

## The quantity and quality of anti-PRP induced by the new Indonesian DTwP-HB-Hib vaccine compared to the Hib vaccine given with the DTwP-HB vaccine

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### Abstract

**Background** A phase II study of DTwP-HB-Hib vaccine compared to Hib (monovalent) vaccine given simultaneously with DTwP-HB vaccine has been done following the success of phase I study in infants, where the new DTwP-HB-Hib has excellent safety profiles and antibody responses in infants.

**Objective** To evaluate the titer (quantity), avidity, and bactericidal capacity (quality of anti-polyribosylribitol phosphate/anti-PRP), of a new combined Bio Farma DTwP-HB-Hib (pentavalent) vaccine, compared to the Hib monovalent vaccine given simultaneously with the DTwP-HB vaccine (DTwP-HB+Hib).

**Methods** The study was a prospective, randomized, open label, phase II trial. Subjects aged 6-11 weeks were allocated according to the randomization list. The pentavalent group received the DTwP-HB-Hib vaccine, while the monovalent group received the Hib monovalent and DTwP-HB vaccines separately. Immunizations were given in three doses with 28-day intervals. Blood specimens were taken before the first dose and 28 days after the last dose. We evaluated anti-PRP titers quantity (geometric mean antibody concentration/GMC) and seroprotection, followed by avidity and bactericidal (quality) testing. Titer and avidity of anti-PRP were tested using a modified version of the improved Phipps ELISA. Bactericidal capacity was evaluated using a Hib killing assay. Immune responses against other antigens in the vaccine were reported separately.

**Results** One hundred five subjects in the pentavalent group and 106 subjects in the Hib monovalent group were tested for anti-PRP titers. Only 102 specimens for each group were available for bactericidal testing, due to insufficient volume for testing. Both vaccines induced similar anti-PRP titers, for GMC and seroprotection. Avidity increases were 82.9% and 76.4% in the pentavalent and Hib monovalent groups, respectively. Bactericidal activities were 94.1% and 89.2%, respectively. Both avidity and bactericidal activity were not significantly different between groups.

**Conclusion** DTwP-HB-Hib vaccine induced anti-PRP quantity

and quality comparable to those of the Hib monovalent vaccine given simultaneously with the DTwP-HB vaccine. [Paediatr Indones. 2017;57:262-8; doi: <http://dx.doi.org/10.14238/pi57.5.2017.262-8>].

**Keywords:** avidity; anti-PRP; bactericidal; DTwP-HB-Hib; immunization; titer

**H**aemophilus influenzae type b (Hib) causes infection with predominant manifestations of pneumonia, meningitis, and other invasive diseases. These infections occur primarily in children under 2 years of age, particularly in infants before Hib vaccinations became available.<sup>1,2</sup> In Asia, 23% of pneumonia cases are caused by Hib, while other etiologies include pneumococcus, staphylococcus, streptococcus, and viruses.<sup>3</sup> In Indonesia, pneumonia and meningitis cause an

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estimated 15.5% and 8.8%, respectively, of all deaths recorded in children under five years of age.<sup>4,5</sup>

The World Health Organization (WHO) has recommended worldwide incorporation of the Hib vaccination into all routine infant immunization programs, given after 6 weeks of age, preferably as a diphtheria-tetanus-pertussis (DTP)-based combination to allow for rapid integration into existing DTP vaccination schedules.<sup>2</sup> A phase I study comparing the Hib monovalent and the new DTwP-HB-Hib vaccines showed that both were immunogenic and well tolerated when administered as a single injection (Hib) in adults or as a primary dose (DTwP-HB-Hib) in infants, with 28-day intervals between doses.<sup>6,7</sup>

We aimed to evaluate the quantity of anti-PRP titer (GMC & seroprotection), avidity, and bactericidal capacity (quality of anti-PRP), of the new combined *Bio Farma* DTwP-HB-Hib (pentavalent) vaccine, compared to the currently approved Hib monovalent vaccine given simultaneously with the DTwP-HB vaccine (DTwP-HB+Hib).

## Methods

The subjects were recruited for a prospective, randomized, open label, phase II study on the combined DTwP-HB-Hib vaccine. The study was conducted at 3 primary health centers in Bandung from July 2011 to January 2012. Subjects' parents provided written informed consent before enrollment. The study was conducted in accordance with the Helsinki Declaration and Good Clinical Practice Guidelines, with approval of the Ethics Review Committee of Universitas Padjadjaran Medical School.

The study population was comprised of healthy infants aged 6-11 weeks at the time of enrollment, of gestational age 37-42 weeks at delivery, with a birth weight of 2,500 – 4,000 g, and had received a single dose of monovalent hepatitis B vaccine at 0-7 days after birth, as proven by written vaccination documentation. At the time of enrollment, subjects were assigned to one of two vaccine groups by permuted-block randomization.

We administered the primary vaccinations to Indonesian infants according to the *Expanded Program on Immunization* (EPI) schedule, at 6, 10, and 14

weeks of age, after a birth dose of hepatitis B vaccine, as recommended by the WHO. Anti-PRP titers and avidity will be tested using ELISA, bactericidal activities will be tested using Hib bactericidal assay. The safety and immune response against other antigens in the vaccine were published separately.

The new liquid DTwP-HB-Hib (pentavalent) vaccine was produced by *Bio Farma*. This vaccine has 5 antigens, with each 0.5 mL dose containing > 30 IU purified diphtheria toxoid, > 60 IU purified tetanus toxoid, > 4 IU inactivated *Bordetella pertussis*, 10 µg recombinant HBsAg, and 10 µg Hib/PRP conjugated to tetanus toxoid. As a control, the DTwP-HB vaccine, also manufactured by *Bio Farma*, contains 4 antigens, with similar amounts, except for hepatitis B which had only 5 µg HBsAg per dose (0.5 mL). The Hib monovalent vaccine was imported (registered in Indonesia) and also contained 10 µg Hib/PRP conjugated to tetanus toxoid per dose. Vaccines were administered at 6, 10, and 14 weeks of age, with 28-day intervals between doses. One group received the new DTwP-HB-Hib combination vaccine, while the other group received the DTwP-HB and Hib vaccines simultaneously. Vaccines were given intramuscularly in the external anterolateral region of the thigh.

Four-mL blood specimens were collected before the first vaccine dose and 28 days after the third dose to evaluate antibody responses. All assays were performed at the *Bio Farma Clinical Trial Laboratory*. The titer of antibodies to PRP were measured by the *Improved Phipps* enzyme-linked immunosorbent assay (ELISA), a competitive ELISA for measuring serum antibody levels to *Haemophilus influenzae type b*.<sup>8,10</sup>

Tests were done in duplicate, and a control serum was added to monitor variations between test plates. Anti-PRP antibody reference lot 1983 from the US *Federal Drug Association* (FDA) was used in all plates. Anti-PRP antibody concentration of >0.15 µg/mL was considered to be the minimum protection threshold titer and a concentration of >1.0 µg/mL was regarded as the long-term protection threshold titer.

The percentage of protection (seroprotection) was calculated and differences between groups were evaluated using Chi-square or Fisher's tests. The GMCs with 95%CI were calculated by taking the log-transformation of individual concentrations and calculating the anti-log of the mean of these transformed values. Exploratory analyses were

performed to compare GMCs between the vaccine groups using Mann-Whitney test.

Avidity was tested using a modified ELISA, which included the use of isothiocyanate, a chaotropic agent.<sup>8</sup> After coating the plate with PRP antigen and incubating overnight, serum specimens were prediluted to a concentration of 0.5µg/mL anti-PRP antibodies. Ammonium thiocyanate at concentrations of 0.8, 0.4, 0.2, 0.1, and 0.05M were added to some wells of the plate, but not all, in order to later obtain an avidity index. The reactions were stopped when the well without ammonium thiocyanate reached an optical density of 1.0. Antibody avidity was expressed as the avidity index corresponding to the molar concentration of ammonium thiocyanate required to produce a 50% reduction in absorbance.<sup>9</sup> Differences in percentages of subjects with increased avidity between groups were evaluated using Chi-square or Fisher's tests.

The bactericidal assay was used to evaluate the capacity of anti-PRP antibodies present in the serum to bind and activate complement, leading to the killing of the bacteria. The Hib strain Hib-CB33 was cultivated, harvested, and diluted to a concentration of around 104 CFU/mL. Eleven, serial, two-fold dilutions of serum to be tested (starting at 1:4) were mixed with 3-4 week rabbit complement and 25 µg of bacteria. After 45 minutes of incubation, the number of surviving bacteria was determined by plating 5µg onto chocolate agar and counting the colonies after plate incubation for 18 hours. The serum bactericidal titer was defined as the inverse of the highest dilution that led to > 50% bacterial killing, and was compared to the negative control serum. The cut-off value was 4 BT (bactericidal titer).<sup>9</sup> The percentage of subjects with > 4 BT was calculated, and the difference between groups was evaluated using Chi-square test. Serum bactericidal antibody (SBA) geometric mean titers (GMT) were also calculated, and the differences between groups were evaluated using Mann-Whitney test.

## Results

Of the 220 subjects recruited and randomly allocated to one of two vaccine regimens, only 211 subjects were available for immunogenicity analysis in phase I.<sup>10</sup>

Likewise, in this study not all subjects were available for all assays. Anti-PRP titer was measured in 2012; avidity was evaluated in 2013; and the bactericidal assay was done in 2015, due to reagent availability. The number of subject samples available for each assay is shown in **Table 1**. Fewer specimens were available for the bactericidal assay due to lack of volume. The minimum required sample size was 100 per group, hence, the actual number of subjects' specimens fulfilled the minimum requirement.

Anti-PRP antibody measurements were based

**Table 1.** Number of subjects' specimens available for each assay

Assays	DTwP-HB-Hib (n)	DTwP-HB+Hib (n)	Total (N)
Anti-PRP ELISA	105	106	211
Avidity ELISA	105	106	211
Bactericidal assay	102	102	204

on GMC and percentage of infants with titers of >0.15 µg/mL and >1.0 µg/mL, are presented in **Table 2**. Before and after vaccination, there were no significant differences between the DTwP-HB-Hib and DTwP-HB+Hib groups, with regards to mean GMC, and percentage of subjects with anti-Haemophilus B conjugate (PRP-T) >0.15 µg/mL, and anti-PRP-T >1.0 µg/mL.

The number and percentage of subjects with increased avidity is shown in **Table 3**. Both groups showed similar results, in terms of anti-PRP avidity.

As shown in **Table 4**, the DTwP-HB-Hib group had more subjects with bactericidal activity (titer > 4) than the DTwP-HB+Hib group, but the difference was not significant [94.1% vs. 89.2%, respectively; (P=0.205)]. The SBA GMTs were also not significantly different between groups, both before and after immunization.

## Discussion

**Table 2.** Anti-PRP antibody concentrations by group, pre- and post-vaccination

Description	Pre-vaccination		P value	Post-vaccination		P-value
	DTwP-HB-Hib (n=105)	DTwP-HB +Hib (n=106)		DTwP-HB-Hib (n=105)	DTwP-HB+Hib (n=106)	
GMC, IU/mL (95%CI)	0.988 (0.925 to 1.055)	1.075 (0.9990 to 1.167)	0.989	12.612 (9.689 to 16.421)	11.663 (8.962 to 15.81)	0.876
Anti-PRP-T ≥ 0.15 µg/mL						
n	30	28		103	105	
%SP (95%CI)	28.6 (20.8 to 37.8)	26.4 (19.0 to 35.5)	0.726	98.1 (93.3 to 99.5)	99.1 (94.8 to 99.8)	0.621
Anti-PRP-T ≥ 1.0 µg/mL						
n	14	17		101	101	
%SP (95%CI)	13.3 (8.1 to 21.1)	16.0 (10.3 to 24.2)	0.579	96.2 (90.6 to 98.5)	95.3 (89.4 to 98.0)	1.00

DTwP-HB-Hib: diphtheria-tetanus-whole cell pertussis-hepatitis B-Haemophilus influenza type b vaccine; DTwP-HB+Hib: diphtheria-tetanus-whole cell pertussis-hepatitis B vaccine given simultaneously with Hib vaccine; n = number of available observations; % SP = seroprotection rate

**Table 3.** Anti-PRP antibody avidity by group

Avidity description	DTwP-HB-Hib (n=105)	DTwP-HB+Hib (n=106)	P value
Avidity increased, n(%)	87 (82.9)	81 (76.4)	0.245*
No avidity, n(%)	2 (1.9)	2 (1.9)	1.0**
Avidity constant, n(%)	9 (8.6)	14 (13.21)	0.325*
Avidity decreased, n(%)	7 (6.67)	9 (8.49)	0.617*

\* Chi-square test; \*\* Fisher's exact test.

**Table 4.** Bacterisidal activity by group

Description	DTwP-HB-Hib (n=102)	DTwP-HB+Hib (n=102)	P value
Titer ≥ 4, n(%)	96 (94.1)	91 (89.2)	0.205*
GMT before immunization, BT (95%CI)	0	1.013 (0.987 to 1.039)	0.320**
GMT after immunization, BT (95%CI)	36.041 (27.321 to 47.534)	25.456 (18.471 to 5.075)	0.160**

\* Chi-square test; \*\* Fisher's exact test.

The main objective of this study was to compare the quantity and quality of anti-PRP antibodies induced by the DTwP-HB-Hib pentavalent combination vaccine to the Hib monovalent vaccine given simultaneously with the DTwP-HB vaccine (DTwP-HB+Hib), as the primary vaccination in infants who had received a dose of hepatitis B vaccine at birth. After the primary series of vaccines was given, 98.1% of the DTwP-HB-Hib group and 99.1% of the DTwP-HB+Hib group had anti-PRP antibody titers above the conservative threshold of protection of 0.15 µg/mL. In addition, 96.2% of the DTwP-HB-Hib group and 95.3% of the

DTwP-HB+Hib group had titers above 1.0 µg/mL. Our previous study showed that the immune response to Hib in the DTwP-HB-Hib pentavalent combination was not significantly different from the group that received separate administration of the monovalent Hib vaccine.<sup>10</sup>

Anti-PRP antibody avidities were similar in the pentavalent and Hib monovalent groups (P=0.245 to 1.0). Also, while the pentavalent group had a higher percentage of subjects (94.1%) with titer>4 (bactericidal activity), it was not significantly different from the monovalent Hib group (89.2%) (P=0.205). Nor were GMTs significantly different between groups,

both before and after immunization ( $P=0.320$  and  $0.160$ ).

A 2009-2010 study in India in 661 infants aged 6 to 8 weeks using pentavalent combination vaccines with a 1-month interval between doses, reported anti-PRP titers of 100% seroprotection.<sup>11</sup> In addition, Hla *et al.* used a pentavalent vaccine given in 1-month intervals to 608 infants aged 6 weeks and showed similar results to ours: 100% short-term protection (anti-PRP  $> 0.15 \mu\text{g/mL}$ ) and 95% long-term protection (anti-PRP  $> 1 \mu\text{g/mL}$ ).<sup>12</sup> Another Indian study in 165 infants at 6, 10, and 14 weeks of age also found similar results to our study: at one month after the third vaccination, the percentages of infants who achieved the predefined protective antibody levels were 100% Hib short-term ( $\geq 0.15 \mu\text{g/mL}$ ) and 95% Hib long-term ( $\geq 1.0 \mu\text{g/mL}$ ) protection.<sup>13</sup> These three studies were conducted without control groups.

A Latin American study used pentavalent vaccines in 1,000 infants. Statistical comparisons following primary vaccination showed that, in terms of antibody response to the PRP antigen, the combined DTwP-HB/Hib vaccine was clinically non-inferior to the licensed DTwP-HB and Hib vaccines.<sup>14</sup> Furthermore, Rao *et al.* compared the new pentavalent vaccine to two groups: those who received the DTwP-HB+Hib vaccine (separate injections) and another registered pentavalent vaccine. They found that 98.32% of subjects in the new pentavalent vaccine trial group had seroprotective anti-PRP-T IgG antibody concentrations of  $\geq 0.15 \mu\text{g/mL}$ , as compared to 100% and 98.94% of subjects in DTwP-HB+Hib and the other registered pentavalent vaccine groups, respectively.<sup>15</sup>

We found that the avidity increases were not significantly different between the pentavalent and Hib monovalent groups [82.86% vs. 77.14%, respectively ( $P=0.245$ )]. In contrast, a German study conducted in 90 infants with an immunization schedule at 3, 4, and 5 months using the DTaP-HB-Hib vaccine compared to separate injections given simultaneously showed significantly lower avidity in the combination vaccine group ( $P<0.0001$ ).<sup>9</sup> A Myanmar study in 50 infants immunized at 6, 10, and 14 weeks of age compared several concentrations of Hib vaccines ( $10 \mu\text{g/mL}$  and  $2.5 \mu\text{g/mL}$  of a whole cell-based vaccine) showed avidity that was not significantly

different ( $P=0.239$ ).<sup>8</sup> In these two previous studies, anti-PRP avidity was not influenced by the whole cell-based pertussis vaccine, but, decreased by acellular pertussis base vaccine.<sup>9</sup> In our study both the pentavalent and Hib monovalent groups had subjects with avidity decreases of 6.67% and 8.49%, respectively ( $P=0.617$ ). Some subjects also showed constant avidity before and after immunization, in both groups. Most subjects with non-increased avidity showed high titer concentrations of anti-PRP before immunization. A possible explanation for this phenomenon is the presence of maternal antibodies, which may prevent an immune response after immunization. The presence of maternal antibodies in the subjects before immunization may have also decreased the avidity after immunization, because this maternal antibody would eventually reduce significantly in time, especially after immunization at age 14 weeks or above.<sup>16,17,18</sup>

In our study, both groups also showed similar bactericidal activity ( $P=0.205$ ). A study in Germany was conducted in 90 infants, with an immunization schedule of 3, 4, and 5 months using the DTaP-HB-Hib vaccine compared to separate injections given simultaneously. They also had not significantly different bactericidal activities.<sup>9</sup> The Myanmar study in 50 infants immunized at 6, 10, and 14 weeks of age comparing several concentrations of Hib vaccines ( $10 \mu\text{g/mL}$  and  $2.5 \mu\text{g/mL}$  in a whole cell-based vaccine) also showed bactericidal activities which were not significantly different between groups.<sup>9</sup>

Some of our subjects had high anti-PRP and low SBA titers after immunization. Discrepancies can be expected because the SBA assay measures functional antibodies, regardless of isotype, to the whole organism, whereas anti-PRP IgG simply measures total IgG against capsular polysaccharide, regardless of antibody avidity. At the same time, infants with low anti-PRP IgG and high SBA titers were identified. There are several possible explanations for this observation. SBA titer may be the result of IgM and IgG, while we did not measure serum IgM alone. Another explanation for the discrepancy could be that the SBA assay measured functional activity against whole bacteria, and some infants may have naturally acquired functional antibodies against Hib subcapsular antigens. However, the ELISA measured only the concentration of anti-PRP (Hib polysaccharide capsule).<sup>19</sup>

Regarding the serum bactericidal antibody GMT, the pentavalent combination vaccine group reached 36.041 BT, while the separate injection group reached 25.456 BT (not significantly different,  $P=0.160$ ). Townsend et al. found that SBA GMT in infants also reached 31 BT, similar to the SBA GMT of the trial vaccine.<sup>19</sup>

In conclusion, this study demonstrates that the new combined pentavalent DTP-HB-Hib vaccine induces comparable anti-PRP antibody titers, avidity, and bactericidal activity (anti-PRP quality) to the Hib monovalent vaccine given simultaneously with the DTwP-HB vaccine.

### Funding

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### Conflict of Interest

None declared.

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