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Immunogenicity and reactogenicity of a combined fully liquid DTPw–HepB–Hib pentavalent vaccine in healthy infants: no clinically relevant impact of a birth dose of hepatitis B vaccine

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SUMMARY

Objectives: In this open-label, non-randomized phase II study, the safety and immunogenicity of a fully liquid diphtheria–tetanus–whole cell pertussis–hepatitis B–*Haemophilus influenzae* type b (DTPw–HepB–Hib) combination vaccine (Quinvaxem®) were assessed in infants who had or had not received a birth dose of hepatitis B (HepB) vaccine.
Study design: Two groups of infants, 'HepB at birth' ($n = 110$) and 'no HepB at birth' ($n = 108$), were enrolled and received a primary vaccination course using a 2–4–6 months schedule.
Results: Seroprotection/seroconversion rates of >95% were achieved against all antigens included in the combination vaccine for both study groups. Although significantly higher anti-hepatitis B virus ($p < 0.001$) and anti-tetanus ($p = 0.031$) antibody titers were achieved in group 'HepB at birth' when compared with group 'no HepB at birth', the proportion of 'no HepB at birth' subjects achieving protective titers was non-inferior to the proportion of subjects in group 'HepB at birth'. The birth dose of HepB vaccine did not seem to influence the safety pattern of the DTPw–HepB–Hib combination vaccine.
Conclusions: The present study demonstrated that the fully liquid DTPw–HepB–Hib vaccine was safe and immunogenic when administered using a 2–4–6 months immunization schedule, regardless of whether or not infants had received a dose of HepB vaccine at birth.

1. Introduction

The combined vaccine against diphtheria, tetanus and pertussis (DTP) is the core vaccine in childhood immunization programs and is included in the Expanded Programme on Immunization (EPI).[1–3] In addition, the World Health Organization (WHO) has recommended the inclusion of vaccination against hepatitis B virus (HBV) since 1992 and *Haemophilus influenzae* type b (Hib) since 1997 in the EPI.[4–6] In regions where HBV infection is endemic, hepatitis B vaccination (HepB vaccination) in infancy has been shown to be highly effective in preventing HBV infection, chronic carriage, and primary liver cancer.[7,8]

Four licensed pentavalent diphtheria–tetanus–whole cell pertussis–hepatitis B–*H. influenzae* type b (DTPw–HepB–Hib) combination vaccines are currently prequalified by the WHO.[9–15] The earlier generations of these vaccines consist of a DTPw–HepB component and a separate lyophilized Hib component that has to be reconstituted before use, while newer ones are available as fully liquid suspensions. Quinvaxem® is a fully liquid DTPw–HepB–Hib combination vaccine; it is ready to use and therefore eliminates additional on-site handling procedures. Quinvaxem has been shown to be safe and immunogenic when administered in a 2–3–4 months primary vaccination schedule,[16] and was licensed and prequalified by the WHO in 2006.

Since the highest risk of HBV transmission is from a hepatitis B surface (HBs) and/or hepatitis B envelope (HBe) antigen-positive mother to her newborn child, it is recommended that infants in highly endemic areas receive a HepB vaccine dose within 12 h of birth, followed by two additional doses.[17] Inclusion of HepB vaccine in combination childhood vaccines will help to increase the coverage of HepB immunization and minimize the number of doctor visits and injections received by the infant. However, the use of HepB-containing combination vaccines results in infants receiving an additional, fourth dose of HepB vaccine. The safety and

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efficacy of a four-dose schedule with monovalent HepB vaccine has previously been demonstrated.[18–21] It has also been shown that a single dose of HepB vaccine at birth has no adverse impact on immunogenicity and safety of DTP–HepB-containing vaccines administered according to a variety of schedules.[22–25]

The objective of the current study was to assess the immunogenicity, tolerability, and safety of a primary vaccination course with the DTPw–HepB–Hib vaccine, when administered in a 2–4–6 months schedule to infants who had, or had not, received a birth dose of a monovalent HepB vaccine. According to the national EPI schedule in Argentina, the primary immunization course, as applied in this study, was to be followed by a DTPw–Hib vaccination at 18 months of age. Although it was not part of the study procedure, all children were followed-up to ensure they received this fourth dose.

2. Methods

2.1. Study design

The current study was a non-randomized, open-label, single-centre study conducted between April 2005 and May 2006 at the Hospital de Niños Ricardo Gutierrez in Buenos Aires, Argentina. Infants between 6 and 12 weeks of age, and free of obvious health problems as assessed by the investigator, participated in the study. Infants who were born before 37 weeks of gestation, with a birth weight below 2500 g, or who had previously received any dose of Hib and/or DTP vaccines were excluded from the study.

A single dose of HepB vaccine given as soon as possible after birth, regardless of the infection status of the mother, is part of the national EPI in Argentina. Because the assignment to treatment groups was dependent on the HepB vaccination status of subjects before study enrolment, the allocation was not randomized. In order to ensure that enough infants were eligible for the group 'no HepB at birth', the following procedure was used: pregnant women were screened for HBV infection, and those testing negative were invited to have their child participate in group 'no HepB at birth' of the study. In order for infants to be included in this group, the mothers had to agree not to have their child vaccinated with HepB vaccine at birth. Children brought to the study centre for the usual EPI vaccination who had received one dose of HepB vaccine at birth were eligible for enrolment into group 'HepB at birth' of the study. It was planned to have 220 subjects enrolled into the study, in a 1:1 ratio in the two groups according to their HepB vaccination status.

Three doses of the DTPw–HepB–Hib vaccine were administered at 2, 4, and 6 months of age according to the schedule adopted by Argentina. The vaccine was administered intramuscularly into the right thigh. Blood samples for evaluation of the primary objective of this study were obtained prior to the first vaccination (baseline), and 1 month after the third vaccination. An additional sample was taken just prior to the third vaccination (data not shown). Oral polio vaccine was concomitantly administered to all infants.

The study protocol with all amendments and informed consent forms were approved by the appropriate independent ethics committee, and the study was performed in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines (GCP) and local laws. Written informed consent to participate in the study was obtained from the parent(s)/legal guardian(s) of all subjects before enrolment into the study.

2.2. Vaccines

Each DTPw–HepB–Hib fully liquid vaccine dose contained 0.5 ml: ≥30 IU diphtheria toxoid (D), ≥60 IU tetanus toxoid (T), ≥4 IU inactivated whole cell *Bordetella pertussis* (Pw), 10 μg *H. influenzae* type B (Hib) polyribosyl ribitol phosphate (PRP) oligosaccharide conjugated to approximately 25 μg of CRM_{197} protein, and 10 μg purified HBV surface antigen (HBsAg). The D, T, Pw, and Hib antigens were identical to the antigens used in Quattvaxem™ (Novartis Vaccines and Diagnostics, formerly Chiron Vaccines, Siena, Italy) and the HBsAg was identical to the antigen used in Hepavax-Gene® (Crucell Berna Biotech Korea Corp., formerly Green Cross Vaccine Corporation). The DTPw–HepB–Hib vaccine was developed and manufactured by Crucell Berna Biotech Korea Corp. Production was performed according to Good Manufacturing Practice (GMP) guidelines.

2.3. Immunogenicity assessment

Blood samples were analyzed by enzyme-linked immunosorbent assays (ELISAs) for the presence of antibodies. HBV seroprotection was defined as an anti-HBs antibody level of ≥10 IU/l.[26] Anti-diphtheria and anti-tetanus antibodies at a concentration of ≥0.1 IU/ml were considered protective.[27,28] Antibodies against *B. pertussis* were determined using a whole-cell ELISA.[29] Since a correlate for seroprotection against *B. pertussis* has not yet been established, seroconversion was defined as either titer levels ≥20 ELISA IU (EIU) or a 4-fold increase over baseline titers. Antibodies against Hib PRP were measured by ELISA,[30] and seroprotection rates were assessed according to the two commonly used cut-off levels: anti-PRP ≥0.15 μg/ml and ≥1.0 μg/ml.

Anti-diphtheria toxoid, anti-tetanus toxoid, anti-PRP and anti-HBs assays were performed at Novartis Vaccines and Diagnostics (formerly Chiron Vaccines), Clinical Serology, Marburg, Germany. The anti-*B. pertussis* antibody ELISA was performed at the University of Turku, Department of Medical Microbiology, Turku, Finland.

2.4. Safety and reactogenicity assessment

Solicited local adverse events (AEs; tenderness, erythema and induration) and systemic AEs (fever, rash, change in eating habits, sleepiness, unusual crying, persistent crying, irritability, vomiting and diarrhea) were recorded by the subject's parent(s) or legal guardian(s) on a diary card for a 7-day period after each vaccination. Fever was defined as an axillary temperature ≥38.0 °C[31] and persistent crying as unaltered crying >3 h.[32] Subjects were monitored by study personnel for 1 h after each vaccination, and the parent(s) or legal guardian(s) were further contacted by telephone at 2–3 days and again at 7–8 days after each vaccination to assess the daily axillary temperature, the occurrence of AEs, and the use of concomitant medication. Unsolicited AEs and serious adverse events (SAEs) were recorded throughout the study period.

2.5. Statistical analysis

The primary objective of this study was to assess non-inferiority of the anti-HBs seroprotection rate induced by the HBV component of DTPw–HepB–Hib in subjects who had received a HepB vaccination at birth ('HepB at birth') compared to subjects who had not received a HepB vaccination at birth ('no HepB at birth'). In addition, seroprotection rates for the other antigens included in the combination vaccine (seroconversion rates for *B. pertussis*) were also assessed in both study groups. Comparison between the two study groups was based on a non-inferiority limit of 10% (lower limit of the two-sided 90% confidence interval (CI) >−10%).

Calculations of geometric mean titers (GMTs) were performed by taking the anti-$\log_{10}$ of the mean of the $\log_{10}$ titer transformations. The GMTs were calculated together with the corresponding 95% CIs (normal approximation).

The sample size was calculated based on the primary objective assuming a reference seroprotection to HBs of 95% and a non-

Table 1
Study population and demographic characteristics

	HepB at birth		No HepB at birth	
	n	%	*n*	%
Number of subjects enrolled/vaccinated	110	100	108	100
Evaluated[a]				
Safety	107	97.3	103	95.4
ATP	103	93.6	100	92.6
Demographic characteristics (safety population)				
Male/female	61/46	57.0/43.0	53/50	51.5/48.5
Mean age, months (range)	2.1 (1.4–2.7)		2.0 (1.5–2.8)	
Mean weight, kg (range)	5.4 (3.7–7.6)		5.3 (3.9–6.9)	

HepB, hepatitis B vaccine; ATP, according-to-protocol.
[a] Percentages based on all vaccinated subjects.

inferiority limit of 10%. Thus, a sample size of 93 evaluable subjects per group was required to demonstrate, with a power of 90% and a one-sided significance level of 5%, the non-inferiority between subjects having received HepB vaccine at birth and subjects not having received HepB vaccine at birth. Considering possible dropouts, the sample size was increased by a suitable amount of 15% for a total of 220 eligible subjects to be allocated to the study groups according to HepB vaccination status. Sample size estimation was performed using the Anderson–Hauck correction for proportions. SAS 8.1 software (SAS Institute, Cary, NC, USA) was used for the statistical analysis.

3. Results

3.1. Subjects and demographics

Of 260 infants screened, 218 infants were enrolled in the study and received at least the first DTPw–HepB–Hib vaccination (Table 1). Parents declined participation for general reasons, because of blood extraction, travel or not having access to a phone. For eight of the infants enrolled, no follow-up safety data were provided. Thus, the safety population included 210 subjects. Fourteen vaccinated infants did not complete the study period. Among these, two subjects discontinued due to AEs (one subject experienced hypertonia and metabolic acidosis and the other subject died of sudden infant death syndrome), consent was withdrawn for three subjects, six subjects moved from the study area, and three were lost to follow-up. In addition, one subject was excluded from the immunogenicity analysis because the fourth study visit was outside the specified time window. Consequently, data from 203 infants (HepB at birth, $n = 103$; no HepB at birth, $n = 100$) included in the according-to-protocol (ATP) population were used for the primary immunogenicity analysis. The demographic characteristics of the two study groups (safety population) were similar (Table 1).

3.2. Immunogenicity

All but one subject (99.0%, 90% CI 94.7–100%) in the 'HepB at birth' group and 97.0% (90% CI 91.5–99.4%) in the 'no HepB at birth' group had seroprotective anti-HBs antibody levels (≥10 IU/l) 1 month after the third dose. The difference in seroprotection rates between the two groups was 2.0% (90% CI −1.2–5.3%). Since the lower confidence limit of the group difference is above −10.0%, non-inferiority between the groups can be concluded and the primary study objective was met (Table 2).

Similarly, there were no significant differences between the groups in the analyses of the other antigens. All subjects (100%) in

Table 2
Seroprotection and seroconversion rates at baseline (prior to the first vaccination) and 1 month after the third vaccination with DTPw–HepB–Hib in infants who had received a single dose hepatitis B vaccine at birth (HepB at birth) or not (no HepB at birth); ATP population

	Baseline		Post-third vaccination	
	HepB at birth, $n = 103$	No HepB at birth, $n = 100$	HepB at birth, $n = 103$	No HepB at birth, $n = 100$
Hepatitis B				
% SP	20.8	5.0	99.0	97.0
Absolute difference (90% CI)			2.0 (−1.2, 5.3)[a]	
Hib (anti-PRP ≥0.15 μg/ml)				
% SP	51.5	43.4	98.0	99.0
Absolute difference (90% CI)			−1.0 (−3.8, 1.9)[a]	
Hib (anti-PRP ≥1.0 μg/ml)				
% SP	12.6	14.1	87.1	89.9
Absolute difference (90% CI)			−2.8 (−10.2, 4.7)	
Diphtheria				
% SP	75.7	78.6	96.1	100
Absolute difference (90% CI)			−3.8 (−7.6, −0.0)[a]	
Tetanus				
% SP	99.0	100	100	100
Absolute difference (90% CI)			0.0 (−2.2, 2.3)[a]	
Pertussis				
% SC	2.0	0.0	95.1	98.0
Absolute difference (90% CI)			−2.9 (−7.1, 1.4)[a]	

ATP, according-to-protocol; CI, confidence interval; % SC, seroconversion rate (pertussis only); % SP, seroprotection rate.
[a] Non-inferiority concluded if the lower limit of the two-sided 90% CI is >−10% for the absolute difference between the groups.

Table 3
Geometric mean titers at baseline (prior to the first vaccination) and 1 month after the third vaccination with DTPw–HepB–Hib in infants who had received a single dose hepatitis B vaccine at birth (HepB at birth) or not (no HepB at birth); ATP population

	Baseline		Post-third vaccination	
	HepB at birth, $n = 103$	No HepB at birth, $n = 100$	HepB at birth, $n = 103$	No HepB at birth, $n = 100$
Hepatitis B				
GMT (IU/l)	5.25	3.54	560.99	281.31
95% CI	4.28, 6.44	2.90, 4.32	439.96, 715.3	221.92, 356.58
p-Value[a]	0.007		<0.001	
Hib				
GMT (μg/ml)	0.162	0.153	9.564	13.494
95% CI	0.123, 0.213	0.113, 0.208	6.665, 13.724	9.701, 18.769
p-Value[a]	0.787		0.165	
Diphtheria				
GMT (IU/ml)	0.292	0.336	0.997	0.905
95% CI	0.209, 0.407	0.239, 0.471	0.812, 1.224	0.753, 1.088
p-Value[a]	0.556		0.486	
Tetanus				
GMT (IU/ml)	1.980	2.335	5.353	3.832
95% CI	1.627, 2.408	1.937, 2.815	4.346, 6.594	3.063, 4.794
p-Value[a]	0.228		0.031	
Pertussis				
GMT (EIU)	4.04	4.22	45.01	43.25
95% CI	3.50, 4.66	3.70, 4.82	40.79, 49.68	39.17, 47.76
p-Value[a]	0.657		0.572	

ATP, according-to-protocol; GMT, geometric mean titer; CI, confidence interval; IU, international units; EIU, ELISA international units.
[a] Two-sided p-values are based on 90% CI calculated using normal approximation.

both groups were protected against tetanus, while for diphtheria 96.1% where protected in the 'HepB at birth' group compared to 100% in the 'no HepB at birth' group. Seroconversion to *B. pertussis* was achieved in 95.1% of the subjects in the 'HepB at birth' group compared to 98.0% in the 'no HepB at birth' group. When the anti-PRP ≥0.15 μg/ml cut-off level was applied, 98.0% of the subjects in the 'HepB at birth' group were protected against Hib compared with 99% in the 'no HepB at birth' group, demonstrating non-inferiority after the third vaccination, as with tetanus and diphtheria. However, non-inferiority could not be demonstrated when the anti-PRP ≥1.0 μg/ml cut-off level was applied (87.1% of the subjects in the 'HepB at birth' group were protected compared with 89.9% in the 'no HepB at birth' group).

The 'HepB at birth' group had significantly higher anti-HBs GMTs at all time-points of the analysis. After completion of the primary vaccination course, the GMT of the 'HepB at birth' group (560.99 IU/l, 95% CI 439.96–715.3 IU/l) was approximately double that of the 'no HepB at birth' group (281.31 IU/l, 95% CI 221.92–356.58 IU/l). Although statistically significant, the difference between the groups is not considered clinically relevant, as titers are well above the seroprotective level in both groups and also a very large increase in GMT over the baseline (approximately 110-fold in the 'HepB at birth' group and approximately 80-fold in the 'no HepB at birth' group) was shown. After the third vaccination, the anti-tetanus GMTs were significantly higher for the 'HepB at birth' group than for the 'no HepB at birth' group ($p = 0.03$). All other GMTs were similar between the two study groups (Table 3).

3.3. Safety and reactogenicity

There were no marked differences in the rates, severity, or duration of solicited AEs after vaccination with DTPw–HepB–Hib between the infants who had received a birth dose of HepB vaccine and those who had not. The number of subjects reporting fever was

Table 4
Rate of subjects with solicited local and systemic events reported from first to third vaccination; safety population

	First vaccination		Second vaccination		Third vaccination	
	HepB at birth $n = 106$ (100%)	No HepB at birth $n = 102$ (100%)	HepB at birth $n = 103$ (100%)	No HepB at birth $n = 101$ (100%)	HepB at birth $n = 103$ (100%)	No HepB at birth $n = 101$ (100%)
Local						
Erythema	24 (22.6)	32 (31.4)	25 (24.3)	27 (26.7)	37 (35.9)	25 (24.8)
Induration	47 (44.3)	48 (47.1)	40 (38.8)	37 (36.6)	51 (49.5)	30 (29.7)
Tenderness	94 (88.7)	93 (91.2)	78 (75.7)	79 (78.2)	69 (67.0)	63 (62.4)
Systemic						
Change in eating habits	34 (32.1)	37 (36.3)	23 (22.3)	19 (18.8)	17 (16.5)	22 (21.8)
Diarrhea	18 (17.0)	10 (9.8)	10 (9.7)	11 (10.9)	9 (8.7)	7 (6.9)
Fever[a]	24 (22.6)	29 (28.4)	15 (14.6)	19 (18.8)	24 (23.3)	29 (28.7)
Irritability	91 (85.8)	89 (87.3)	73 (70.9)	71 (70.3)	69 (67.0)	58 (57.4)
Rash	2 (1.9)	2 (2.0)	1 (1.0)	2 (2.0)	7 (6.8)	0 (0)
Sleepiness	49 (46.2)	49 (48.0)	30 (29.1)	30 (29.7)	20 (19.4)	28 (27.7)
Unusual crying	1 (0.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Persistent crying[b]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Vomiting	6 (5.7)	9 (8.8)	5 (4.9)	6 (5.9)	8 (7.8)	7 (6.9)

Results are n (%). HepB, hepatitis B vaccine.
[a] Axillary temperature ≥38 °C.
[b] Unaltered crying lasting >3 h.

slightly higher among the 'no HepB at birth' group (52.9%) compared with the 'HepB at birth' group (40.6%). During the course of vaccination, the rates of most AEs tended to decrease. The majority of AEs were reported within 1 day post-vaccination and resolved within 2 to 4 days.

Tenderness was the most frequently reported local AE, but decreased throughout the course of vaccination (Table 4). Most events of erythema and induration were classified as clinically insignificant, and only minor differences were observed for the incidence rates after each injection. All local AEs were considered vaccination-related by definition. Irritability was the most frequently reported systemic AE, but the occurrence decreased from 86.5% to 62.3% from the first to the third injection. No incidents of persistent crying were reported, and unusual crying was reported only for one subject after the first vaccination.

There were no solicited vaccine-related SAEs reported during the study period. One incident of sudden infant death syndrome was reported, which the investigator assessed as being unrelated to vaccination. Another 24 SAEs judged as unrelated to the study vaccine were reported for 17 subjects; all recovered without sequelae. There was one incidence of bronchiolitis that was classified as vaccine-related by the investigator.

4. Discussion

The availability of multivalent vaccines plays an important role in ensuring an increased compliance in the infant vaccination programs recommended by the WHO. The acceptance of multivalent vaccines largely depends on (1) their adaptability to various vaccination schedules used in various countries; (2) the demonstration that the included antigens do not negatively influence each other's immunogenicity; (3) the inclusion of antigens that do not interfere with concomitant or preceding vaccinations; and (4) the demonstration that the vaccine is safe and well tolerated.

The present study showed that regardless of whether a birth dose of HepB vaccine was administered or not, the fully liquid pentavalent combination vaccine DTPw–HepB–Hib was immunogenic and well tolerated when administered using a 2–4–6 months immunization schedule. Similar results have been achieved with other multivalent vaccines in infants who had, or had not, received HepB at birth.[22,23] There was no influence of the birth dose of HepB vaccine on the seroprotection or seroconversion rates against HBV, Hib, diphtheria, tetanus, and *B. pertussis* after the primary immunization course. Seroprotection and seroconversion rates against HBV, Hib ($\geq$0.15 μg/ml), diphtheria, tetanus, and *B. pertussis* were also comparable to rates achieved with the same vaccine after shorter intervals between doses,[16] as well as to the immunogenicity reported for an alternative pentavalent vaccine.[12,13] Only in the evaluation where the anti-PRP $\geq$1.0 μg/ml cut-off level was applied, was a slightly lower seroprotection rate seen in both groups, as reported in a previous study.[16] However, the rate is comparable with results achieved in a similar study with an alternative multivalent vaccine,[23] as well as with the anti-PRP $\geq$1.0 μg/ml seroprotection rates achieved with a commercially available monovalent Hib vaccine, which can be used in combination with another multivalent vaccine for primary immunization during the first year of life.[33]

As expected, infants who had received HepB vaccination at birth achieved higher anti-HBs antibody GMTs after the primary vaccination course. The difference in anti-HBs antibody titers was, however, not likely to be clinically significant, since long-term epidemiologic studies have shown that infants who achieve anti-HBs antibody titers >10 IU/l are protected for at least 10 to 20 years.[34] Similarly, the difference in anti-tetanus titers between the two study groups in the present study was concluded to be without clinical significance because titers in both groups were well above the seroprotective level.

The additional dose of a monovalent HepB vaccine at birth did not result in a clinically significant increase in AEs, which was in agreement with other studies.[18–22] There was no marked impact of the HepB vaccine birth dose on incidence rates, severity, or duration of solicited local and systemic AEs, indicating that DTPw–HepB–Hib was safe when administered to infants, whether or not they had received a birth dose of HepB vaccine. The slightly increased incidence of fever in the 'no HepB at birth' group was also observed in a similar study using another pentavalent vaccine.[12] Although the overall incidence of local and systemic AEs was high in this study, the clinical significance of these events was most likely low, because the severity of the majority of local and unsolicited AEs was considered mild. This was supported by the fact that the incidence rate of AEs decreased during the vaccination course.

In conclusion, the current study demonstrated that the fully liquid pentavalent combination vaccine DTPw–HepB–Hib was immunogenic and well tolerated when administered in a 2–4–6 months immunization schedule, regardless of whether infants had or had not received a birth dose of HepB vaccine.

5. Conflict of interest

This study was supported by a grant from Crucell, Berne, Switzerland. During the time of the study, Christian Herzog, Ursula Aeberhard, Ursula Yela, and Christian Spyr were employed by Crucell, the study sponsor and the manufacturer of Quinvaxem. Anne Katrin Hilbert is employed by Novartis Vaccines, the co-development partner for Quinvaxem, the vaccine evaluated in the study.

6. Ethical approval

The study protocol with all amendments and informed consent forms were approved by the appropriate independent ethics committee, and the study was performed in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines (GCP) and local laws. Written informed consent to participate in the study was obtained from the parent(s)/legal guardian(s) of all subjects before enrolment into the study.

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