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Research Article

CLINICAL TRIAL OF TETANUS TOXOID CONJUGATED VI POLYSACCHARIDE TYPHOID VACCINE IN INFANTS AND YOUNG CHILDREN

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ABSTRACT

Typhoid fever remains a common and serious disease in population living in countries south of Globe. Vi polysaccharide typhoid vaccine (Vi polysaccharide) and oral typhoid vaccine are available in the market but still a large population particularly infants and children below 5 years remain uncovered. These vaccines conferred protection in 78% in 5 years and older vaccines only effective for a shorter span of 2-3 years particularly in children in age group 2-5 years with protection levels less than 60% and the Vi polysaccharide does not induce a booster response. Bio-Med (P) Ltd. (BMPL) has developed the technology for manufacture of the Tetanus toxoid Vi polysaccharide conjugated typhoid vaccine (Vi-TT) as Peda Typh™ as per standard set by the WHO TRS 840 (1994). It is recommended to administer as intramuscular injection. Three consecutive batches of vaccines submitted to the Central Drug Laboratory CRI, Kasauli (H.P.) have been found to be of standard quality and thus was approved for clinical trial. Open Multicentric, controlled and comparative Phase III clinical trial was conducted to assess safety, immunogenicity in 3 months to 2 years at 3 centers (Bhavnagar, Hyderabad and Meerut). All serum samples after 4 weeks of vaccination with Vi-TT or Vi polysaccharide were found to have more than 4 fold increase in IgG antibody titers in 100% of the infants and older children. The vaccine was found to be safe, immunogenic and protective.

Keywords: Tetanus toxoid Vi conjugated typhoid vaccine, clinical trial in infants and older children, Vi conjugated typhoid vaccine, typhoid vaccine.

INTRODUCTION

Typhoid fever remains a common and serious disease in population living in countries South of Globe. More effective vaccines e.g. Vi polysaccharide typhoid vaccine (Vi Polysaccharide) and oral typhoid vaccine are available since past decade, still a large population particularly infants and children below 5 years remain uncovered. These vaccines conferred protection in 78% in 5 year and older vaccines only. Further, the Vi Polysaccharide has been shown to be effective for a shorter span of 2-3 years,

particularly in children in age group 2-5 years, with protection levels less than 60% (Kossaczka *et al.,* 1999). Thus re-vaccination is required at regular intervals. In addition, the Vi Polysaccharide does not induce a booster response (Szu *et al.,* 1987). Recent studies have indicated high incidence of typhoid fever in infants above 6 months age and children in 2-5 year age group (Sinha *et al.,* 1999; Saha *et al.,* 2003).

Typhoid in infants often remains unrecognized due to atypical clinical picture. Use of more sensitive media and bone marrow samples for typhoid bacteria isolation has resulted in identification of more cases (Gilman *et al.,* 1975; Hoffman *et al.,*1986).

Monsoon months had the highest disease occurrences 44.62% and post-monsoon 24.85% (PLOS Neglected Tropical Disease, 2013). Hygiene is very difficult to maintain for infants and toddlers. Extra precautions are taken for use of antibiotics in children. The commonly used antibiotics to treat Typhoid fever have become ineffective due to development of resistant *Salmonella typhi* organisms. The cost of treatment of typhoid fever patient is very high especially if hospitalization becomes necessary.

Robbins *et al.*(2008) explained the reasons for typhoid fever relapses following withdrawal of treatment or sometimes thereafter to the patient remaining in area where active re-infection could occur as well as S. Typhi does not circulate in the blood stream in large enough quantities to shed enough Vi antigen for immune apparatus to respond. They advocated vaccination with Vi antigen type vaccine immediately after clinical recovery. Hence control of disease by vaccination becomes a natural choice (Bahl *et al.,* 2004). Infants less than two years have remained unprotected by hitherto available typhoid vaccines e.g. whole cell vaccines, Oral (Murphy *et al.,* 1991) or Vi polysaccharide (Acharya *et al.,* 1987). The Vi polysaccharide vaccine does not elicit immune response in children less than 2 years of age because of its age related `T' cell independent immunologic properties, which are similar to other polysaccharide vaccines (Szu *et al.,*1987).

Since all available vaccines to-date are recommended for children above 2-5 years in age, scientists have been working on vaccines which could be used to vaccinate infants (Szu *et al.,* 1987). Majority of workers have attempted to use one or the other useful protein derived by rDNA technology e.g. rEPA of *Pseudomonas aeruginosa* (Feng *et al.,* 2001), B subunit of cholera exotoxin (Szu *et al.,*1989).

The present study was undertaken to assess the immunogenicity and safety in infants and children of a new Tetanus toxoid conjugated Vi polysaccharide typhoid vaccine developed at BMPL. This new technology has avoided the use of recombinant proteins for conjugation. The vaccine has passed the required safety and immunological parameters in animals and has been found to be of standard quality by the Central Drugs Laboratory, CRI, Kasauli, H.P. The clinical trials were carried out at three centers in India to evaluate the immunogenicity and safety of Vi-TT in comparison to Vi Polysacch. manufactured by BMPL by conducting clinical trial phase III in human volunteers.

Vaccines using a variety of proteins conjugated with Vi antigen of S.Typhi have been developed and tested in

laboratory animals. Recombinant non-toxic *Pseudomonas aeruginosa*–Vi antigen of S.Typhi vaccine (Vi-rEPA) manufactured on experimental basis has been tested in large scale clinical trial in Vietnam on 2 year and older children. The vaccine proved to be efficacious in >91% observed over 4 years (Lanh *et al.,* 2003).

MATERIALS AND METHOD

Clearance and Approval

The study protocols were approved by the ethics committees of medical colleges located in three widely separated zones of India (L.L.R.M. Medical College, Meerut, U.P., Gandhi Medical College, Secunderabad, A.P. and Medical College, Bhavnagar, Gujarat). This was followed by grant of necessary permission for conducting clinical trials – Phase III by the Drugs Controller General of India in July 2007.

A multicentric, open, controlled, comparative clinical trial phase III for Vi - TT manufactured by BMPL was undertaken at the following centers located in widely separated zones of India in September 2007. 1. L.L.R.M. Medical College, Meerut, U.P. (LLRM). 2. Medical College, Bhavnagar, Gujarat (MCB). 3. Gandhi Medical College, Hyderabad, A.P. (GMC).

Vaccines

Vi Polysaccharide is presented in single dose vial as 0.5 ml clear sterile solution containing purified Vi polysaccharide of S. Typhi–25 µg in isotonic saline. The vaccine has ≤ 0.25% phenol as preservative. It is to be given as intramuscular/subcutaneous injection to children above 2 years of age.

Vi –TT is presented in single dose vials as 0.5 ml clear to slightly turbid sterile solution containing purified polysaccharide of S. Typhi–5 µg conjugated to 5 µg tetanus toxoid protein in isotonic saline. It contains no preservatives and is recommended to administer as intramuscular injection in healthy infants above 3 months of age and older subjects. Vi polysaccharide meets the requirement of World Health Organization (Requirement for Vi polysaccharide typhoid vaccine, 1994).

Tetanus toxoid used in the manufacture of the vaccine, meets the requirement of Indian Pharmacopoeia. Vi-TT Tetanus toxoid was treated with adipic acid dihydrazide (ADH) and covalently conjugated to Vi polysaccharide by carbodimide– mediated coupling. Vaccine was manufactured in cGMP facilities complying with schedule 'M' of Government of India and WHO GMP requirements.

Subjects

A total of 206 healthy volunteers both male and female of different age groups participated in the trial after full checking by the medical doctors for perfect clinical health status. All children and parents were explained the purpose of the clinical trial, the types of vaccine being used for injection, their potential side effects and benefits. The consent forms were duly filled in and signed by the parents/guardians (Table 1). Three consecutive batches of Vi-TT were used. All vaccines were grouped in Group A (Vi-TT) and Group B (Vi-Polysaccharide).

Study Protocol

Design: Multicentric, open, controlled and comparative.

Objective: To evaluate safety and immunogenicity of Tetanus toxoid Vi

polysaccharide conjugated typhoid vaccine (Vi-TT) in comparison with plain Vi polysaccharide typhoid vaccine (Vi-polysacch.), manufactured by BIOMED (P) Ltd. (BMPL) as Peda Typh™ and Bio Typh™ respectively, by conducting Phase III clinical trial in healthy infants (3 months – 2 year) and children (2 years & older).

Method: Volunteers were vaccinated, reactogenicity recorded, pre-immune and post immune paired serum samples were tested for IgG anti-Vi polysaccharide antibody concentration by ELISA.

Blood samples (2 ml) were taken by vein puncture before vaccination on '0' day (pre-immune) and 4 weeks after vaccination (post- immune). Serum was separated and stored at or below -20^{o}C until testing.

Volunteer Records

A detailed volunteer record file (VRF) was maintained which contained consent form, a detailed demographic profile including age, sex, address, medical history and general examination.

Adverse reactions were noted as per protocol on every visit subsequent to vaccination.

Clinical Evaluation

All volunteers were clinically examined to assess for safety parameters and the results duly recorded.

1. Local side effects at the site of vaccination- Pain, erythema and inflammation were recorded 30 minutes, 4-6 hours and 48 hours after vaccination using scale of adverse reactions as:

(a). Excellent – no local or systemic reactions. (b). Good – local reaction (inflammation) ≤ 5 cm in diameter and axillary temperature ≤ 38.3^{o}C. (c). Fair – local reaction (inflammation) > 5 cm in diameter and axillary temperature ≤ 38.3^{o}C (d). Bad – local reaction (inflammation) > 5 cm in diameter and axillary temperature ≥ 38.3^{o}C.

2. Systemic side effects: Fever, diarrhea, vomiting and any other side effects were recorded 30 minutes, 4-6 hours and 48 hours of post-vaccination. In addition to the above, in children below 5 years of age excessive crying and refusal to take food was also recorded.

EVALUATION OF IMMUNOGENICITY

Paired serum samples from all the 3 centers were analyzed at the Department of Pathology & Microbiology, LLRM Medical College, Meerut. IgG antibodies against Vi polysaccharide of S. Typhi were estimated by Enzyme linked immunosorbent assay (ELISA) performed on paired serum samples from each volunteer: Microtiter plates (Nunc Maxisorp^{TM}) were coated with Vi polysaccharide covalently conjugated to bovine serum albumin via adipic acid dihydrazide linker) diluted in phosphate buffer saline, pH 7.4 @ 2 µg per well. Sera were assayed for IgG antibodies using anti human IgG – HRP conjugate in PBS with 1% bovine serum albumin at 1:10,000 dilution. The anti-Vi IgG Standard Reference (lyophilized, 0.2 ml/vial) consisted of serum sample of an adult vaccinated with Vi polysaccharide typhoid vaccine. It was assigned a value of 118 ELISA units calibrated with the Reference sera used by (Kossaczka *et al.,* 1999) (one ELISA unit is approximately equivalent to

0.1 µg of IgG anti Vi / ml). The measure of optical density was done in ELISA reader at 492 nm wavelength with appropriate controls.

Immune response to vaccine was defined as ≥ 4 fold rise in anti-Vi polysaccharide antibody level i.e. O.D. of 1:400 dilution of post-immune sera should be greater than O.D. of 1:100 dilution of pre-immune sera (Requirement for Vi polysaccharide typhoid vaccine, 1994 and Klugman *et al.,* 1996). For the dilutions of standard reference Vi polysaccharide IgG antibodies (1:100 → 1:51, 200) plotted a curve of absorbance against IgG Vi polysaccharide antibodies. From the value of absorbance obtained for the test serum samples, the corresponding value of IgG can then be read of the standard curve. Analysis of results was done by program of ELISA for windows version 2.0 of the CDC, US Department of Health & Human Services, Atlanta, U.S.A. (Tielde *et al.,* 1979; Draper *et al.,* 1998).

RESULTS

The clinical trial of Vi-TT Vaccine (BMPL) conducted at three centers Bhavnagar, Hyderabad and Meerut) using 3 consecutive batches proved to be safe and immunogenic in 100% of the subjects. The immune response was more than four fold in infants (3 months and older), 2-5 years old and older children in all the 3 study centers where 3 consecutively manufactured batches of the Vi-TT vaccine was used. Hence the immunogenicity results were combined for statistical analysis. They are at GMT (95% confidence interval) for children below two years of age 80.43 (69.08–93.64) and children above 2 years of age at GMT (95% confidence interval) 62.57 (53.26–73.50). Statistical analysis of serological response to recombinant nontoxic exoprotein of *Pseudomonas aeruginosa* Vi polysaccharide conjugate vaccine (Vi-rEPA) conjugated typhoid vaccine tested in Vietnam trials (Feng *et al.,* 2001) and the Vi-TT vaccine are statistically similar. Tetanus toxoid proved a superior protein for conjugation with Vi since it was used in less than 25% quantities than in Vi-rEPA (Feng *et al.,* 2001). Large quantities of Vi-TT have been used without any break in immunity over past 18 months. By ELISA test, antibody titers were higher in 5 years and older children than in 2-5 years old group, who were vaccinated with Vi-polysaccharide.

A total of children 169 and 37 were given Vi-TT designated as Group A, and Vi Polysaccharide designated as Group B, respectively. There were no serious adverse events leading to premature withdrawals from the study in any of the 3 centers of study. Local reactions were confined to mild transient pain in a small fraction of the vaccines of 3 months and older children which also resolved without sequelae. The observations have been summarized in Table 2 and 3.

The data presented in Table 2 and 3 indicated that the local reactions were confined to mild transient pain in a small number of volunteers. Fever, outside the set limits of ≥ 38.3^{o}C was not recorded in any one. Thus Vi-TT was found to be well tolerated. All the volunteers Showed an excellent and good tolerance based on the global scale of assessment of adverse

reactions. There was no reaction noted 30 minutes post-vaccination. None of the volunteers had any local or systemic side effects up to 48 hours of vaccination. From the above it is concluded that the Vi-TT is statistically as safe and safer than Vi Polysaccharide. The above data were compared with those reported by (Feng *et al.,* (2001) for recombinant protein r-EPA. The data had p-value of P = 0.623 (Exact) which indicates Vi-TT is as safe as Vi-rEPA used by the NIH, USA workers.

All serum samples after 4 weeks of vaccination with Vi-TT or Vi Polysaccharide were found to have more than 4 fold increases in IgG antibody titers in 100% percent of the infants and older children. The data have been presented in Table 4 and 5 for Vi-TT and Vi Polysaccharide respectively. The vaccines are considered as immunogenic and protective as per standards set by the WHO (WHO Technical Report, 1994). The immune response was higher in infants above 3 months – 2 years old than in children older than 2 years.

The data in Table 5 for immune response to Vi Polysaccharide indicate lower titers in 24-60 months old children where compared to those more than 60 months. The results of ELISA on paired pre and post serum samples were analyzed (Table 6). The immune response of Vi-TT was similar in children below two years age and older children at all the 3 centers and therefore analyzed after combining them [Geometric mean (95% confidence interval) 80.43 (69.08 – 93.64)] and children above two years of age [Geometric mean (95% confidence interval) 62.57 (53.26 – 73.50)].

The results of ELISA on pre & post immunization paired serum samples were analyzed statistically. Pre-IgG ELISA values in Vi-TT and Vi Polysacch. groups were comparable for all the three clinical trial study centers. Therefore, Pre-IgG values were pooled and compared between the two groups. Post IgG values were similar in both the groups for all the three clinical trial centres separately and the three centers combined together. Being an equivalence study, 95% confidence interval for both the groups were compared. Since the 95% confidence interval was overlapping, it is concluded that the difference in post IgG values for Vi-TT and Vi Polysacch. are similar and statistically not significant. On comparison with data presented by (Feng *et al.,* 2001) with the use of recombinant proteins rEPA conjugated Vi-TT (Vi rEPA), the post IgG Geometric mean (25^{th}, 75^{th} percentile) for Vi-TT [75-52 (51-74-113.32)] and Vi-rEPA (N.I.H., U.S.A.) [72.9 (50.7-124)] are statistically similar.

Table 1. Age and Gender ratio of volunteers at the 3 different centers of the study

Center	Gender Ratio		Age Distribution		
	Male	Female	≥ 3-24 Months	> 24-60 Months	> 60 Months or more
LLRM	31	28	43	9	7
MCB	41	27	21	23	24
GMC	42	37	21	22	36

Table 2. Local and systemic side effects on 3 medical centers of the study (Group A vaccinated with Vi-TT, Group B vaccinated with Vi Polysacch.

Parameters	LLRM		MCB		GMC	
Total Children	Group A	Group B	Group A	Group B	Group A	Group B
	52	7	56	12	61	18
Pain	5	1	10	3	4	3
Erythema	1	1	0	1	0	1
Inflammation	0	0	0	0	0	0
Fever (>38.3^{o}C)	0	0	2	0	1	1
Excessive crying	1	0	0	0	0	0
Refusal to take food	0	0	0	0	0	0
Diarrhea	0	0	0	0	0	0
Vomiting	0	0	1	0	0	0
Any other	0	0	0	0	0	0

Table 3. Local and systemic side effects on 3 centers of study based on the scale of adverse reactions – Group A – Vi-TT, Group B – Vi Polysacch.

Parameters	LLRM		MCB		GMC	
	Group A	Group B	Group A	Group B	Group A	Group B
Total Children	52	7	56	12	61	18
Excellent	44	6	46	9	57	15
Good	8	1	10	3	4	3
Fair	0	0	0	0	0	0
Bad	0	0	0	0	0	0

The data presented in the Table 3 were analyzed statistically in totality as under:-

Parameters	Vi-TT	Vi Polysacch.	p-value
Pain	19/169	7/37	P = 0.203
Erythema	1/169	3/37	P = 0.019 (Exact test)
Inflammation	0/169	0/37	----------
Fever (> 38.3^{o}C)	3/169	1/37	P = 0.55

From the above it is concluded that the Vi-TT is statistically as safe and safer than Vi Polysaccharide.

Table 4. Results of IgG anti Vi antibody titers in ELISA units at 3 medical centers of study. Volunteers vaccinated with 3 consecutive batches of Vi-TT – one at one center.

GROUP 'A'

Parameters	LLRM		MCB		GMC	
Age of volunteers	Pre immune	Post immune	Pre immune	Post immune	Pre immune	Post immune
> 3-24 Months	2.985	87.908	2.663	107.882	2.684	110.71
> 24-60 Months	3.420	79.76	2.964	90.329	2.950	70.586
> 60 Months	4.72	129.672	3.026	106.26	3.124	54.012

Table 5. Results of IgG anti Vi antibody titers in ELISA units at 3 medical centers of study. Volunteers vaccinated with one batch of Vi Polysaccharide.

GROUP 'B'

Parameters	LLRM		MCB		GMC	
Age of volunteers	Pre immune	Post immune	Pre immune	Post immune	Pre immune	Post immune
> 24-60 Months	4.841	71.981	---	---	2.685	51.06
> 60 Months	3.843	130.111	3.518	71.594	3.004	79.413

Table 6. Results of ELISA on pre and post immunization serum samples on statistical analysis Geometric Mean (95% confidence interval):

Center(s)	Group(s)	Pre IgG	Post IgG
GMC	Vi-TT	2.43 (1.96-3.02)	60.38 (55.08-72.81)
	(n=54)	2.67 (2.11-3.3.8)	61.96 (44.7-85.84
	Vi-Polysacch.	----------	----------
	(n=18)	p = NS	p=NS
LLRM	Vi –TT	2.32 (1.77-3.04)	78.75 (66.16-93.73)
	(n=43)	3.84 (2.38-6.19)	78.81 (33.12-193.39)
	Vi Polysacch.	----------	----------
	(n=6)	p = NS	p = NS
MCB	Vi-TT (n=47)	2.47 (2.08-2.92)	75.53 (60.62-94.10)
	Vi Polysacch.	2.78 (0.96-8.03)	53.95 (20.46-142.24)
	(n=5)	----------	----------
		p = NS	p = NS
Total	Vi-TT	2.41 (2.13-2.73)	70.32 (62.86-78.66)
	(n=144)	2.90 (2.35-3.57)	63.59 (48.68-83.07)
	Vi Polysacch.	----------	----------
	(n=29)	p = NS	p = NS

NS : Statistically Not Significant ($p > 0.05$)

DISCUSSION

Vi polysaccharide typhoid vaccine induces only short lived, antibody response (lasting for 2-3 years) in children 2-5 years old with efficacy of $\leq$ 60% when compared with efficacy of > 78% in 5 years and older. In adults reinjection restores the level of antibodies but does not induce booster response. This age related and T cell independent immunogenic properties are similar to most polysaccharide vaccines (Klugman *et al.,* 1996). In view of the above Vi polysaccharide vaccines were recommended for 5 years or older children initially. In the present studies also, we observed that immune response in 2-5 years old children is weaker than the group over 5 years to Vi-Polysaccharide Vaccine.

The immune response was similar and in 100% children at 3 study centers using consecutively manufactured batches of Vi-TT. This observation proves which only 5 µg each of Tetanus Toxoid and Vi Polysaccharide was used. This indicates superiority of Vi-TT over r-EPA-Vi in that Vi-TT is equally effective and inducing immune reaction even though it has less than 25% of active Vi antigen.

Lanh *et al.* (2013), reported that the rEPA-Vi conjugate vaccine induced booster immune reaction in children studied 4 weeks after second injection, levels of serum IgG Vi antibodies had increased by a factor of 10 or more. The booster response was similar to other vaccines like *Haemophilus influenzae* type b-tetanus conjugate vaccine. Based on these observation, two injection schedule of Vi-TT at internal of one month or more in infants has been recommended by BMPL, it is expected to ensure full protection against severe natural challenge of S. Typhi. Infants kept in relatively good sanitary conditions,

could be expected to be protected even after one injection. A booster at 2 years age or thereafter would be expected to confer lifelong immunity as has been postulated by Robbins (Robbins *et al.*, 2008). A large use of Vi-TT vaccine all over India during past 18 months without any adverse reactogenicity and immunity breaks confirms its safety and efficacy.

The use of Vi-TT to vaccinate infants in countries where Typhoid fever is prevalent will go a long way in protecting the most susceptible and vulnerable infants. We can expect that intensive use of conjugated vaccine will be able to break the cycle of S. Typhi infection in regions where it is prevalent (Keitel *et al.,* 1994).

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