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POTENCY TESTING OF NOVEL DTP GROUP OF VACCINES

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ABSTRACT

Conventionally, the production of DTP group of vaccines involves the growth of toxicogenic strains of *C. diphtheria*, *C. tetani* and *B. pertussis* on media of animal origin e.g. casein digests or meat extracts. These media pose various risks such as Bovine Spongiform Encephalopathy (BSE), microbial contamination and allergic reactions. To avoid such risks, media containing nutrients of vegetative origin are substituted, which support the growth of the pathogenic bacteria. The present study involves the potency testing of DTP group of vaccines produced on such vegetative media. The lethal challenge method and antibody induction method were the two methods used to determine the potency of this group of vaccines. The potency of these DTP vaccines was found to be well within limits set by the Indian Pharmacopoeia, 2007.

Keywords: Antibody induction method, DTP vaccine, Indian Pharmacopoeia, Lethal challenge method, Potency.

INTRODUCTION

Potency assay is an essential step in the quality control of vaccines for human use according to WHO Expert Committee on Biological Standardization.[1,2] The aim of potency testing is to ensure that the vaccine under test will provide protection when used in human subjects. For checking the potency of DTP group of vaccines two methods are used: (i) Challenge Method and (ii) Antibody Induction Method (AIM).[3] Irrespective of the method used, induction of an antitoxin response in laboratory animals and the measurement of that response are two vital steps of the method in assessing the potency of the DTP group of vaccines.[1-4]

In both of the above mentioned methods, induction of the immune response is by immunization of the animals with a suitable antigenic preparation.[5,6] In the challenge method, the induction is only once i.e. animal is immunized at one occasion only, whereas in the antibody induction method, the animal is immunized on two occasions.[7] In the challenge method, measurement of the induced response is done by challenging the immunized animals with a suitable toxin preparation 28 days after immunization; while in AIM, measurement of the induced response is done by some suitable antibody titration method performed on the sera raised from the immunized animals.[8,9]

For diphtheria and tetanus component and for pertussis component, challenge method was performed by subcutaneous lethal challenge method[10] and intracerebral lethal challenge method, respectively.[11-13] These methods are more accurate and economical than the previous method namely, intradermal challenge method, in which a large number of animals were required and also involved multiple intradermal challenges on the depilated skin of animals, which causes severe pain to the animals.[14,15] Challenge method is preferable, as animal immunization is done only once and the same animals are used for induction and measurement of the immune response to the vaccine.[16,17]

The antibody induction method was carried out for the determination of the diphtheria and tetanus components, in which the tetanus and diphtheria antibodies were estimated in the individual guinea pig sera by using the toxin neutralization test (*in-vivo*)[18-20] This method which is recommended by I.P., 2007 is most valid, precise and requires only a smaller number of animals. It is also an attractive alternative for potency testing, because assay of more than one toxoid component in a group of animals can be carried out and it also provides a greater amount of quantitative information i.e. the amount of antitoxin induced by antigen.[2,3,21]

MATERIALS AND METHODS

Vaccine Samples

DTP, DT, TT, Pentavalent (DTP/IPV/Hib) and Quadruple vaccine (DTP/IPV) were obtained from Central Drug Laboratory (CDL) of Central Research Institute (CRI), Kasauli (HP). DTP group of vaccines were produced by growing the strains of pathogenic bacteria on media of vegetative origin which is a better substitute for nutrients of animals origin. The toxin obtained/generated was converted to toxoid by the addition of formalin. Crude toxoid obtained was concentrated and purified to obtain final product. All vaccines were stored at temperature of 2- 8°C.

Toxins and Standard Toxoids

Diphtheria Toxin, Tetanus toxin, In House Reference Standard of Diphtheria toxoid (208 IU/vial), In House

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Reference Standard of Tetanus Toxoid (310 IU/vial), National Reference Standard for Diphtheria Antitoxin (10 IU/ml), National Reference Standard for Pertussis Vaccine (120 IU/vial) and National Reference Standard for Tetanus Antitoxin (10 IU/ml) were obtained from Central Drug Laboratory (CDL) of Central Research Institute (CRI), Kasauli (HP).

Animals

Guinea pigs (250-350 g) and Swiss albino mice (13-22 gms) were used for the potency testing of vaccines. They were housed under standard conditions (Temperature: 28 ± 2°C, Relative humidity: 50 ± 2%, 12 hr light / dark cycle) and provided with standard pellet diet and water *ad libitum.* The study was conducted in the Central Drug Laboratory, Central Research Institute, Kasauli, Dist. Solan, Himachal Pradesh. All the procedures were approved and carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Lethal Challenge Method

Potency Testing of Diphtheria Component by Subcutaneous Lethal Challenge Method: Animals were divided into twelve groups each containing 16 animals. Guinea pigs in Groups I, II and III were immunized subcutaneously with 1.0 ml of each dilution (1:10, 1:20 and 1:40) of reference standard, respectively. Guinea pigs in Groups IV, V & VI; VII, VIII & IX; and X, XI & XII were inoculated subcutaneously with 1.0 ml of each dilution (1:40, 1:80 and 1:160) of DTP, DT and Pentavalent (DTP/IPV/Hib) vaccine, respectively. The response to single dose of vaccine was determined 28 days (4 weeks) after inoculation and were challenged subcutaneously with1.0 ml of the Diphtheria Toxin.[3]

Further, as positive control animals were grouped into four groups each containing 2 guinea pigs. Animals in group I, II, III and IV were inoculated subcutaneously with 1.0 ml of each dilution of 100 LD_{50} toxin as 1:18, 1:900, 1:1800 and 1:3600, respectively. Challenged guinea pigs were observed twice a day for 5 days and death and survival of animals were recorded daily. LD_{50} of the challenge culture was estimated by the Reed and Muench Method.[22] The potency of the test vaccine was estimated by Probit Analysis.

Potency Testing of Tetanus component by Lethal Challenge Method: Animals were divided into twelve groups each containing 16 animals. Animals in Groups I, II and III were immunized subcutaneously with 0.5 ml of each dilution (1:5, 1:10 and 1:20) of reference standard, respectively. Animals in Groups IV, V & VI; VII, VIII & IX; and X, XI & XII were inoculated subcutaneously with 1.0 ml of each dilution (1:6.8, 1:15.6 and 1:31.2) of DTP, DT and TT vaccine, respectively.

The response of the mice to single dose of vaccine was determined 28 days (4 weeks) after inoculation and all immunized mice were challenged subcutaneously with 0.5 ml of the Tetanus Toxin.[3] Further, as positive control animals were grouped into four groups each containing 8 mice. Animals in Groups I, II, III and IV were inoculated subcutaneously with 0.5 ml of each dilution of 100 LD_{50} toxin as 1:3250, 1:162500, 1:325000 and 1:650000, respectively. Challenged mice were observed twice a day for 5 days and death and survival of animals were recorded daily. LD_{50} of the challenge culture was estimated by the Reed and Muench Method.[22] The potency of the test vaccine was estimated by Probit Analysis.

Potency Testing of Pertussis Component by Lethal Challenge Method: Animals were divided into twelve groups each containing 20 animals. Mice in Groups I, II and III were immunized intraperitoneally with 0.5 ml of each dilution (1:4, 1:20 and 1:100) of the National Reference Standard (NRS), respectively. Animals in Groups IV, V & VI, VII, VIII & IX and X, XI & XII were inoculated intraperitoneally with 0.5 ml of each dilution (1:5, 1:25 and 1:125) of DTP, Quadruple (DTP/IPV) and Pentavalent (DTP/IPV/Hib) vaccine, respectively. The response of the animals to single dose of vaccine was determined 14 days (2 weeks) after inoculation and all groups of mice were challenged with 0.03 ml of challenge suspension (100000 Org./0.03ml) intracerebrally using a 1/4th inch needle fitted to a 0.25 ml sterile syringe.[3] Further, as positive control animals were grouped into five groups each containing 10 mice. Animals in group I, II, III, IV and V were inoculated intracerebrally with 0.03 ml of each dilution containing 10,0000, 10,000, 2000, 400 and 80 Org. respectively. The mice were observed daily for 14 days. Mice which died within 3 days were excluded from the test and records were kept. Deaths were recorded daily upto the 14th day. LD_{50} of the challenge culture was estimated by the Reed and Muench Method[22] and the potency of the test vaccine was estimated by Probit Analysis.

Antibody Induction Method

Potency Testing of Diphtheria Component by Antibody Induction Method: 1.0ml of 1/100 dilution of vaccine in physiological saline was inoculated subcutaneously into 10 normal and healthy guinea pigs weighing between 250-350 g. A second dose of the same dilution was injected into each guinea pig after an interval of 4 weeks (IP, 2007). Between the 2nd and 3rd week after the 2nd dose, all the animals were bled under anesthesia, by cardiac puncture. Blood was allowed to clot and sera was separated individually by centrifugation at 2000 rpm for 10 minutes and stored at 2-8°C until tested.

Estimation of Diphtheria antibodies in the individual guinea pig sera by intradermal test (in-vivo): The serum of each guinea pig was tested for diphtheria antitoxin by intradermal Toxin Neutralization Test using Lr/100 dose of diphtheria toxin at 4 different levels such that it contains 1, 2, 3 & 4 IU/ml. National Reference Standard of Diphtheria Antitoxin was also run in parallel as the working standard. The unitage of guinea pig sera was tested at different dilutions, separately. The toxin-antitoxin mixtures were made using Lr/100 dose of toxin with Standard Diphtheria Antitoxin (DAT) and with different serum samples collected from ten immunized guinea pigs. Both the mixtures were incubated at room temperature for 1 hour. For inoculation of mixture of Toxin- Standard Diphtheria antitoxin, animals were divided into five groups each containing one animal. 0.2 ml of each mixture of Toxin- Standard antitoxin was inoculated intradermally into animal in group I and local reaction was recorded after 48 hours. Similarly, animals in Groups II, III, IV and V were also inoculated mixtures and local reaction was recorded. The Lr/100 dose of standard diphtheria antitoxin which produces Schick type reaction (12×14 mm) at 0.01 IU/ml was taken as standard value. Guinea pigs were grouped into ten groups each containing one animal.[3] Animal in group I was inoculated with a dose of 0.2 ml of each of mixture of Toxin- Serum sample (collected from immunized guinea pigs) in the depilated skin intradermally. Diphtheria antitoxin content was

determined at four different unitages, namely 1, 2, 3 & 4 IU/ml by inoculating mixtures into the shaved skin of guinea pigs at various spots not less than 2.5 cm apart . Similarly, animals in Groups II, III, IV, V, VI, VII, VIII, IX and X were inoculated 0.2 ml intradermally with each of Toxin-Serum sample mixture. All the injection sites were examined after 48 hours and erythematous reaction was recorded. The unitage of the sample was estimated in comparison with standard antitoxin. The geometric mean of 10 guinea pigs sera was calculated.

Potency Testing of Tetanus Component by Antibody Induction Method: 1.0 ml of 1/20 dilution of vaccine in physiological saline was inoculated subcutaneously into 9 normal and healthy guinea pigs weighing between 250-350 gm. A second dose of the same dilution was injected into each guinea pig after 4 weeks interval (IP, 2007). All the animals were bled at 2nd week under anesthesia by cardiac puncture. The blood was incubated at 22°C for 1 hour. The blood was allowed to clot and sera was separated aseptically by centrifugation at 2000 rpm for 10 minutes and stored at 2- 8°C until tested.

Estimation of Tetanus antibodies in immunized guinea pig sera by Toxin Neutralization Test (in-vivo): The serum of each guinea pig was tested for tetanus antitoxin by Toxin Neutralization Test using L+/10 dose of tetanus toxin. National Reference Standard of Tetanus Antitoxin was also run in parallel as the working standard. The unitage of guinea pig sera was tested at different dilutions, separately. The toxin- antitoxin mixtures were made using L+/10 dose of toxin with Standard Tetanus Antitoxin (TAT) and with different serum samples collected from nine immunized guinea pigs. All the mixtures were incubated at room temperature for 1 hour. For inoculation of mixture of Toxin- Standard Tetanus antitoxin, animals were divided into five groups each containing 6 mice weighing between 17- 22 gms.[3] 0.5 ml of each mixture of Toxin- Standard antitoxin was inoculated subcutaneously into animals in group I. Animals were observed daily for 5 days and tetanus symptoms and death were recorded. Similarly, animals in groups II, III, IV and V were also inoculated with mixtures and tetanic reaction was recorded for 5 days. The L+/10 dose of standard tetanus antitoxin kills 50% of test animals at 0.1 IU/ml and this was taken as standard value. For inoculation of mixture of Toxin- Serum samples (collected from immunized guinea pigs) animals were grouped into nine groups each containing 6 mice. Mice in group I were inoculated subcutaneously with a dose of 0.5 ml of each of mixture of Toxin- Serum samples. The animals were observed daily for 5 days and tetanus symptoms and death were recorded. Similarly, animals in group II, III, IV, V, VI, VII, VIII, IX and X were inoculated 0.5 ml subcutaneously with each of Toxin- Serum sample mixture and were observed daily for 5 days and tetanus symptoms and death were recorded. The antibody titre of the test serum was estimated in comparison with the results of standard tetanus antitoxin.

RESULTS AND DISCUSSION

Lethal Challenge Method

Potency Testing of Diphtheria Component by Subcutaneous Lethal Challenge Method: In the Groups I, II and III, where animals were immunized with different dilutions (1:10, 1:20 and 1:40, respectively) of the International Reference Standard (IRS) and challenged with 1.0 ml of diphtheria toxin, a cumulative survival of 31, 15 and 03 and cumulative deaths of 00, 04 and 17 were observed, resulting in mortality ratios of 0.00, 0.21 and 0.85, respectively. In the Groups IV, V & VI, VII, VIII & IX and X, XI & XII, where animals were immunized with each dilution (1:40, 1:80 and 1:160) of the DTP, DT and Pentavalent vaccine (DTP/IPV/Hib) and challenged with 1.0 ml of diphtheria toxin, the cumulative survival was found to be 35, 20 & 06, 29, 15 & 02 and 33, 17 & 04 and cumulative deaths were 01, 03 & 13, 02, 05 & 19 and 00, 03 & 15, resulting in mortality ratios of 0.03, 0.13 & 0.68, 0.06, 0.25 & 0.90 and 00, 0.15 & 0.79, respectively (Table **1**). By using Probit Analysis, potency of DTP, DT & Pentavalent vaccine (DTP/IPV/Hib) was found to be 98.01, 73.39 & 90.65 IU/Dose, respectively.

Table 1. Effect of various dilutions of vaccine samples on cumulative survival and mortality; and mortality ratios following lethal diphtheria challenge method.

Product Details	Group	Dilution	Observations (Mortality)					Survival	Mortality	Mortality Ratio		
			1st Day	2nd Day	3rd Day	4th Day	5th Day			CS	CD	MR
IRS	I	1:10	-	-	-	-	-	16	00	31	00	0.00
	II	1:20	1	1	2	-	-	12	04	15	04	0.21
	III	1:40	12	1	-	-	-	03	13	03	17	0.85
DTP	IV	1:40	1	-	-	-	-	15	01	35	01	0.03
	V	1:80	1	1	-	-	-	14	02	20	03	0.13
	VI	1:160	7	3	-	-	-	06	10	06	13	0.68
DT	VII	1:40	2	-	-	-	-	14	02	29	02	0.06
	VIII	1:80	1	2	-	-	-	13	03	15	05	0.25
	IX	1:160	14	-	-	-	-	02	14	02	19	0.90
Pentavalent vaccine (DTP/IPV/ Hib)	X	1:40	-	-	-	-	-	16	00	33	00	0.00
	XI	1:80	2	1	-	-	-	13	03	17	03	0.15
	XII	1:160	9	3	-	-	-	04	12	04	15	0.79

n= 16 per group; ***IRS*** *- International Reference Standard;* ***DTP*** *- Diphtheria Tetanus and Pertussis Vaccine;* ***DT*** *- Diphtheria and Tetanus Vaccine;* ***Pentavalent vaccine (DTP/IPV/Hib)*** *– Diphtheria, Tetanus and Pertussis Vaccine/ Inactivated Polio Vaccine/ Haemophilus influenza type b;* ***CS*** *- Cumulative Survival;* ***CD*** *- Cumulative Death;* ***MR*** *-Mortality Ratio.*

LD_{50} of Challenge Toxin: In the Groups I, II, III and IV, where animals were challenged with various dilutions of the diphtheria toxin (1:18, 1:900, 1:1800 and 1:3600), a cumulative survival of 0, 0, 1 and 3 and cumulative deaths of 7, 3, 1 and 0 were observed, resulting in mortality ratios of 1, 1, 0.5 and 0, respectively. Percentage mortality was found to be 100, 100, 50 and 00 for groups I, II, III and IV, respectively (Table **2**). The LD_{50} of the vaccine was found to be 7.849×10^{-4}.

Potency Testing of Tetanus component by Lethal Challenge Method: In the Groups I, II and III, where animals were immunized with various dilutions (1:5, 1:10 and 1:20, respectively) of the International Reference Standard (IRS) and challenged with 1.0 ml of tetanus toxin,

a cumulative survival of 26, 11 and 04 and cumulative deaths of 01, 10 and 22 were observed, resulting in mortality ratios of 0.03, 0.47 and 0.84, respectively.

In the Groups IV, V & VI, VII, VIII & IX and X, XI & XII, where animals were immunized with each dilution (1:6.8, 1:15.6 and 1:31.2) of the DTP, DT and TT vaccine and challenged with 1.0 ml of tetanus toxin, the cumulative survival was found to be 35, 20 & 07, 34, 19 & 07 and 35, 19 & 06 and cumulative deaths were 00, 03 & 12, 01, 05 & 14 and 00, 03 & 13, resulting in mortality ratios of 0, 0.13 & 0.63, 0.02, 0.20 & 0.66 and 0.03, 0.13 & 0.0, 0.06, 0.13 & 0.68, respectively (Table **3**). By using Probit Analysis, potency of DTP, DT & TT vaccine was found to be 64.93, 56.83 & 61.25 IU/Dose, respectively.

Table 2. Effect of various dilutions of vaccine samples on survival, mortality and mortality ratios following diphtheria challenge for LD_{50} determination.

Group	Dilution of Toxin	LD_{50}/ml	Observations (Mortality)					Survival	Mortality	Mortality Ratio			% Mortality
			1st Day	2nd Day	3rd Day	4th Day	5th Day			CS	CD	MR	
I	1:18	100	2	-	-	-	-	0	2	0	7	1	100
II	1:900	2	-	1	-	1	-	0	2	0	3	1	100
III	1:1800	1	-	-	-	-	1	1	1	1	1	0.5	50
IV	1:3600	0.5	-	-	-	-	-	2	0	3	0	0	00

n= 2 per group

CS *- Cumulative Survival;* ***CD*** *- Cumulative Death;* ***MR*** *- Mortality Ratio*

Table 3. Effect of various dilutions of vaccine samples on survival, mortality and mortality ratios following tetanus challenge method.

Product	Group	Dilution	Observations (Mortality)					Survival	Mortality	Mortality Ratio		
			1st Day	2nd Day	3rd Day	4th Day	5th Day			CS	CD	MR
IRS	I	1:5	-	1	-	-	-	15	01	26	01	0.03
	II	1:10	5	2	1	1	-	07	09	11	10	0.47
	III	1:20	7	1	2	2	-	04	12	04	22	0.84
DTP	IV	1:6.8	-	-	-	-	-	16	00	36	00	0.00
	V	1:15.6	1	1	1	-	-	13	03	20	03	0.13
	VI	1:31.2	3	2	2	2	-	07	09	07	12	0.63
DT	VII	1:6.8	1	-	-	-	-	15	01	34	01	0.02
	VIII	1:15.6	1	2	1	-	-	12	04	19	05	0.20
	IX	1:31.2	2	2	4	1	-	07	09	07	14	0.66
TT	X	1:6.8	-	-	-	-	-	16	00	35	00	0.00
	XI	1:15.6	-	2	1	-	-	13	03	19	03	0.13
	XII	1:31.2	4	3	3	-	-	06	10	06	13	0.68

n=16 per group

IRS *- International Reference Standard;* ***DTP*** *- Diphtheria Tetanus and Pertussis Vaccine;* ***DT*** *- Diphtheria and Tetanus Vaccine;* ***TT****- Tetanus Toxoid;* ***CS*** *- Cumulative Survival;* ***CD*** *- Cumulative Death;* ***MR*** *- Mortality Ratio*

LD_{50} of Challenge Toxin: Amongst the Groups I, II, III and IV, where animals were challenged with various dilutions of the tetanus toxin (1:3250, 1:162500, 1:325000 and 1:650000), a cumulative survival of 00, 00, 02 and 10 and cumulative deaths of 30, 14, 06 and 00 was observed, resulting in mortality ratios of 1.00, 1.00, 0.75 and 0.00, respectively. Percentage mortality was found to be 100, 100, 75 and 0 for Groups I, II, III and IV, respectively (Table **4**). The LD_{50} was found to be 2.44466×10^{-6}.

Table 4. Effect of various dilutions of vaccine samples on survival, mortality and mortality ratios following tetanus challenge for LD_{50} determination.

Group	Dilution of Toxin	LD_{50}/ml	Observations (Mortality)					Survival	Mortality	Mortality Ratio			% Mortality
			1st Day	2nd Day	3rd Day	4th Day	5th Day			CS	CD	MR	
I	1:3250	200	8	-	-	-	-	0	8	0	30	1	100
II	1:162500	4	-	1	7	-	-	0	8	0	14	1	100
III	1:325000	2	-	1	3	2	-	2	6	2	6	0.75	75
IV	1:650000	1	-	-	-	-	-	8	0	10	0	0	0

n = 8 per group

CS *- Cumulative Survival;* ***CD*** *- Cumulative Death;* ***MR*** *- Mortality Ratio*

Potency Testing of Pertussis Component by Lethal Challenge Method: In the Groups I, II and III, animals were immunized with various dilutions of the National Reference Standard (NRS), namely 1:4, 1:20 and 1:100, respectively and challenged with 10×10^4 Org./0.03ml of *B. pertussis* strain. Animals in groups I, II and III, showed a cumulative survival of 30, 14 and 04 and cumulative deaths of 04, 14 and 30, resulting in mortality ratios of 0.11, 0.5 and 0.88, respectively.

In the Groups IV, V & VI, VII, VIII & IX and X, XI & XII, where animals were immunized with each dilution (1:5, 1:25 and 1:125) of the DTP, Quadruple vaccine (DTP/IPV) and Pentavalent vaccine (DTP/IPV/Hib) and challenged with 10×10^4 Org./0.03ml of *B. pertussis* strain, the cumulative survival was found to be 30, 13 & 04, 30, 14 & 04 and 30, 14 & 05 and cumulative deaths were 03, 12 & 27, 03, 13 & 29 and 04, 14 & 29, resulting in mortality ratios of 0.09, 0.48 & 0.87, 0.09, 0.48 & 0.87 and 0.11, 0.5 & 0.85, respectively. By using Probit Analysis, potency of DTP, Quadruple (DTP/IPV) and Pentavalent vaccine (DTP/IPV/Hib) was found to be 5.43, 5.25 & 5.29 IU/Dose, respectively. (Table **5**)

LD_{50} of Challenge Toxin: In the Groups I, II, III, IV and V, where animals were challenged with various number of organisms per dose of the *B. pertussis* strain (100000, 10000, 2000, 400 and 80 organisms/dose), they showed a cumulative survival of 00, 00, 01, 03 and 10 and cumulative deaths of 40, 30, 20, 11 and 03, resulting in mortality ratios of 1.00, 1.00, 0.9524, 0.7857 and 0.231, respectively. The percentage mortality was found to be 100, 100, 95.24, 78.57 and 23.1 for groups I to V, respectively (Table **6**). The LD_{50} was found to be 174.582.

Table 5. Effect of various dilutions of vaccine samples on survival, mortality and mortality ratios following pertussis challenge method.

Product	Group	Dilution	Non-Specific Mortality		Observations (Specific Mortality)												Survival	Mortality	Mortality Ratio		
			1	2	3	4	5	6	7	8	9	10	11	12	13	14			CS	CM	MR
NRS	I	1:4	-	-	-	2	1	-	112	-	-	-	-	-	-	-	16	04	30	04	0.11
	II	1:20	-	-	-	2	3	3		1	-	-	-	-	-	-	10	10	14	14	0.50
	III	1:100	-	-	-	4	5	3		2	-	-	-	-	-	-	04	16	04	30	0.88
DTP	IV	1:5	-	-	-	2	-	1	-	-	-	-	-	-	-	-	17	03	30	03	0.09
	V	1:25	-	1	-	2	3	2	1	1	1	-	-	-	-	-	09	09	13	12	0.48
	VI	1:125	-	-	-	5	4	2	2	1	-	-	-	-	-	-	04	15	04	27	0.87
Quadruple (DTP/IPV)	VII	1:5	-	-	1	1	1	-	1	-	-	-	-	-	-	-	16	03	30	03	0.09
	VIII	1:25	-	-	-	3	2	3	-	22	-	-	-	-	-	-	10	10	14	13	0.48
	IX	1:125	-	-	-	5	4	4	1		-	-	-	-	-	-	04	16	04	29	0.87
Pentavalent (DTP/IPV/ Hib)	X	1:5	-	-	-	136	1	1	-	1	-	-	-	-	-	-	16	04	30	04	0.11
	XI	1:25	1	-	-		3	21	11	1	-	-	-	-	-	-	09	10	14	14	0.5
	XII	1:125	-	1	-		6			1	-	-	-	-	-	-	05	15	05	29	0.85

n= 2 per group; **NRS-** National Reference Standard; **DTP-** *Diphtheria Tetanus* and *Pertussis*; **IPV-** Inactivated Polio Vaccine; **Hib-** *Haemophillus Influenza* type b; **CS-** Cumulative Survival; **CD-** Cumulative Death; **MR-** Mortality Ratio.

Table 6. Effect of various dilutions of vaccine samples on survival, mortality and mortality ratios following pertussis challenge for LD_{50} determination.

Group	Organisms/ dose	Non Specific Mortality		Observations (Specific Mortality)												Survival	Mortality	Mortality Ratio			% Mortality
		1	2	3	4	5	6	7	8	9	10	11	12	13	14			CS	CM	MR	
I	100000	-	-	-	1	4	3	1	-	1	-	-	-	-	-	00	10	00	40	1	100
II	10,000	-	-	-	1	2	3	2	1	-	1	-	-	-	-	00	10	00	30	1	100
III	2,000	-	-	-	2	1	2	1	2	1	-	-	-	-	-	01	09	01	20	0.95	95.24
IV	400	-	-	-	-	1	1	2	1	1	1	-	1	-	-	02	08	03	11	0.79	78.57
V	80	-	-	-	-	-	1	1	-	-	1	-	-	-	-	07	03	10	03	0.23	23.1

n= 10 per group.

Antibody Induction Method (alternative method) Potency Testing of Diphtheria Component by Antibody Induction Method:

Effect of different mixtures of toxin and standard diphtheria antitoxin on local reaction in antibody induction method: The dilution of the toxin which produces a positive Schick type reaction (15×15 mm) is taken as the Lr/100 test dose of diphtheria antitoxin. Lr/100 is the minimum amount of toxin which when combined with 0.01 I.U. of standard antitoxin in a volume of 0.2 ml causes a local skin reaction that is just visible.

The animals in Group I, II, III, IV and V, were tested for unitage of standard diphtheria antitoxin as 0.012, 0.011, 0.010, 0.009 and 0.008, by inoculating different mixtures of toxin and antitoxin (0.000126 and 0.012; 0.000126 and 0.011; 0.000126 and 0.010; 0.000126 and 0.009; and 0.000126 and 0.008). The local reaction was measured after 48 hours and was found to be nil, 5×5 mm, 12×14 mm, 18×18 mm and 22×22 mm, respectively. The Lr/100 dose of standard diphtheria antitoxin produced Schick type reaction (12×14 mm) at 0.01 IU/ml and this was taken as standard value (Table **7**).

Table 7. Effect of different toxin mixtures and standard diphtheria antitoxin on local reaction in antibody induction method in animals.

Group	Unitage Tested (IU/ml)	Dose injected in each spot			Local reaction measured in mm after 48 hours (in mm)
		Toxin (ml)	Antitoxin Unit (ml)	Volume (ml)	
I	0.012	0.000126	0.012	0.2	--
II	0.011	0.000126	0.011	0.2	5 × 5
III	0.010	0.000126	0.010	0.2	12 ×14
IV	0.009	0.000126	0.009	0.2	18 × 18
V	0.008	0.000126	0.008	0.2	22 × 22

n= 1 per group

Effect of different mixtures of toxin and diphtheria antitoxin samples on local reaction in antibody induction method: For animals in Groups I to X, the diphtheria antitoxin content in serum samples was determined at four different unitages, namely 1, 2, 3 & 4 IU/ml, by inoculating mixtures of toxin and antitoxin (0.000126 and 0.01) at different sites not less than 2.5 cm apart. The local reactions were observed after 48 hours and were compared with standard, which developed a local reaction measuring 12×14 mm; and the diphtheria antitoxin content was found to be 3, 4, 4, 2, 4, 4, 3, 4, 3 and 4IU/ml, respectively. The geometric mean of 10 guinea pig sera was found to be 3.4 IU/ml (Table **8**). As per the requirements of IP 2007, a test vaccine passes the potency assay of diphtheria component if the geometric mean of 10 guinea pigs sera is ≥ 2 IU of Diphtheria antitoxin per ml. Thus the test vaccine under consideration meets the requirements of IP, 2007 and passes the potency assay of diphtheria component.

Potency Testing of Tetanus Component by Antibody Induction Method:

Effect of different mixtures of toxin and standard tetanus antitoxin on tetanic reactions in antibody induction method: The minimum dose of toxin, which kills 50% of the test animals showing symptoms of tetanic paralysis/death, is taken as the L+/10 dose of the toxin. L+/10 is the minimum amount of toxin which when combined with 1 I.U. of antitoxin kills 50% of test animals of a defined weight in 5 days**.**

In Groups I, II, III, IV and V animals were tested for unitage of standard tetanus antitoxin as 0.12, 0.11, 0.10, 0.09 and 0.08, by inoculating different mixtures of toxin and Standard Tetanus Antitoxin (0.12 and 0.5; 0.11 and 0.5; 0.10 and 0.5; 0.09 and 0.5; 0.08 and 0.5). All the animals in Groups I and II were found to be alive and healthy with no titanic symptoms; whereas in Group III two animals were alive and healthy; one animal suffered from moderate and the other from severe titanic symptoms and the remaining two animals died at the end of 4th or 5th day of observation. In the Group IV all the animals died on the 3rd or 4th day and in a matter of 2 days in Group V. The L+/10 dose of standard tetanus antitoxin kills 50% of test animals at 0.1 IU/ml and this was taken as standard value (Table **9**).

Table 8. Effect of different mixtures of toxin and diphtheria antitoxin on local reaction in antibody induction method in animals.

Group	Unitage tested For (IU/ml)	Dose injected in each spot			Local reaction measured (in mm) after 48 hours	Content of Diphtheria toxin (IU)
		Toxin (ml)	Antitoxin Unit (ml)	Volume (ml)		
I	a = 1 units b = 2units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = 8×8 d = 20×20	3 IU
II	a = 1 units b = 2units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = nil d = nil	4 IU
III	a = 1 units b = 2units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = 5×5 d = 8×5	4 IU
IV	a = 1 units b = 2units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = 18×18 d = Necrosis	2 IU
V	a = 1 units b = 2units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = nil d = nil	4 IU
VI	a = 1 units b = 2units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = nil d= 8×8	4 IU
VII	a = 1 units b = 2units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = 10×10 d = 20×18	3 IU
VIII	a = 1 units b = 2units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = nil d = nil	4 IU
IX	a = 1 units b = 2 units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = 12×12 d = Necrosis	3 IU
X	a = 1 units b = 2 units c = 3 units d = 4 units	0.000126	0.01	0.2	a = nil b = nil c = nil d = 5×5	4 IU
Local reaction measured for standard (in mm) after 48 hours = 12x14						

Table 9. Effect of different mixtures of toxin and standard tetanus antitoxin on tetanic reactions in antibody induction method in animals.

Group	Unitage Tested (IU/ml)	Dose injected in each animal			Observation				
		Toxin (ml)	Antitoxin Unit (ml)	Volume (ml)	1	2	3	4	5
I	0.12	0.0047	0.12	0.5	L	L	L	L	L
					L	L	L	L	L
					L	L	L	L	L
					L	L	L	L	L
					L	L	L	L	L
					L	L	L	L	L
II	0.11	0.0047	0.11	0.5	L	L	L	L	L
					L	L	L	L	L
					L	L	L	L	L
					L	L	L	L	L
					L	L	L	L	L
					L	L	L	L	L
III	0.10	0.0047	0.10	0.5	L	L	L	L	L
					L	L	L	L	L
					L	L	t	t	tt
					L	L	t	tt	ttt
					L	L	t	tt	D
					L	L	tt	D	-
IV	0.09	0.0047	0.09	0.5	L	t	ttt	D	-
					L	t	ttt	D	-
					L	tt	ttt	D	-
					L	tt	ttt	D	-
					L	tt	D	-	-
					L	ttt	D	-	-
V	0.08	0.0047	0.08	0.5	L	D	-	-	-
					t	D	-	-	-
					t	D	-	-	-
					t	D	-	-	-
					t	D	-	-	-
					t	D	-	-	-

n= 6 per group; **L-** Live & Healthy; **t -** mild tetanus; **tt -** moderate tetanus; **ttt -** severe tetanus; **D -** death.

Effect of different mixtures of toxin and tetanus antitoxin (from different samples) on tetanic reactions in antibody induction method: Animals in Groups I, II, III, IV, V, VI, VII, VIII to IX, inoculated subcutaneously with a dose of 0.5 ml of each of mixture of Toxin- Serum samples (serum sample numbers 1, 2, 3, 4, 5, 6, 7, 8 and 9, respectively) were found to be alive and healthy and showed no tetanus symptoms during the observation period of 5 days. Thus the tetanus antitoxin content in serum was found to be 0.5 IU/ml (Table **10**).

Table 10. Effect of different mixtures of toxin and tetanus antitoxin (from different samples) on tetanic reactions in antibody induction method in animals.

Serum Sample No.	Group	Unitage Tested (IU/ml)	Dose injected in each animal			Observations				
			Toxin (ml)	Antitoxin Unit (ml)	Volume (ml)	1	2	3	4	5
1	I	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
2	II	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
3	III	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
4	IV	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
5	V	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
6	VI	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
7	VII	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
8	VIII	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
9	IX	0.5	0.0047	0.10	0.5	L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L
						L	L	L	L	L

n= 6 per group; **L-** Live & Healthy: **t -** mild tetanus; **tt -** moderate tetanus; **ttt -** severe tetanus, **D -** death.

Thus the results revealed that serum of nine guinea pigs contained not less than 0.5 IU of tetanus-antitoxin per ml. Hence, the test sample met the requirements of IP, 2007.

Potency assay of DTP group of vaccines shows the best result in terms of linearity, accuracy, precision and quality rather than the previous methods of production.[21] All DTP group of vaccines were prepared by using strains of *C. diphtheria*, *C. tetani* and *B. pertussis* and grown on media containing proteinaceous material of vegetative origin such as proteins from soy beans, cotton seeds, potatoes, etc. for 38- 48 hours.[9] When optimal level of toxin was reached it was harvested and stored at 37°C for 4-6 weeks with formalin (0.6%) for complete detoxification of toxin. Crude toxoid obtained was concentrated and purified and final product was obtained.[5] Nutrients of vegetative origin are substitute for media containing nutrients of animal origin (casein digest or meat extract) and support growth of the pathogenic bacteria. This media completely removes the risk of animal peptides derived contamination, such as Bovine Spongiform Encephalopathy (BSE), microbial contamination and allergic reactions, being transmitted into humans in any subsequent therapeutic or prophylactic applications.

The potency of diphtheria and tetanus component was

estimated by subcutaneous lethal challenge method[10] and of pertussis component by intracerebral lethal challenge method.[11-13] These methods are more accurate and economical than previous method, intradermal challenge method in which at large number of animals were required and involved multiple intradermal challenges on the depilated skin of animals, which causes severe pain to the animals. Potencies of the test samples were calculated to be 98.01, 73.39 & 90.65 IU/Dose and 64.93, 56.83 & 61.25 IU/Dose for diphtheria and tetanus components, which were well above the permissible lower limit which is 30 and 40 IU/SHD. As per IP 2007, a test sample passes the potency test for diphtheria and tetanus component (by lethal challenge method) if it contains not less than 30 and 40 IU/SHD. Thus, the vaccines were found to pass the test. Potencies of test samples for pertussis component were calculated to be 5.43, 5.25 & 5.29 IU/SHD, which were above the minimum permissible limit (more than 4 IU/SHD). As per the requirement set by IP, 2007, a vaccine that contains not less than 4IU/SHD of pertussis component shall pass the test for potency.[3] The vaccines thus passed the test for potency of pertussis component.

An alternative method antibody induction method for diphtheria and tetanus components was carried out in which of tetanus and diphtheria antibodies were estimated in the individual guinea pigs sera by toxin neutralization test (*in-vivo*) is recommended by IP, 2007.[3,18] As per IP, 2007 requirements, the test vaccine passes the potency test of Diphtheria component, according to which the geometric mean of 10 immunized guinea pigs sera must be $\geq$ 2 IU/ml. The geometric mean of ten immunized guinea pigs for the test vaccines was calculated to be 3.5 IU of diphtheria antitoxin/ml, which thus served the criteria set by IP Thus the vaccine samples passed the test for the potency of diphtheria component. As per the specifications mentioned in IP 2007 the content of tetanus- antitoxin should not be less than 0.5 IU/ml.[3] The study revealed a content of 0.5 IU of tetanus-antitoxin per ml in the serum of nine guinea pigs. As the samples had a concentration above the minimum level specified, they passed the test (as per the requirements of IP, 2007).

CONCLUSION

In the present study the potency tests, which were used for the quality control of vaccines, are more economical, simpler and show the best result in terms of linearity, accuracy, precision and quality for the vaccines rather than the other previous methods. After analyzing the tests employed in the present project, the potency was found to be well above the acceptable limit as per the requirements of IP, 2007. The results obtained thus fulfilled the requirements for safe and efficacious vaccines which can be used in vaccination programs or therapeutic treatments.

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