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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Safety evaluation in mice of the childhood immunization vaccines from two south-eastern states of Nigeria

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PEER REVIEW

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Comments
The study evaluated the vaccines that are utilized in routine immunization program in Imo and Abia States, Nigeria. The methods employed to test the vaccines in mice are body weight change test, leukopenic promoting toxicity test, and leukopenic toxicity tests in mice before and after immunization of the mice.
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ABSTRACT

Objective: To check the effects of the vaccines on the hematopoietic system and weight of mice after immunization.

Methods: The study was done with the Expanded Programme on Immunization vaccines donated by the Ministries of Health of Abia and Imo States of Nigeria. The vaccines were collected from the cold-chain stores and transported in vaccine carriers to the cold-chain facility in Nnamdi Azikiwe University Teaching Hospital within 3 hours of collection. They were used to immunize a total of 160 mice. The Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi of Anambra State, Nigeria approved the protocol.

Results: Mice body weight changes test showed that the mice all had increased body weight at Days 3 and 7 post-immunization and none died during the 7 d post-immunization observation. The percentage weight gains of the mice compared with the control were 69%, 70%, 64%, 63%, 65% and 68% for oral polio vaccine, diphtheria-pertussis-tetanus, bacillus Calmette–Guérin, measles, yellow fever and hepatitis B vaccines respectively collected from Imo State. The mice immunized with oral polio vaccine, pentavalent, bacillus Calmette–Guérin, measles, yellow fever and hepatitis B vaccines collected from Abia State had 123%, 114%, 121%, 116%, 142% and 119% weight gain respectively compared with the control. Leukocytosis promoting toxicity test showed that none of the vaccines was able to induce proliferation of leukocytes up to ten folds. Leukopenic toxicity test showed that all the vaccines had an leukopenic toxicity test value higher than 80% of the control (physiological saline).

Conclusions: The vaccine samples tested were safe and did not affect the hematopoietic system adversely. The storage conditions of the vaccines in the States' cold-chain stores had not compromised the safety of the vaccines.

KEYWORDS
Safety evaluation, Vaccines, Routine immunization, Imo and Abia, Nigeria

1. Introduction

Vaccine safety relates to the adverse event following immunization with the vaccine[1], and the most common cause of vaccine-related adverse events is human error which stems from logistics problems. Hence, all attempts should be taken to retain the safety

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Article history:
Received 1 Sep 2014
Received in revised form 9 Sep, 2nd revised form 11 Sep 2014
Accepted 16 Oct 2014
Available online 29 Oct 2014

of the vaccine. Allergic reactions to vaccine components, although rare, are important issue to consider[2]. Safety is an obligatory requirement for a vaccine, be it human or animal. Adverse effects can have a strong negative effect on immunization coverage, as the benefits of vaccination may not be directly clear for individual vaccinees, but adverse effects are immediately apparent. Vaccine safety is maintained by developing consistent production processes, extensive safety testing and post-marketing surveillance. Safety tests are also done to prevent more serious adverse events. Potential safety problems of attenuated vaccines are reversion to wild type. The potential risk of inactivated vaccines is that they are not killed or detoxified completely[3]. Vaccine safety is a prime concern for the public, manufacturers, immunization providers and recipients of vaccines[4].

Just like other pharmaceutical products, no vaccine is completely safe, which is being prone to adverse effects. This safety question is heightened when the vaccines poorly stored. Being biological products, they deteriorate faster when are poorly stored and so become unsafe for use in immunization programme. While almost all known vaccine adverse events are minor and self-limited, some vaccines have been associated with very rare but serious health effects[5-7].

The study sets to determine how safe the childhood immunization vaccines in use in Abia and Imo States of Nigeria are.

2. Materials and methods

2.1. Animals used and method of identification

This study used 70 albino mice grouped randomly into 7 with each group containing 10 mice. The commonly used methods for labeling the animals used in biomedical research are temporary identification method and permanent identification method[8,9]. Temporary identification methods (employed in this study) involved the use of permanent markers to mark the tail, fur or skin of the animals. The marks were renewed every 2 days. Permanent identification method includes tattooing, use of ear punches, microchip transponders and ear tags. The temporary identification method was used in this study and the marks were renewed every 2 days. All animals [(30±5) days old] were treated in accordance with the guidelines to promote the wellbeing of animals used for scientific purposes[10,11].

2.2. Determination of the toxicity and safety of the vaccines

The toxicity and safety of the vaccines were determined using modified Haruka *et al.*[12] method and described under the individual tests. Modifications were done by the researchers.

2.2.1. Animal body weight changes test

This is a general safety test for vaccines. A volume of one human dose of test vaccine was injected into the peritoneum [except for oral polio vaccine (OPV) which was by oral route] of each of the 10 mice used in this study and observed daily for 7 d. Vaccines were considered non-toxic if they meet the following requirements[13-16]: (a) the total weight of the mice 3 d post-vaccination is equal to or more than the initial weight, (b) at the end of 7 d post-vaccination, the average weight gain per mouse in the vaccine group was not less than 60% of the control group, and (c) not more than 5% of the animals died during the test period and none should show any sign of ill health within the 7 d.

2.2.2. Leukocytosis promoting toxicity test using experimental animal

This tests whether or not the vaccine is toxic to the hematopoietic system. A volume of one human dose of test vaccine is injected into the peritoneum (except for OPV which was by oral route) of 10 mice. Before the immunization and 3 d after, 20 μL blood samples of the mice were collected in sterile Eppendoff tube using heparinized capillary tube inserted just below the eye ball, diluted with Turk's solution (380 μL) and leukocytes [white blood cells (WBC)] count done using hemocytometer. The standard criterion of safety is that the mean WBC count in peripheral blood 3 d after injection should not be greater than 10 times before injection[12,17].

2.2.3. Leukopenic toxicity test in vaccinated animals

This also tests the toxicity of the vaccines to the hematopoietic system using the general safety test and the leukopenic toxicity test (LTT). Eighty mice were selected and grouped into A to H with each group containing 10 mice. Groups A to F were given a volume of one human dose of bacillus Calmette–Guérin (BCG), OPV, hepatitis B vaccine (HBV), measles vaccine (MV), yellow fever vaccine (YFV) and diphtheria-pertussis-tetanus (DPT) or pentavalent vaccine respectively by intra-peritoneum (except for OPV which was by oral route). Group G was given cyclophosphamide (400 mg/kg)[18] by gavage to serve as positive control while Group H was given 500 μL of normal saline to serve as negative control. Cyclophosphamide for injection was reconstituted with the 0.9% sodium chloride injection to make a stock solution of 20 mg/L (Biochem Pharmaceutical Industries, India–Lot #: KB116010). After 18 h, 20 μL blood samples of the mice were collected in sterile Eppendoff tube using heparinized capillary tube inserted just below the eye ball, diluted with Turk's solution (380 μL) and leukocytes count done using hemocytometer. The vaccine is considered safe if it shows a leukopenic toxicity value greater than or equal to 80% of the leukopenic toxicity of the control[12,14,19].

$$\text{Leukopenic toxicity value } (LT_{value}) = \frac{\text{WBC count (test or control)}}{\text{WBC count (toxic substance)}}$$

2.3. Ethical issues

The work described in this article was approved by the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi (Approval #: NAUTH/CS/66/Vol.4/220)

2.4. Statistical analysis

The results were analyzed using GraphPad Prism[20]. The statistical tests used were: unpaired student's *t*-test, analysis of variance (One-way and Two-way ANOVA), Dunnett's test of multiple comparison and Bartlett's test for equal variances. All *P* values reported are for a one-tailed test. $P<0.05$ was considered significantly different.

3. Results

All the mice used (or immunized) were observed for 7 d, but none showed any abnormality, signs of ill health or died within the period. There was increase in the mean body weight in all the animals as seen in Table 1.

Table 1
Animal body weight changes test for vaccines from Imo State.

Vaccines	Day after vaccination	Mice body weight (g)			Weight gain compared to control (%)	*P* value
		Total weight	Mean weight (n=10)	Mean weight gain		
OPV	0	146.40	14.64	0.00		
	3	156.00	15.60	0.96		0.2212
	7	211.60	21.16	6.52	69.35	<0.0001*
DPT	0	165.30	16.53	0.00		
	3	177.40	17.74	1.21		0.1667
	7	230.70	23.07	6.54	69.87	<0.0001*
BCG	0	202.30	20.23	0.00		
	3	215.00	21.50	1.27		0.2144
	7	265.40	26.54	6.31	63.90	0.0013*
Measles	0	169.20	16.92	0.00		
	3	178.90	17.89	0.97		0.1863
	7	231.80	23.18	6.26	62.90	<0.0001*
YFV	0	177.50	17.75	0.00		
	3	193.00	19.30	1.55		0.1870
	7	241.00	24.10	6.35	64.94	0.0007*
HBV	0	168.10	16.81	0.00		
	3	182.00	18.20	1.39		0.1270
	7	232.80	23.28	6.47	68.05	<0.0001*
Control NS	0	188.00	18.80	0.00		
	3	198.40	19.84	1.04		0.2865
	7	226.50	22.65	3.85		0.0244*

Control NS: Control using 0.9% normal saline. Day 0 means before vaccination. *P<0.05. P value is for student's t-test.

Comparing the mean body weight due to the vaccines with their initial weights using the student's t-test, it showed that at 3 d post-vaccination, all the mice immunized had non-significant weight gain (P>0.05). All the vaccines passed the compendia and World Health Organization recommendations for abnormal toxicity test of 3 d post-vaccination.

At 7 d post-vaccination, all the mice had significant weight gain (P<0.05) compared with their initial weights, and the % weight gains (of the animals immunized with BCG, measles and yellow fever vaccines) were barely greater than 60% compared to mean weight gain of control. These batches of the vaccines, although passed the test, must be consumed soonest so that their safety will not be compromised.

Two-way ANOVA of the results of the animal body weight changes test showed that both the vaccine type and post-vaccination day significantly affected the weight of the animals by 13.28% and 36.43% respectively (P<0.0001). However, the vaccine type did not have the significant effect at all values affected by post-vaccination day (P=0.9885) as the interaction of the two treatments only accounted for 0.95% of the total variance.

The leukocytosis promoting toxicity test showed that none of the vaccines produced up to 10-fold increase of the WBC in the animals (Table 2). This compared well with the control. Most of the vaccine, except BCG, produced approximately 3-fold increase in leukocyte count 3 d post-vaccination–similar to the physiological saline used as control. The BCG vaccines, like the physiological saline used as control, produced a 2-fold leukocyte increase. A t-test analysis showed that the increase in leukocyte count produced by the test vaccines and the control were all significant (P<0.05). The Dunnett's multiple comparison test showed non-significant difference in the effects of the vaccines when compared with the control.

Table 2
Leukocytosis promoting toxicity test for vaccines from Imo State.

Vaccines	Day of vaccination	Leukocyte count ($\times 10^9$/L)		WBC increased by X folds	*P* value
		Total WBC	Mean WBC (n=10)		
OPV	0	49.55	4.955	3.02	<0.0001*
	3	149.55	14.955		
DPT	0	35.65	3.565	2.71	<0.0001*
	3	96.90	9.690		
BCG	0	65.65	6.565	2.36	0.0009*
	3	155.15	15.515		
Measles	0	54.90	5.490	2.68	<0.0001*
	3	147.40	14.740		
YFV	0	54.35	5.435	2.75	<0.0001*
	3	149.65	14.965		
HBV	0	47.05	4.705	3.13	<0.0001*
	3	147.25	14.725		
Control NS	0	61.25	6.125	2.36	<0.0001*
	3	144.55	14.455		

Day 0 means before vaccination. Control NS: Control using 0.9% normal saline. *P<0.05. P value is for student's t-test.

A Two-way ANOVA of the results also showed that both the vaccine type and post-vaccination day significantly affected the leukocyte count by 5.75% and 65.78% respectively (P=0.0004, P<0.0001 respectively) (Table 2). The vaccine type did not have the significant effect at all values affected by post-vaccination day–the interaction accounting for only 1.31% of the total variations. This meant that the interactions of the two treatments did not significantly affect the leukocyte count (P=0.4192).

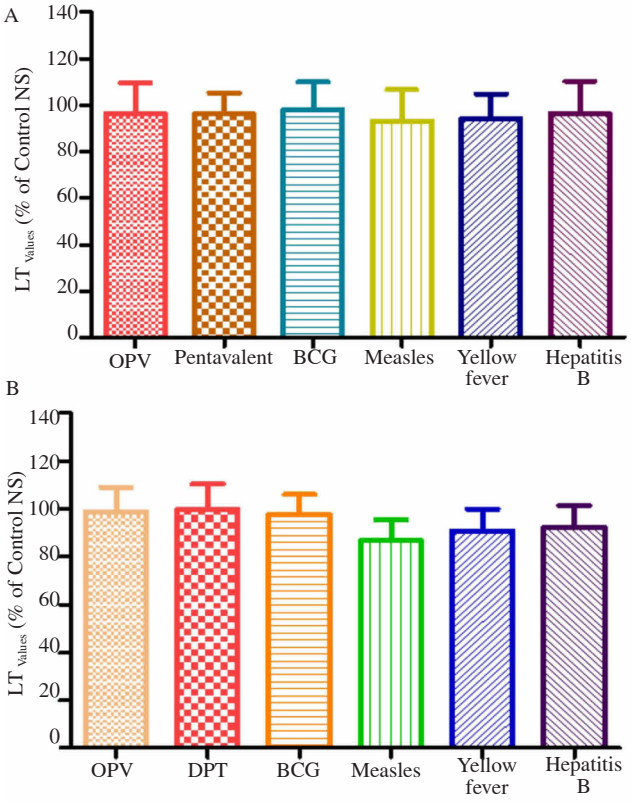

Figure 1. Leukopenic test for vaccines from Abia (A) and Imo (B) States.

The LTT showed that the vaccines and the 0.9% sodium chloride had the LT_{values} of 2.703, 2.778, 2.707, 2.343, 2.498, 2.578 and 2.832 respectively in the mice used. The LT_{value} of each of the vaccine was higher than the 80% LT_{value} of 0.9% sodium chloride (Figure 1A). One-way ANOVA of the mean leukocyte counts showed significant difference (P<0.000 1) between the cyclophosphamide and the vaccines. Bartlett's test for equal variances also showed significant difference (P=0.002 5).

Dunnett's multiple comparison test showed significant differences in the mean leukocyte count due to the cyclophosphamide compared with those due to the vaccines and the sodium chloride (P<0.05). However, a comparison of the counts due to the sodium chloride with those due to the vaccines showed no significant difference for the vaccines (P>0.05) and the Bartlett's test for equal variances revealed that the variances were different (P=0.023 6).

One-way analysis of the results of the LTT showed that the leukocyte count was significantly affected by all the treatments (P<0.000 1)–the vaccines, the normal saline and the cyclophosphamide. The Bartlett's test for equal variances also showed that the variances were significant difference (P=0.003 2). Dunnett's multiple comparison test showed that the effects of the vaccine type on the leukocyte count were significantly different compared with the effect of the leukopenic agent-cyclophosphamide. Nevertheless, the vaccines effects on the leukocyte count did not differ significantly, showing that the vaccines were as safe as the physiological saline (P=0.080 1).

Table 3

Animal body weight changes test for vaccines from Abia State.

Vaccines	Day after Vaccination	Mice body weight (g)			Weight gain compared to control (%)	P value
		Total weight	Mean weight (n=10)	Mean weight gain		
OPV	0	195.30	19.53	0.00		
	3	211.20	21.12	1.59		0.081 4
	7	255.10	25.51	5.98	123.13	<0.000 1*
Pentavalent	0	194.50	19.45	0.00		
	3	195.70	19.57	0.12		0.445 3
	7	252.10	25.21	5.76	114.93	<0.000 1*
BCG	0	171.80	17.18	0.00		
	3	178.80	17.88	0.70		0.230 8
	7	231.00	23.10	5.92	120.90	<0.000 1*
Measles	0	173.70	17.37	0.00		
	3	179.80	17.98	0.61		0.211 5
	7	231.70	23.17	5.80	116.42	<0.000 1*
YFV	0	186.10	18.61	0.00		
	3	188.80	18.88	0.27		0.374 3
	7	251.00	25.10	6.49	142.16	<0.000 1*
HBV	0	163.80	16.38	0.00		
	3	173.20	17.32	0.94		0.050 0
	7	222.40	22.24	5.86	118.66	0.000 4*
Control	0	166.40	16.64	0.00		
	3	174.00	17.40	0.76		0.088 7
	7	193.20	19.32	2.68		<0.000 1*

Day 0 means before vaccination. *P<0.05. P value is for student's t-test.

None of the animals used in the test died or showed any sign of ill health throughout the 7 d post-vaccination observation. None showed weight loss. Comparing the mean body weights at pre- and post-vaccination days, Table 3 shows that there was generally non-significant weight gain in all the animals immunized with the vaccines under study on 3 d post-vaccination, but there were significant weight gains in all the mice on 7 d post-vaccination (P<0.05). A comparison of the variances in the mean body weight also at the pre- and post-vaccination days showed no significant difference (P>0.05). All the vaccines produced more than 100% weight gain on 7 d post-vaccination in the immunized mice compared to the control. This was an excellent result compared to the vaccines procured from the previous statement.

The vaccines were all still within the acceptable safety as shown by the test. Two-way ANOVA of the result of animal body weight changes test showed that the animals' weight was significantly affected by both the vaccine type and the post-vaccination period (P<0.000 1) but not the interactions of the two (P<0.093 8). The vaccine type affected the weight by 17.18%, the post-vaccination period caused 50.2% of the changes while the interactions of the two accounted for 3.01% of the total variance.

The Two-way ANOVA of the result of the leukocytosis promoting test showed that the leukocyte count was significantly affected by the vaccine type, post-vaccination period and the interactions of the two. Vaccine type accounted for 8.94% (P<0.000 1), post-vaccination period accounted for 49.19% (P<0.000 1) while the interactions of the two accounted for 5.51% (P=0.000 61) of the variations in the leukocyte count (Table 4).

The Table 4 shows the leukocytosis promoting toxicity test for the vaccines under study as compared with 0.9% sodium chloride. The increase in the leukocyte count produced by the vaccines was very high comparable with that of the sodium chloride as none increased the count by 10-folds. The highest increase was by 2-folds which were produced by the OPV, measles and yellow fever vaccines. A t-test analysis to compare the effects produced by the individual vaccine with leukocyte counts before the vaccine administration showed significant differences at pre- and post-vaccination periods (P<0.05). Dunnett's multiple comparison test to compare the effects of the vaccines with the 0.9% sodium chloride showed no significant difference (P>0.05). Therefore, the increases in the leukocyte produced by the vaccines were comparable to that produced by 0.9% sodium chloride.

Table 4

Leukocytosis promoting toxicity test for vaccines from Abia State.

Vaccines	Day after vaccination	Leukocyte count ($\times 10^9$/L)		WBC increased by X folds	P value
		Total WBC	Mean WBC (n=10)		
OPV	0	55.65	5.565		
	3	118.15	11.815	2.12	<0.000 1*
Pentavalent	0	74.50	7.450		
	3	129.50	12.950	1.74	<0.000 1*
BCG	0	75.40	7.540		
	3	129.35	12.935	1.72	0.000 6*
Measles	0	57.70	5.770		
	3	115.85	11.585	2.01	<0.000 1*
YFV	0	74.10	7.410		
	3	160.55	16.055	2.17	<0.000 1*
HBV	0	74.25	7.425		
	3	140.15	14.015	1.89	<0.000 1*
Control NS	0	72.10	7.210		
	3	89.60	8.960	1.24	<0.000 1*

Day 0 means before vaccination. Control NS: Control using 0.9 % normal saline. *P<0.05. P value is for student's t-test.

The LTT showed that the vaccines and the 0.9% sodium chloride had the LT_{values} of 3.200, 3.537, 3.0967, 3.030, 3.020, 3.126 and 3.766 respectively. The LT_{value} of the vaccines was higher than the 80% LT_{value} of sodium chloride (Figure 1B). Dunnett's multiple comparison test to compare the leukocyte count due to the vaccines and the sodium chloride showed no significant difference at $P>0.05$. Also, a comparison of the leukocyte count due to the vaccines with the count due to the cyclophosphamide showed marked significant differences ($P<0.05$).

One-way analysis of the results of the LTT showed that the leukocyte count was significantly affected by all the treatments ($P<0.000\,1$)–the vaccines, the normal saline and the cyclophosphamide. The Bartlett's test for equal variances was also in concordance with the previous statement ($P=0.001\,2$). The Dunnett's multiple comparison test showed that the effects produced by the vaccines did not differ significant from each other and from the normal saline. However, the effects differed significantly from that produced by the leukopenic agent-cyclophosphamide. The vaccines were therefore judged to be safe and non-toxic, at least, to the hematopoietic systems.

4. Discussion

The results of this study showed that the vaccines were safe because each did not cause lower mean mouse weight at 3 d post-vaccination and at 7 d post-vaccination, the percent (%) weight gain compared to mean weight gain of control for each vaccine was above 60%. However, the samples from Abia State have better values for the percent (%) weight gain compared to mean weight gain of control. This could mean that the samples from Abia were better stored and had complete absence of residual toxin and/or chemicals, degradation products in the vaccines which results from poor vaccine storage. Perkins *et al.* showed that the pertussis-containing vaccines that lower mice body weight could cause adverse reactions if administered to children[21]. The results of abnormal toxicity test correlates well with vaccine toxicity[22].

The leukocytosis promoting toxicity test showed that the vaccines produce much less than 10-folds increase of the WBC in the vaccinated mice. The increase in the leukocytes due to the vaccines compares well with that due to the control 0.9% sodium chloride showing that the vaccines did not cause any hematopoietic damage. These are contrary to the findings of von Elten *et al.* when they demonstrated that pneumococcal vaccines cause leukocytosis and fever in vaccinated individuals[23].

LTT is a test to investigate whether or not a vaccine is capable of decreasing the number of eukocytes in vaccinated individuals thereby placing them at the risk of infection. The statistical analysis (Dunnett's multiple comparison test) of this study showed that the vaccines were similar to the non-toxic reference (0.9% sodium chloride) used and significantly different ($P<0.05$) from the toxic reference (the cyclophosphamide). Therefore, they are safe as they are not capable of causing leucopenia in vaccinated individuals. However it should be understood that live vaccines are contraindicated in immuno-compromised individuals for the risk of infections and increases leucopenia[24].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study is part of the doctorial research work undertaken by Oli AN. This research was funded by the Canadian Commonwealth Scholarship Program administered by the Canadian Bureau for International Education and African Doctoral Dissertation Research Fellowship award offered by the African Population and Health Research Center in partnership with the International Development Research Centre (Grant No. ADDRF Award 2012-2014 ADF 020). States' Ministries of Health donated the vaccines.

Comments

Background

Vaccines stimulate human immune system by mimicking a natural infection. The introduction and widespread use of vaccines have resulted in dramatic declines in the morbidity, disability, and mortality caused by many infectious diseases in the world. Although vaccines are developed in accordance with the highest standards of safety, licensed vaccines should be continually monitored for safety and efficacy before and after immunization.

Research frontiers

This study aims at checking the effects of the vaccines on the hematopoietic system and weight of mice after immunization.

Related reports

Childhood immunization schedule (covering children from birth through age 6 years) immunizes children with vaccines that protect young children against over 10 pathogens and strives to protect children at the youngest, and the most vulnerable age. The post-marked surveillance of licensed vaccines are important for the safety of vaccines. The mouse is chosen as the test species because of its acceptance as a predictor of toxic changes in man.

Innovations and breakthroughs

The study evaluated the safety of six vaccine products that are currently being used for childhood immunization program in two states of Nigeria, tested the vaccine products by simple and practicable methods, and provided important information about the safety of the vaccines.

Applications

The methods used in the study to evaluate the safety of the licensed vaccines are simple and affordable for developing countries.

Peer review

The study evaluated the vaccines that are utilized in routine immunization program in Imo and Abia States, Nigeria. The methods employed to test the vaccines in mice are body weight change test, leukopenic promoting toxicity test, and leukopenic toxicity tests in

mice before and after immunization of the mice.

References

[1] Stinchfield PK, Froese-Fretz A. Vaccine safety communication: the role of the pediatric nurse. *J Spec Pediatr Nurs* 2001; **6**(3): 143-146.

[2] Pickering LK, Baker CJ, Long SS, McMillan JA. *Report of the committee on infectious diseases*. 27th ed. Elk Grove: American Academy of Pediatrics; 2006.

[3] Metz B, Hendriksen CFM, Jiskoot W, Kersten GFA. Reduction of animal use in human vaccine quality control: opportunities and problems. *Vaccine* 2002; **20**(19-20): 2411-2430.

[4] Centers for Disease Control and Prevention. Vaccine safety. DeKalb County: Centers for Disease Control and Prevention. [Online] Available from: http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/safety.pdf [Accessed on 17th November, 2013]

[5] Bohlke K, Davis RL, Marcy SM, Braun MM, DeStefano F, Black SB, et al. Risk of anaphylaxis after vaccination of children and adolescents. *Pediatrics* 2003; **112**(4): 815-820.

[6] Slade BA, Leidel L, Vellozzi C, Woo EJ, Hua W, Sutherland A, et al. Postlicensure safety surveillance for quadrivalent human papillomavirus recombinant vaccine. *JAMA* 2009; **302**(7): 750-757.

[7] Levin A. Vaccines today. *Ann Intern Med* 2000; **133**: 661-664.

[8] Hoogstraten-Miller S, Burns W, Leja DL, National Human Genome Research Institute (U.S.). *Training in basic biomethodology for laboratory mice.* Bethesda: National Numan Genome Research Institute; 2004.

[9] Marcel I, Perret-Gentil. *Mouse biomethodology handout: laboratory animal resources center*. San Antonio: the University of Texas at San Antonio; 2013.

[10] Australian Government, National Health and Medical Research Council. Guide lines to promote the wellbeing of animals used for scientific purposes: the assessment and alleviation of pain and stress in research animals. Canberra: Australian Government, National Health and Medical Research Council; 2008. [Online] Available from: https://www.nhmrc.gov.au/guidelines/publications/ea18 [Accessed on 10th October, 2014]

[11] Animal Welfare Information Center. Animal welfare act and regulations. Beltsville: United States Department of Agriculture; 2013. [Online] Available from: http://awic.nal.usda.gov/government-and-professional-resources/federal-laws/animal-welfare-act [Accessed on 31st October, 2011]

[12] Momose H, Mizukami T, Ochiai M, Hamaguchi I, Yamaguchi K. A new method for the evaluation of vaccine safety based on comprehensive gene expression analysis. *J Biomed Biotechnol* 2010; doi: 10.1155/2010/361841.

[13] World Health Organization. WHO technical report series No. 941, Annex 6, Recommendations for whole-cell pertussis vaccine. Geneva: World Health Organization; 2007. [Online] Available from: http://www.who.int/biologicals/publications/trs/areas/vaccines/whole_cell_pertussis/Annex%206%20whole%20cell%20pertussis.pdf [Accessed on 8th October, 2014]

[14] World Health Organization. Manual of laboratory methods for testing of vaccines used in the WHO Expanded Programme on Immunization. Geneva: World Health Organization; 1997. [Online] Available from: http://whqlibdoc.who.int/hq/1997/WHO_VSQ_97.04_(parts1-2).pdf [Accessed on 15th October, 2014]

[15] Council of Europe. *European pharmacopoeia*. 5th edition. Strasbourg: Council of Europe; 2005.

[16] Indian Pharmacopoeia Commission. Diphtheria and tetanus and whole cell pertussis vaccine (Adsorbed). *Indian Pharmacopoeia* 2007; **3**(3): 744-757.

[17] National Institute of Infectious Diseases. Minimum requirements for biological products. Tokyo: National Institute of Infectious Diseases; 2006. [Online] Available from: http://www.nih.go.jp/niid/MRBP/files/seibutsuki english.pdf [Accessed on 5th October 2013]

[18] Khan MA, Owais M. Toxicity, stability and pharmacokinetics of amphotericin B in immunomodulators tuftsin-bearing liposomes in a murine model. *J Antimicrob Chemother* 2006; **58**: 125-132.

[19] Chino F. The views and policy of the Japanese control authorities on the three Rs. *Dev Biol Stand* 1996; **86**: 53-62.

[20] GraphPad Prism [computer program]. Version 5.00. San Diego (CA): GraphPad Software Inc.

[21] Perkins FT, Sheffield F, Miller CL, Skegg JL. The comparison of toxicity of pertussis vaccines in children and mice. *Symp Ser Immunobiol Standard* 1970; **13**: 141-149.

[22] Mizukami T, Masumi A, Momose H, Kuramitsu M, Takizawa K, Naito S, et al. An improved abnormal toxicity test by using reference vaccine-specific body weight curves and histopathological data for monitoring vaccine quality and safety in Japan. *Biologicals* 2009; **37**(1): 8-17.

[23] von Elten KA, Duran LL, Banks TA, Banks TA, Collins LC, Collins LC. Systemic inflammatory reaction after pneumococcal vaccine: a case series. *Hum Vaccin Immunother* 2014; **10**: 1767-1770.

[24] Centers for Disease Control and Prevention. Recommendations of the Advisory Committee on Immunization Practices (ACIP): use of vaccines and immune globulins in persons with altered immunocompetence. DeKalb County: Centers for Disease Control and Prevention; 1993. [Online] Available from: http://www.cdc.gov/mmwr/PDF/rr/rr4204.pdf [Accessed on 10th October, 2014]