked epitope suppression caused by preferential responses of memory B-
420 cells following secondary exposure to vaccine components. The memory B-cells thus
421 outcompete naïve B-cells for access to the *Bordetella* epitopes. An alternative explanation is
422 that some protection is provided by the DTaP2 vaccine and that vaccine failures in the DTaP2
423 vaccine group may have milder or less prolonged disease than those in the DT group and thus
424 have reduced responses to non-vaccine antigens.
425
426 We also had access to seroepidemiology samples collected in 1997 from children who had not
427 received a pertussis vaccination as well as samples collected in 2007 when either DTaP2 or
428 DTaP3 had been in use for about 11 years. Reverse cumulative distribution curves in Figure 4
429 show that the distribution of IgG concentrations obtained is the same for anti-Fim2 in both
430 years and for Fim3 in 2007, whilst lower anti-Fim3 concentrations were obtained in 1997. The

close similarity of the IgG concentration distributions was also clear from the percentage of individuals with anti-Fim2 and anti-Fim3 IgG concentrations in various ranges (Table 4). The observation that the distributions were the same shows that individuals had an equal likelihood of exposure to Fim2 or Fim3 in 2007, despite the majority of case isolates serotyped as Fim3 only. This is clear evidence that *B. pertussis* is able to express both Fim2 and Fim3 during infection. Expression of both fimbrial genes is affected by a polyC tract in the promoter region (12) with an optimal polyC tract length required for expression. PolyC tract length can be altered by slipstrand mispairing during DNA replication. This may occur in vivo at a higher rate than previously thought and this genetic flexibility may be an advantage to the organism.

Thus, in this study, we have characterized the immune response to separate Fim2 and Fim3 using a panel of sera from Sweden Trial I. This has allowed the anti-Fim2 and anti-Fim3 IgG concentrations to be determined following vaccination with either DT, DTwP, DTaP2 or DTaP5. We have also determined the responses in the same children before, during and following an episode of pertussis disease. Thus the priming potential of DTaP5 and DTwP for anti-Fim2 and anti-Fim3 IgG responses was determined. As expected, due to Fim2 strains predominating at this time there was a greater response to Fim2 than Fim3. However, DTwP vaccinated children produced an equal boost response to both Fim2 and Fim3 suggesting that either there were more circulating Fim3 strains than among those isolated and serotyped or this vaccine provides a very efficient prime for response to this antigen. This also suggests that there is scope for reformulating acellular vaccines to improve the priming provided by the Fim3 component. The distribution of anti-Fim2 and anti-Fim3 IgG concentrations in seroepidemiology samples obtained in 1997 and 2007 showed a very similar distribution of IgG concentrations against both

antigens, except that anti-Fim3 concentrations in 1997 were lower. This observation strongly suggests that *B. pertussis* expresses Fim2 and Fim3 during infection irrespective of the predominant serotype of case isolates.

Acknowledgements

Studies at Public Health England were funded by the National Institute for Health Research Centre for Health Protection Research.

482 **REFERENCES**

483 1. **Klein NP, Bartlett J, Fireman B, Rowhani-Rahbar A, Baxter R**. 2013. Comparative
484 effectiveness of acellular versus whole-cell pertussis vaccines in teenagers. Pediatrics
485 **131:**1716-22.

486 2. **Tartof SY, Lewis M, Kenyon C, White K, Osborn A, Liko J, Zell E, Martin S, Messonnier**
487 **NE, Clark TA, Skoff TH.** 2013. Waning immunity to pertussis following 5 doses of DTaP.
488 Pediatrics **131:** 1047-52.

489 3. **Hallander HO, Nilsson L, Gustafsson L.** 2011. Is adolescent pertussis vaccination
490 preferable to natural booster infections? Expert Rev Clin Pharmacol. **4**:705-11.

491 4. **Rodríguez ME, Hellwig SM, Pérez Vidakovics ML, Berbers GA, van de Winkel GJ**. 2006.
492 *Bordetella pertussis* attachment to respiratory epithelial cells can be impaired by
493 fimbriae-specific antibodies. FEMS Immunol. Med. Microbiol. **46**:39-47.

494 5. **Heck DV, Trus BL, Steven AC.** 1996. Three-dimensional structure of *Bordetella pertussis*
495 fimbriae. J Struct Biol. **116**:264-9.

496 6. **Mooi FR,Avest A, and van der Heide HG**. 1990. Structure of the *Bordetella pertussis*
497 gene coding for the serotype 3 fimbrial subunit. FEMS Microbiol Lett. **54:**327-31.

498 7. **Packard ER, Parton R, Coote JG, Fry NK.** 2004. Sequence variation and conservation in
499 virulence-related genes of *Bordetella pertussis* isolates from the UK. J. Med. Microbiol.
500 **53:**355-65.

501 8. **Tsang RS, Lau AK, Sill ML, Halperin SA, Van Caeseele P, Jamieson F, Martin IE.** 2004.
502 Polymorphisms of the fimbria fim3 gene of *Bordetella pertussis* strains isolated in
503 Canada. J. Clin. Microbiol. **42**:5364-7.

9. **Preston NW**. Essential immunogens in human pertussis: the role of fimbriae. 1985. Dev Biol Stand. **61**:137-41.
10. **Robinson A, Ashworth LA, Irons LI**. 1989. Serotyping *Bordetella pertussis* strains. Vaccine. **7**:491-4.
11. **WHO**, WHO Expert Committee on Biological Standardization, 30th Report. 1979. Technical Report Series. **638**:61-65.
12. **Chen, Q, Decker KB, Boucher PE, Hinton D, Stibitz S.** 2010. Novel architectural features of *Bordetella pertussis* fimbrial subunit promoters and their activation by the global virulence regulator BvgA. Mol Microbiol. **77**:1326-40.
13. **Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J**. 1996. A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N Engl J Med. **334**:349-55.
14. **Olin P, Rasmussen F, Gustafsson L, Hallander HO, Heijbel H.** 1997. Randomised controlled trial of two-component, three-component, and five-component acellular pertussis vaccines compared with whole-cell pertussis vaccine. Ad Hoc Group for the Study of Pertussis Vaccines. Lancet **350**:1569-77.
15. **Nilsson L and Rydevik G.** 2010. Twelve year report. Pertussis surveillance in Sweden. [cited 2010; Available from: http://www.smittskyddsinstitutet.se/upload/Publikationer/smirapport-01-2010.pdf.
16. **Hallander, HO, Advani A, Donnelly D, Gustafsson L, Carlsson RM**. 2005. Shifts of *Bordetella pertussis* variants in Sweden from 1970 to 2003, during three periods marked by different vaccination programs. J Clin Microbiol. **43**:2856-65.

17. **Medical Research Council**. 1956. Vaccination against whooping cough. Relation between protection in children and results of laboratory tests. British Med. J. **2**: 454-462.
18. **Cherry, JD, Gornbein J,Heininger U, Stehr K.** 1998. A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. Vaccine **16:**1901-6.
19. **Meade BD,Deforest A, Edwards KM, Romani TA, Lynn F, O'Brien CH, Swartz CB, Reed GF, Deloria MA**. 1995. Description and evaluation of serologic assays used in a multicenter trial of acellular pertussis vaccines. Pediatrics. **96**:570-5.
20. **Storsaeter J, Hallander HO, Gustafsson L, Olin P**. 1998. Levels of anti-pertussis antibodies related to protection after household exposure to Bordetella pertussis. Vaccine. **16**:1907-16.
21. **Alexander F, Matheson M, Fry NK, Labram B, Gorringe AR.** Antibody responses to individual *Bordetella pertussis* fimbrial antigen Fim2 or Fim3 following immunization with the five-component acellular pertussis vaccine or to pertussis disease. Clin Vaccine Immunol. **19**:1776-83
22. **Hallander HO, Ljungman M, Jahnmatz M, Storsaeter J, Nilsson L, Gustafsson L**. 2009. Should fimbriae be included in pertussis vaccines? Studies on ELISA IgG anti-Fim2/3 antibodies after vaccination and infection. APMIS. **117**:660-71.
23. **Hallander HO**. Microbiological and serological diagnosis of pertussis. 1999. Clin Infect Dis. **28** Suppl 2:S99-106.
24. **Reizenstein E, Hallander HO, Blackwelder WC, Kühn I, Ljungman M, Möllby R**. 1995. Comparison of five calculation modes for antibody ELISA procedures using pertussis serology as a model. J Immunol Methods **183**:279-90.

548 25. **Reed GF, Meade BD, Steinhoff MC.** 1995. The reverse cumulative distribution plot: a
549 graphic method for exploratory analysis of antibody data. Pediatrics **96**:600-3.
550 26. **Edwards KM, Meade BD, Decker MD, Reed GF, Rennels MB, Steinhoff MC, Anderson**
551 **EL, Englund JA, Pichichero ME, Deloria MA.** 1995. Comparison of 13 acellular pertussis
552 vaccines: overview and serologic response. Pediatrics **96**:548-57.
553 27. **Kallonen, T, and He Q**. 2009. *Bordetella pertussis* strain variation and evolution
554 postvaccination. Expert Rev. Vaccines. **8**:863-75.
555 28. **Litt DJ, Neal SE, and Fry NK**. 2009. Changes in genetic diversity of the *Bordetella*
556 *pertussis* population in the United Kingdom between 1920 and 2006 reflect vaccination
557 coverage and emergence of a single dominant clonal type. J. Clin. Microbiol. **47**:680-8.
558 29. **Poolman JT, and Hallander HO.** 2007. Acellular pertussis vaccines and the role of
559 pertactin and fimbriae. Expert Rev. Vaccines. **6**:47-56.
560 30. **Heikkinen, E, Xing DK, Olander RM, Hytönen J, Viljanen MK, Mertsola J, He Q**. 2008.
561 *Bordetella pertussis* isolates in Finland: serotype and fimbrial expression. BMC Microbiol.
562 **8**:162.
563 31. **Cherry, JD, Heininger U, Richards DM, Storsaeter J, Gustafsson L, Ljungman M,**
564 **Hallander HO**. 2010. Antibody response patterns to *Bordetella pertussis* antigens in
565 vaccinated (primed) and unvaccinated (unprimed) young children with pertussis. Clin.
566 Vaccine Immunol. **17**:741-7.

567
568
569

Figure legends

Figure 1. Reverse cumulative distribution curves of anti-Fim2 and anti-Fim3 IgG concentrations one month following the third dose of A; DTaP5 or B; DTwP vaccine.

Figure 2. Reverse cumulative distribution curves of post disease onset anti-Fim2 (A) and Fim3 (B) ELISA IgG concentrations in sera from Sweden trial I from children previously vaccinated with three doses of either DTaP5, DTaP2, DTwP or DT vaccines.

Figure 3. Children in fimbriae 2+3-primed, DTaP5 and DTwP, arms. Twenty-nine children with 105 samples, with positive anti-PT serology in Trial I. Upper line shows median values of anti-Fim2 IgG concentration for samples in intervals before, during and after the start of pertussis episode (black dots). Lower line shows median values of anti-Fim3 IgG concentrations (red dots) in the same intervals. X-axis shows duration in months between sample and start of cough in positive episode. Day 0 is day of onset of pertussis cough episode. Markers on the line indicate the median level and duration since onset of cough for samples in the interval.

Figure 4. Reverse cumulative distribution curves of anti-Fim2 and anti-Fim3 ELISA IgG concentrations in sera from seroprevalence studies collected in Sweden in 1997 or 2007. This analysis was performed with available serum samples with measurable anti-Fim2+3 IgG. In 1997 35% of sera had measurable anti-Fim2+3 IgG and 26% in 2007.

593 **Table 1**. Anti-Fim2 and anti-Fim3 IgG in samples from Sweden trial I and anti-Fim2/anti-Fim3
594 ratio one month after dose 3 in a 2, 4 and 6 months vaccination schedule. Values below MLD
595 were set to 1 for anti-Fim2 and to 0.5 for anti-Fim3.
596

Vaccine group	Number of Children	Geometric mean EU/ml	95% confidence interval of GM
DTaP5 anti-Fim2	182	95.8	80.6, 113.8
DTaP5 anti-Fim3	182	14.6	12.4, 17.2
DTaP5 anti-Fim2/anti-Fim3 ratio	182	6.6	5.8, 7.4
DTwP anti-Fim2	146	28.0	23.2, 33.9
DTwP anti-Fim3	146	17.7	15.4, 20.3
DTwP anti-Fim2/anti-Fim3 ratio	146	1.6	1.4, 1.8

597

598 **Table 2**. Anti-Fim2 and anti-Fim3 IgG in pre-disease serum samples from Sweden trial I and anti-

599 Fim2/anti-Fim3 ratio 6 months after dose 3 in a 2, 4 and 6 months vaccination schedule. Values

600 below MLD were set to 1 for anti-Fim2 and to 0.5 for anti-Fim3.

601

Vaccine group	Number of Children	Geometric mean EU/ml	95% confidence interval of GM
DTaP5 anti-Fim2	13	14.0	7.1, 27.4
DTaP5 anti-Fim3	13	3.8	1.8, 8.1
DTaP5 anti-Fim2/anti-Fim3 ratio	13	3.7	2.0, 6.7
DTwP anti-Fim2	12	12.7	7.1, 22.7
DTwP anti-Fim3	12	5.9	3.6, 9.8
DTwP anti-Fim2/anti-Fim3 ratio	12	2.1	1.3, 3.5
DTaP2 anti-Fim2	42	1.1	1.0, 1.3
DTaP2 anti-Fim3	42	1.0	0.8, 1.2
DT anti-Fim2	12	1.1	0.9, 1.3
DT anti-Fim 3	12	0.7	0.5, 1.0

602
603

604 **Table 3** Peak anti-Fim2 and anti-Fim3 IgG concentrations post onset of pertussis in children who
605 had previously received 3 doses of vaccine. The subjects in table 3 represent the 79 subjects in
606 table 2 plus 12 others for whom the pre-exposure samples were no longer available.

607

608
609

Vaccine group	Number of children	Geometric mean EU/ml	95% confidence interval of GM
DTaP5 anti-Fim2	13	189.1	111.8, 319.7
DTaP5 anti-Fim3	13	17.2	7.0, 42.0
DTaP5 anti-Fim2/anti-Fim3 ratio	13	11.0	4.3, 28.2
DTwP anti-Fim2	16	118.9	59.8, 236.8
DTwP anti-Fim3	16	49.4	28.1, 86.7
DTwP anti-Fim2/anti-Fim3 ratio	16	2.4	1.4, 4.3
DTaP2 anti-Fim2	46	4.9	3.8, 6.2
DTaP2 anti-Fim3	46	7.1	5.7, 8.8
DTaP2 anti-Fim2/anti-Fim3 ratio	46	0.7	0.6, 0.8
DT anti-Fim2	16	6.9	3.6, 13.0
DT anti-Fim3	16	13.5	6.5, 28.1
DT anti-Fim2/anti-Fim3 ratio	16	0.5	0.4, 0.7

610

611

612

Table 4. Number of individuals with anti-Fim2 and anti-Fim3 IgG stratified by concentration in seroprevalence sera obtained in 1997 and 2007. The lower bound value is included in each stratification. This analysis was performed with available serum samples with measurable anti-Fim2+3 IgG. In 1997 35% of sera had measurable anti-Fim2+3 IgG and 26% in 2007. Simultaneous confidence intervals were constructed on the proportions of the samples falling into the categories chosen in Table 4, as if they arose from a multinomial distribution (rather than the underlying distribution shown in Figure 4). This showed significant (P<0.01) differences in the proportions arising from anti-Fim2 and anti-Fim3 IgG concentrations in 1997 complementing the Mann-Witney test result.

	1997		2007	
	Anti-Fim2	**Anti-Fim3**	**Anti-Fim2**	**Anti-Fim3**
Total observations	184	184	95	95
Observations with values 4 - <14 EU/ml	62 (34%)	99 (54%)	38 (40%)	39 (41%)
95% confidence interval of the %	27-41%	46-61%	30-51%	31-52%
Observations with values 14 -<100 EU/ml	106 (58%)	73 (40%)	44 (46%)	45 (47%)
95% confidence interval of the %	50-65%	33-47%	36-57%	37-58%
Observations with values >100 EU/ml	10 (5%)	7 (4%)	8 (8%)	8 (8%)
95% confidence interval of the %	3-10%	2-8%	4-16%	4-16%