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Original Article

Comparative Assessment of the Whole-cell Pertussis Vaccine Potency Using Serological and Intracerebral Mouse Protection Methods

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ABSTRACT

One of the most important QC tests of whole-cell pertussis vaccine (WCPV) is potency test. In this regard, mouse protection test (MPT) is the current potency method, which is associated with severe animal distress and large variability in results. The purpose of this study was to assess Pertussis Serological Potency Test (PSPT) as a serological alternative method to intracerebral challenge in MPT assay. In the current study, the potency of three experimental batches of WCPV (1, 2, and 3) and standard vaccine were compared using MPT and PSPT methods. In the MPT method, mice were immunized with tests and standard vaccines. After 2 weeks, they were intracerebrally challenged with Bordetella pertussis strain (18323). The potency was calculated via parallel line analysis based on the numbers of survivors 2 weeks after the challenge. Similar to MPT method, mice were immunized in the PSPT method and bled after 4 weeks. In the next step, sera were titrated by 18323-WCP-ELISA assay and potency values were estimated via parallel line analysis. Pearson correlation test was used to measure the strength of association between MPT and PSPT assay results. The potency values of the experimental laboratory batches 1, 2, and 3 in MPT assays were 11.14, 5.02, and 4.24 Iu/ml, whereas the obtained results of PSPT assays were 10.32, 4.11, and 3.06 Iu/ml, respectively. The correlation of MPT and PSPT results was 0.807. The findings of the present study demonstrated a significant correlation between MPT and PSPT results. The implementation of PSPT was more advantageous, compared to MPT due to its ethical approaches and less variability in results. The PSPT is a promising alternative method for intracerebral challenge. However, additional validation is needed to support the establishment of this method.

Keywords: MPT, PSPT, Potency, i.c. challenge

Évaluation Comparative de l'Activité du Vaccin contre la Coqueluche à l'Aide de Méthodes de Protection Sérologiques et Intracérébrales chez la Souris

Résumé: Le test d'activité est l'un des tests de contrôle de qualité les plus importants du vaccin anticoquelucheux à germes entiers (WCPV). À cet égard, le test de protection de la souris (MPT) est la méthode d'activité actuellement utilisée qui est cependantassociée à une détresse animale et une grande variabilité des résultats. Le but de cette étude était d'évaluer l'efficacité dutest d'activité sérologique de la coqueluche (PSPT) dans le dosage du MPT en tant qu'une potentiellealternative à la provocation intracérébrale. Dans cette étude, les activités de trois lots expérimentaux de WCPV (1, 2 et 3) et de vaccins standard ont été comparées à l'aide des méthodes MPT et PSPT. Dans la méthode MPT, les souris ont été immunisées à l'aide de tests et vaccins standards. Au bout de 2 semaines, les souris ont été soumises à une provocation intracérébrale avec la souche *Bordetella pertussis* (18323). L'activité a été calculée via une analyse de lignes parallèles basée sur le nombre de

souris survivantes deux semaines après la provocation. De même, les souris ont été immunisées selon la méthode PSPT et saignées au bout de 4 semaines. Ensuite, les sérums ont été évalués par dosage 18323-WCP-ELISA et les valeurs d'activité ont été estimées par analyse de lignes parallèles. Le test de corrélation de Pearson a été utilisé pour mesurer le niveau d'association entre les résultats des tests MPT et PSPT. Les valeurs d'activité obtenues par analyses MPT des lots expérimentaux de laboratoire 1, 2 et 3 étaient respectivement de 11,14, 5,02 et 4,24 UI / ml, alors que les résultats obtenus des analyses PSPT étaient respectivement de 10,32, 4,11 et 3,06 UI / ml,. La corrélation des résultats MPT et PSPT était de 0,807. Ces résultats ont démontré une corrélation significative entre les résultats des tests MPT et PSPT. La mise en œuvre du PSPT était plus avantageuse que le MPT en raison de son approche plus éthique et de la moins grande variabilité de cesrésultats. Le PSPT représente donc une méthode alternative prometteuse à l'essai intracérébral. Cependant, une validation supplémentaire est nécessaire pour appuyer la mise en place de cette méthode.
Mots-clés: MPT, PSPT, Activité i.c. Challenge

INTRODUCTION

Immunization against pertussis disease has been globally applied with triple vaccine (DTwP) since 1974 (Black et al., 2010). Based on the requirements of World Health Organization (WHO), it is essential to perform extensive quality control (QC) testing before human vaccines are marketed. One of the most important QC tests of whole-cell pertussis vaccine (WCPV) is a potency test. The mouse protection test (MPT) is a current method for WCPV potency assay developed by Kendrick et al. (1947). The relevance of the test results to human protection was determined by Medical Research Councils in 1956. The WHO has considered MPT as a mandatory method to estimate WCPV potency (Kendrick et al., 1947; Corbel and Xing, 2004). The worldwide MPT implementation in the past decades demonstrated obvious disadvantages of this method, such as severe animal distress, results variability, and excessive use of animals (van der Ark et al., 1994; Chovel et al., 2012; Matos et al., 2012). New approaches of international authorities (e.g., WHO) and compliance with requirements, such as In-Process Quality Control (IPQC), batch to batch consistency approaches, and 3Rs (Replacement, Refinement and Reduction) of Russell and Burch (1959), compelled scientists to look for a new reproducible, ethical, easy, and safe method. In this regard, scholars developed a serological alternative method named Pertussis Serological Potency Test (PSPT) for WCPV potency assay (van der Ark et al., 1994; Van der Ark et al., 1995; van Der Ark et al., 1998; van der Ark et al., 2000). In this method, mice are immunized with the graded dose of tests and WHO standard pertussis vaccines. In the next step, mice sera were obtained instead of intracerebral challenge with Bordetella pertussis (strain 18323). Moreover, the whole antibody titer of immunized mice against the surface antigen of Bordetella pertussis (strain 18323) was assessed by 18323-WC-ELISA method. The potency of pertussis vaccines is estimated based on the comparison of antibody level induced by test and standard vaccines via parallel line analysis (van der Ark et al., 1994; van der Ark et al., 1995; van Der Ark et al., 1998; van der Ark et al., 2000).

MATERIAL AND METHODS

Mice. To conduct the study, healthy NIH/Razi outbred mice were randomly divided into two groups of MPT and PSPT. The weight ranges of mice in the MPT and PSPT were 14-18 g and 20-24 g, respectively. All mice were housed under conventional condition (12-12 hr. light/dark cycle, temperature 20-25 °C, humidity 50%) in Razi Institute Animal Husbandry, Karaj, Iran (van der Ark et al., 1994).

Vaccines. The WHO International Standard of Whole-Cell Pertussis Vaccine (NIBSC code: 94/532) with the potency of 40 international units (IU) per ampoule was used in the current study. Moreover, three experimental laboratory batches of Razi DTP vaccines (1, 2, and 3) were implemented, each of them contained 32 OU/ml of WCPV (van der Ark et al., 1994).

Bordetella pertussis 18323 challenge strain. International Bordetella pertussis 18323 challenge strain (ATCC code: 9797) was used as intracerebral challenge and ELISA plate coating (van der Ark et al., 1994).

Hyperimmune mice serum (HIS). Mice were immunized intraperitoneally (i.p.) on days 0 and 14 with the highest protective dose (1 IU/ml) of WHO International Standard of WCPV (NIBSC code: 94/532) and bled on day 28. After sera were obtained, samples were pooled, aliquoted, and stored at < -20 °C (van der Ark et al., 1994).

Positive control serum (PSPTpc). The WHO reference of Bordetella pertussis mice anti-serum (NIBSC code: 97/642) was dissolved in 0.5 ml sterile distilled water, aliquoted, and stored at < -20 °C.

Negative control serum (PSPTnc). Mice were introduced (i.p.) on day 0 with 0.9% sterile saline and bled on day 28. Afterward, sera were obtained, pooled, aliquoted, and stored at < -20 °C (van der Ark et al., 1994).

Standard serum (PSPTst). Mice were immunized (i.p.) on day 0 with WHO International Standard of WCPV (NIBSC code: 94/532) and bled on day 28. In the next step, sera were obtained, pooled, and stored at < -20 °C.

Intracerebral mouse protection test (i.c. MPT). The MPT was performed based on the WHO guidelines (TRS 800-1990). To this end, five-fold serial dilutions (1/8, 1/40, and 1/200 of single human dose equal to 2, 0.4, and 0.08 OU per 0.5 ml, respectively) of test vaccines (1, 2, and 3) and five-fold serial dilutions (1/8, 1/40, and 1/200 of 8 IU/ml equal to 1, 0.2, and 0.04 IU/ml, respectively) of the WHO International Standard of WCPV (NIBSC code: 94/532) were applied in 0.9% sterile saline solution. The groups of 16 mice were immunized i.p. with the 0.5 ml of each dilutions and one negative control group was introduced with 0.5 ml of 0.9% sterile saline solution (day 0). Mice were intracerebrally challenged with Bordetella pertussis 18323 challenge strain 2 weeks after the immunization. The numbers of survivors were recorded up to 2 weeks after the challenge, and the potency values of vaccines were estimated by means of CombiStats software (World Health Organization, 2013).

PSPT immunization and serum collection. In this study, the PSPT assay was performed based on van der Ark research in 1994. Similar to MPT group, the groups of 12 mice were immunized i.p. with the 0.5 ml of each dilutions (day 0). One negative control group was introduced i.p. with 0.5 ml of 0.9% sterile saline solution. After 4 weeks, mice were bled by heart puncture. The blood samples of each groups were collected individually in labeled vials and incubated 1 h at 37 °C then stored overnight at 4 °C. Finally, the samples were centrifuged at 800g for 20 min, followed by the separation of supernatant from sediment. In the next step, the product was aliquoted and stored at < -20 °C (van der Ark et al., 1994).

18323-WCP-ELISA. The 18323-WC suspension was obtained from fresh culture (up to 24-30 h) of Bordetella pertussis 18323 strain on Bordet-Gengou agar medium. The suspension was diluted to the concentration of 1 OU/ml in PBS, and 100µl was added to each well in a flat bottom of high binding microtiter plates (JETBIOFIL, 8-strip×12, China). The plate was incubated overnight at 37 °C without cover, which let the well content to evaporate. Next day, the plate was washed 3 times with washing buffer (0.5 gr% w/v bovine serum albumin [BSA, Organon technical, Netherland and 0.05% V/V Tween 20 in PBS, Merck, Germany]) and dried against absorbent papers. The plate was washed and dried at the end of each incubation period and before the addition of next

reagent, as was performed previously. Nonspecific binding sites were blocked with blocking buffer (1 gr% w/v BSA, in PBS), followed by 125µl per well, then covered and incubated for 1h at 37 °C. In the following step, serum samples were diluted in a five-fold serial dilutions range started at 1/1000 and reference serum diluted in a three-fold serial dilutions range started at 1/5000. Moreover, PSPTpc and PSPTnc were respectively diluted at 1/200 and 1/3000 in diluent buffer (0.5% w/v BSA, 0.05% v/v Tween 20 in PBS). The 100 µl of diluted sera were added to each well then incubated for 1h at 37 °C. In next step, a goat anti-mouse IgG, peroxidase conjugated (Nordic Immunology, Netherland) was diluted at 1/6000 in diluent buffer, 100 µl was added to each well, and then incubated for 1h at 37 °C. Afterward, antibody binding to coated suspension was visualized using 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma, USA). The TMB stock substrate solution was prepared by dissolving 1 mg/ml TMB in dimethyl sulfoxide-DMSO (Sigma, USA). The TMB working substrate solution was made by adding the 1/10 dilution of TMB-DMSO stock solution in phosphate-citrate buffer (pH 5.0; Sigma, USA). Immediately before use, 2 µl of fresh hydrogen peroxide 30% (Merck, Germany) was added to 10 ml of substrate/buffer solution, followed by the addition of 100 µl per well TMB working substrate solution and incubation for 10 min at room temperature in a dark place. Finally, the reaction stopped by the addition of 100 µl per well of 2M H2SO4 (Merck, Germany). Absorbance was measured at 450nm using an automatic plate reader (BioTek Instruments, USA; van der Ark et al., 1994).

Statistical methods. CombiStats software (version 4.0) was used for the estimation of MPT potency assay. Parallel line analysis with logarithm transformation was applied for the calculation of PSPT potency assay. Pearson correlation test was used to measure the strength of association between MPT and PSPT assay results. The equality of variance and means were assessed by Levene's test and t-test, respectively (van der Ark et al., 1994).

RESULTS

In PSPT method, the antibody concentration of HIS reference serum was considered 2700 EU/ml. The antibody concentrations of sera in comparison with HIS reference serum using 18323-WC-ELISA and a four parameter fitting analysis were determined at 0, 2200, 1350, 1300, and 2500 EU/ml for PSPTnc, test vaccine A, test vaccine B, test vaccine C, and WHO International Standard of WCPV, respectively (NIBSC code: 94/532; van der Ark et al., 1994; Van der Ark et al., 1995; van Der Ark et al., 1998; Chovel et al., 2012). The values of MPT and PSPT potency for test vaccines were obtained using parallel line analysis with the log transformation of antibody concentrations between the tests and standards (tables 1 and 2).

Sample 1			
Id.	02491001		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	2.53138	11.1486	51.5530
Rel. to Ass.	?	?	?
Rel. to Est.	23.6%	100.0%	462.4%

Sample 2			
Id.	02491002		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	1.11115	5.02765	20.1292
Rel. to Ass.	?	?	?
Rel. to Est.	22.1%	100.0%	400.4%

Sample 3			
Id.	02491003		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	0.916721	4.24922	16.6729
Rel. to Ass.	?	?	?
Rel. to Est.	21.6%	100.0%	392.4%

Table 1. Mouse protection test potency results of three experimental batches of DTP vaccines (1, 2, and 3) estimated by World Health Organization CombiStats software

Pearson correlation coefficient result is displayed in Table 3. The equality of variance and means in the MPT and PSPT groups were assessed by Levene's test and t-test, respectively (tables 4 and 5). The obtained results revealed no significant difference regarding the variability of MPT/PSPT potency results.

DISCUSSION

One of the important and controversial QC tests of WCPV is potency test. Based on WHO-Technical Report Series-800 (1990), relevant studies, and practical experiences of vaccine production centers,

MPT method has some disadvantages in terms of ethics and variability in results.

Table 2. Pertussis Serological Potency Test potency results (IU/ml) of three experimental batches of DTP vaccines (1, 2, and 3) estimated by parallel line analysis with the log transformation of antibody concentrations between the tests and standards of whole-cell pertussis vaccine Scattering plot of MPT and PSPT potency results is shown in Figure 1.

	PSPT		
Samples	Sample 1	Sample 2	Sample 3
Lower limit	3.000	3.930	10.120
Estimate	3.060	4.110	10.320
Upper limit	3.063	4.330	10.410
Av. PSPT	3.041	4.123	10.283

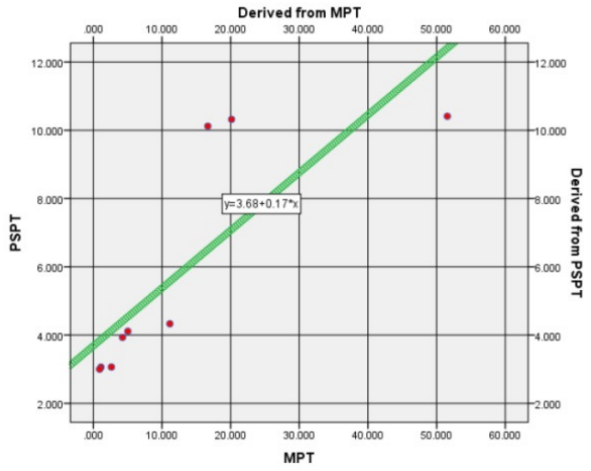

Figure 1. Scattering plot of potency values related to mouse protection test and Pertussis Serological Potency Test obtained from three experimental batches of DTP vaccines (1, 2, and 3) using SPSS software

Accordingly, an attempt has been made to introduce new approaches as supplementary or alternative methods in order to gain more compliance with international requirements (World Health Organization, 1990; van der Ark et al., 1994; Van der Ark et al., 1995; van Der Ark et al., 1998; van der Ark et al., 2000). Accordingly, the replacement of i.c. challenge (MPT) with PSPT serological approach as a combination of in vivo and in vitro method was assessed based on Van der Ark's (1994) guideline in the present study. Unlike studies conducted by van der Ark et al. (1994), van der Ark et al. (1995), van Der Ark et al. (1998), and van der Ark et al. (2000) for immunization of mice, the equal graded doses of WCPV in both MPT and PSPT assays were used in the current study.

Table 3. Pearson correlation coefficient between mouse protection test and Pertussis Serological Potency Test results of vaccines (1, 2, and 3) using SPSS software

Correlations			
MPT	PSPT		
.807**	1	Pearson Correlation	PSPT
.009		Sig. (2-tailed)	
9	9	N	
1	.807**	Pearson Correlation	MPT
	.009	Sig. (2-tailed)	
9	9	N	

**. Correlation is significant at the 0.01 level (2-tailed).

Table 4. Results of Levene's test

Levene's Test for Equality of Variances			
Sig.	F		
.038	5.185	Equal variances assumed	SCOR
		Equal variances not assumed	

Table 5. Obtained results of t-test

		t-test for Equality of Means						
		t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
							Lower	Upper
SCOR	Equal variances assumed	1.234	16	.235	6.788536	5.501714	-4.874576	18.451647
	Equal variances not assumed	1.234	8.701	.250	6.788536	5.501714	-5.722613	19.299684

The omission of dose variation effect on potency results made the researchers to follow such a strategy, and therefore MPT/PSPT result differences would be the reflection of quality not quantity of pertussis vaccines. In the current study, three WCPVs with low, intermediate, and high MPT potency levels were chosen to assess PSPT results in three different conditions. Both genders of mice in MPT and PSPT assays were used to eliminate animal gender influences. As the presence of antibody may interfere MPT/PSPT

assays, Von Hunolstein et al. (2008) controlled the presence of antibody against B. bronchiseptica and B. parapertussis by ELISA before immunization and after bleeding. As demonstrated (van der Ark et al., 1994; Van der Ark et al., 1995; van Der Ark et al., 1998; van der Ark et al., 2000; Matos et al., 2012), the scattering plot of MPT/PSPT potency results showed suitable homogeneity of distribution and relationship between MPT and PSPT results (Figure 1). This means that variation in results between MPT and PSPT had the same direction. The evaluation of homogeneity between MPT and PSPT potency results using Levene's test and t-test) indicated no significant difference in variability of potency results. The obtained results of Pearson correlation test revealed a significant correlation between MPT and PSPT groups ($r=0.807$, $P< 0.05$). This means that humoral immune response (total IgG of immunized mice) induced by the surface antigens of WCPV had a significant correlation with MPT results, and is a suitable parameter for the estimation of WCPV potency assay. In fact, PSPT is the real reflection of humoral immune response to pathogen agent and is an actual indicative for potency estimation. On the contrary, i.c. MPT challenge method is an artificial root of entrance for pathogen agent, and cannot be an effective method for WCPV potency estimation. In a study by Hoonakker et al. (2016), the results were indicative of the importance of PSPT method for WCPV potency assay. However, they believed that the addition of antibody concentration and other parameters of immune system (e.g., T helper cell response and cytokines) contribute to the protection. Therefore, they underscored the importance of the evaluation of this issue. In other words, these are complementary parameters that increase the importance and efficacy of PSPT. The PSPT vs. i.c. MPT is a simple procedure for the acquisition of better reproducibility. Unlike the MPT, the serum samples of PSPT can be assessed in other laboratories and does not require a frequent repetition. The PSPT complying with WHO requirements as in-process quality control, batch to batch consistency approaches (Von Hunolstein et al., 2008), 3Rs ethical viewpoint of Russell and Burch, (1959) in terms of the replacement of intracerebral injection by ELISA and reduction of animal usage and distress (Corbel and Xing, 2004). Regardless of ethical aspect, it is economical to reduce animal use. The PSPT performance is safe for personnel and conforms to European Directorate for the Quality of Medicines and HealthCare-EDQM and European Centre for the Validation of Alternative Methods-ECVAM approaches for substitution of in vivo by in vitro methods (Von Hunolstein et al., 2008). The injection of a challenge dose in MPT has a booster effect, and PSPT removes this interfering effect and supports the improvement of potency results (van der Ark et al., 1994). The obtained results of the current study confirmed a significant correlation between MPT and PSPT assays results in the assessment of whole-cell pertussis vaccine potency. The PSPT method showed more advantageous, including ethical approaches and less variability in results, compared to MPT. The PSPT is a promising alternative or supplementary method for current i.c. challenge test. However, additional data validation in future collaborative studies in vaccine quality control laboratories are needed to support establishment of PSPT-WC-ELISA. In future, PSPT can be simultaneously used with one animal group using single diluted vaccine injection, in multi-parameter tests assay, in combined vaccine such as DTwP (Von Hunolstein et al., 2008). PSPT actually is a combination of mouse weight gain test (MWGT) and lymphocyte promotion test (LPT) which is similarly called mouse toxicity and immunogenicity test (MTI), hence, this capability causes less animal usage and more cost-benefit for this method (van Straaten et al., 2001), but further validation needed for establishment of these tests.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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