

Study on Toxicity Reduction and Potency Induction in Whole-cell *Pertussis* Vaccine by Developing a New Optimal Inactivation Condition Processed on *Bordetella pertussis*

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Abstract

Background: Whooping cough is caused by *Bordetella pertussis*, and it remains a public health concern. Whole-cell *pertussis* vaccines have been commonly employed for expanded immunization. There is no doubt of the efficacy of whole cell *pertussis* vaccine, but it is necessary to improve the vaccine to decrease its toxicity.

Objectives: In this study, an inactivation process of dealing with *pertussis* bacteria is optimized in order to decrease the bacteria content in human doses of vaccines and reduce the vaccine's toxicity.

Materials and Methods: The bacterial suspensions of *pertussis* strains 509 and 134 were divided into 21 sample parts from F₁ to F₂₁ and inactivated under different conditions. The inactivated suspensions of both strains were tested for opacity, non-viability, agglutination, purity, and sterility; the same formulation samples that passed quality tests were then pooled together. The pool of inactivated suspensions were analyzed for sterility, agglutination, opacity, specific toxicity, and potency.

Results: The harvest of both bacterial strains showed purity. The opacity of various samples were lost under different treatment conditions by heat from 8% to 12%, formaldehyde 6% to 8%, glutaraldehyde 6% to 8%, and thimerosal 5% to 8%. Tests on suspensions after inactivation and on pooled suspensions showed inactivation conditions not degraded agglutinins of both strains. The samples of F₂, F₄, F₈, F₁₂, F₁₅, and F₁₇ passed the toxicity test. The potency (ED₅₀) of these samples showed following order F₁₇ > F₁₂ > F₈ > F₁₅, F₄ > F₂, and F₁₇ revealed higher potency compared to other formulations.

Conclusions: It can be concluded that F₁₇ showed desirable outcomes in the toxicity test and good immunogenicity with a low bacterial number content. Consequently, lower adverse effects and good immunogenicity are foreseeable for vaccine preparation with this method.

Keywords: Whole-Cell Vaccines, *Pertussis*, Whooping Cough, Toxicity Test, Potency Test

1. Background

Whooping cough is caused by *Bordetella pertussis*, a Gram-negative bacterium. It is one of the main causes of infant death in the world, and it remains a public health problem even in regions with high coverage vaccination. Whole-cell *pertussis* (wP) vaccines have been encouraged and broadly employed for expanded programs on the immunization of children. They have been used in the world as a component of a combined diphtheria, tetanus, and *pertussis* (DTP) vaccine in national children vaccination plans for many years (1).

The relation of wP vaccine with cases of persistent cerebral injury has not been confirmed (2, 3), since it may be the result of fever, irritability, seizures, hypotension, or local side effects (4). However, the possible side effects result-

ing from the administration of wP vaccines has led to the development of acellular *pertussis* (aP) vaccines in some countries, though wP vaccines are still broadly manufactured and universally employed (5). In comparison to the aP vaccine, which needs vast purification procedures, the wP vaccine is more economical. Furthermore, the resurgence of whooping cough in nations where aP vaccination is high coverage has led to relevant research demonstrating that wP vaccines create superior and continuing immunity compared to aP vaccines. Therefore, it is predicted that wP vaccines will be used for the next years (6-9).

Suspensions of inactivated *B. pertussis* microorganisms are the main immunizing ingredient in wP vaccines, which are produced by culturing suitable *B. pertussis* strains under states that provoke the expression

of the bacteria phenotype of virulent Phase I. The bacteria culture in semi-synthetic or synthetic media has been employed in vaccine preparation for a number of years (10). The bacteria strains are chosen to contain agglutinin serotypes 1, 2, and 3, which are at this time attributed to fimbriae serotypes 1, 2, and 3. Generally, one or more strain of each bacteria serotypes 1, 2 and 1, 3 are employed; however, some manufacturers use a single strain of serotype 1, 2, and 3 (11).

There is no doubt as to the efficacy of a whole cell *pertussis* vaccine, but it is necessary to improve the quality of the vaccine specially to decrease its toxicity (3, 12). In order to decrease the toxicity of wP vaccines, procedures of detoxifying all the toxins of *B. pertussis* organisms without destroying the immunogenicity have been considered (13). On this basis, Gupta et al. evaluated the effects of various chemical agents (such as glutaraldehyde, formaldehyde, acetone, and thimerosal) in different temperature conditions on the inactivation of *B. pertussis* cells (14). Moreover, some studies explained the preparation of a potent and safe *pertussis* vaccine with good stability using the glutaraldehyde inactivation process (15-19).

2. Objectives

In this study, to achieve optimal conditions for preparing a low toxic and highly potent *B. pertussis* whole-cell vaccine, the effects of different chemicals and temperatures were investigated for inactivation of *B. pertussis* micro-organisms. The samples were prepared by killing *B. pertussis* micro-organisms using various concentrations of chemicals with variable durations and temperatures, which were analyzed in mice for intra-cerebral potency and toxicity.

3. Materials and Methods

3.1. Materials

In this study, we used formaldehyde, glutaraldehyde, and thimerosal (Merck, Germany). The *B. pertussis* culture of strains 509 (batch number P93-19, optical density $15, 15 \times 10^9$ bacterial particle/mL) and 134 (batch number P93-20, optical density $15, 15 \times 10^9$ bacterial particle/mL) were obtained from the Razi Vaccine and Sera Research Institute (Alborz, Iran). Freeze-dried, mono-specific *pertussis* agglutinin antiserum for factors 1, 2, and 3 was purchased from NIBSC (UK). All other chemicals were reagent grade.

3.2. Animals

Healthy Saurian strain mice < 5 weeks old from the same stock with weighing 10 to 18 g were used, and the difference in weight between the heaviest and the lightest was not greater than 5 g. They were used for evaluating the intracerebral potency of *B. pertussis* inactivated cells and mice with weight 14 to 16 g in toxicity test. The study was reviewed by the Pasteur institute of Iran, and all animals were handled in accordance with institutional guidelines.

3.3. Bordetella Pertussis Strains

Bordetella pertussis strains 509 (serotypes 1 and 2) and 134 (serotypes 1 and 3) were obtained from the Human bacterial vaccine division of the Razi vaccine and serum research institute, and the strains were used for *pertussis* preparation. *Bordetella pertussis* strain 18323 (serotype 1, 2, and 3) and ATCC 9797 were obtained from the aforementioned institute and employed as a challenge bacteria in the mouse for an intracerebral protection test (MPT) as a current potency test for *pertussis* vaccines.

3.4. Harvesting

Culture suspensions of *B. pertussis* from the fermenter were transferred to the Huading separator. Harvesting was carried out at a separator flow rate of 250 to 300 L/h, 6000 rpm. The ejaculated micro-organism mass was collected in a sterile, non-pyrogen vessel; normal saline was added to adjust optical density (OD) of suspension in the 150 (150×10^9 bacteria particle/mL) to make up the required volume of a pellet.

3.5. Inactivation of B. Pertussis Organisms

The bacterial suspensions of *B. pertussis* strains 509 and 134 were homogenized by shaking at 50 rpm, in $5 \pm 3^\circ\text{C}$ for 1 hour; a 1000 mL sample was taken from each suspension. The samples were then homogenized by shaking at 50 rpm, in $5 \pm 3^\circ\text{C}$ for 1 hour. In this study, 40 mL of each of the 21 samples was prepared from the bacterial suspensions and labelled F_1 to F_{21} . Each part was treated using one of the specified inactivating conditions mentioned (Table 1). The inactivated bacterial suspensions were immediately cooled to below 25°C after completion of the inactivation process and sampled for tests of opacity, non-viability, agglutination, purity, and sterility.

3.6. Pooling of Inactivated Bordetella Pertussis Bacterial Suspensions

The inactivated suspensions of strains 509 and 134 were homogenized by shaking at 50 rpm, in $5 \pm 3^\circ\text{C}$ for 1 hour; thereafter, the same formulation samples were

Table 1. Different Inactivation Formulations of *B. pertussis* Suspensions

Formulation	Heat (°C)	F (%w/v)	G (%w/v)	T (%w/v)	Duration (min)	Shaking (rpm)
F1	56				10	50
F2	56				20	50
F3	56				30	50
F4	37	0.05			24 h	50
F5	37	0.05			36 h	50
F6	37	0.10			24 h	50
F7	37	0.10			36 h	50
F8	37	0.15			24 h	50
F9	37	0.15			36 h	50
F10	22.5		0.025		10	50
F11	22.5		0.025		15	50
F12	22.5		0.05		10	50
F13	22.5		0.05		15	50
F14	22.5		0.075		10	50
F15	22.5		0.075		15	50
F16	37			0.01	24 h	50
F17	37			0.01	36 h	50
F18	37			0.02	24 h	50
F19	37			0.02	36 h	50
F20	37			0.03	24 h	50
F21	37			0.03	36 h	50

Abbreviations: F, formaldehyde; G, glutaraldehyde; T, Thimerosal; min, minute.

pooled together. The mixture of inactivated bacterial suspensions were homogenized by stirring at 50 rpm, in $5 \pm 3^\circ\text{C}$ for 3 hours and then sampled for sterility, agglutination, opacity, specific toxicity, and potency tests.

3.7. Test for Non-Viability

The samples of inactivated bacterial suspensions were tested for absence of *B. Pertussis* viability by inoculating Bordet-Gengou agar slope and incubating at 35°C for 7 days (20).

3.8. Estimation of Opacity Unit

The *B. pertussis* bacterial suspensions' opacity was measured both by visual comparison with the samples of the WHO's 5th International Standard of Opacity (20, 21), which is equivalent to 10 IU of opacity (10×10^9 organisms per mL) (22), and the spectrophotometric method, which evaluates the sample at 590 nm (23). Briefly, serial dilutions of the bacterial suspension were prepared in saline; then, the opacity of the samples were standardized based

on the international reference for opacity. The absorbance of each dilution was measured at 590 nm, and the results were drawn to obtain a calibration curve (Figure 1).

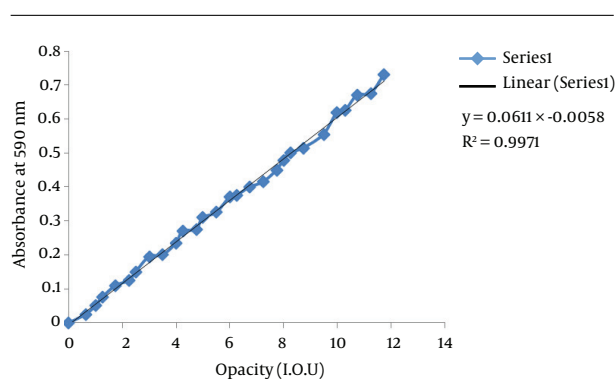


Figure 1. Calibration curve of the correlation between the absorbance of the *B. pertussis* bacteria suspension at 590 nm and the opacity in International Opacity Units (I.O.U.), mean \pm SD, n = 3.

3.9. Estimation of Purity

Before they were killed, the samples of *B. pertussis* bacterial suspensions were analyzed for purity by microscopic evaluation of Gram stained smears and by culture into nutrient agar, Bordet-Gengou agar, and nutrient broth cultures media (24).

3.10. Test for Agglutinin Production

To study the expression of serotype specific fimbriae by *B. pertussis* bacteria and the effects of inactivation conditions on agglutinins, bacterial suspensions were examined before and after inactivation using the slide agglutination assay method (24). Briefly, 1 mL of the *B. pertussis* bacterial suspension was centrifuged, and the sediment was resuspended in 0.1 mL normal saline and homogenized. Then, 10 μ L of the reconstituted sample was mixed with 10 μ L of monospecific antiserum of agglutinins 1, 2 and, 3 separately. The slide gently rotated for 2 to 3 minutes, and agglutination was observed.

3.11. Test for Toxicity

The overall toxicity of the inactivated *B. pertussis* suspensions was performed by a mouse weight gain test (MWGT). Briefly, samples of the inactivated bacteria were diluted by normal saline to 40 opacity unit/mL, and each mouse was injected intraperitoneally with 0.5 mL of the samples. The average weight gain of the animals in the test groups was compared with the control group for 7 days (25, 26).

3.12. Potency Measurement

The potency of inactivated *B. pertussis* bacterial suspensions were measured by the intracerebral mouse protection method. The ED₅₀ quantity for the test samples were estimated by probit analysis (24, 25, 27).

4. Results

4.1. Bacterial Harvest Characteristics

The bacterial harvest of strains 509 and 134 showed purity for cultural staining (a Mercury drop-like appearance on BG slants and no growth in nutrient agar and broth) and morphological specifications of *B. pertussis* (Gram negative, coccobacilli). The bacterial suspension of strain 509 contained agglutinogens 1 and 2, and strain 134 contained agglutinogens 1 and 3. The opacity unit of organisms in the harvests were 150×10^9 /mL for each batch.

4.2. Estimation of Opacity

In this work, the spectrophotometric method for the measurement of the *B. pertussis* bacterial suspensions' opacity was distinguished to be straightforward and was favored; this was because it allowed the estimation of slight variances in opacity, which were indistinguishable by the optical opacity method (Figure 1). The *B. pertussis* suspensions' opacity in various stages (before inactivation, after inactivation, and after pooling of inactivated bacterial suspensions) of work was measured (Table 2).

4.3. Test for Non-Viability

The non-viability test on *B. pertussis* organisms after inactivation showed that the micro-organisms of strains 509 and 134 treated under F₁₀ and F₁₆ conditions were not inactivated, while every other inactivation condition killed bacterial organisms. Therefore, bacterial suspensions of F₁₀ and F₁₆ were eliminated from the study, and other bacterial suspensions of both strains were pooled together for further evaluation and tests.

4.4. Test for Agglutinin

In this study, the expression of serotype specific fimbriae by *B. pertussis* bacteria was examined by slide agglutination assay. In order to evaluate the effects of inactivation conditions on agglutinins, bacterial suspensions were examined after inactivation and pooling for agglutinins (Table 3).

4.5. Test for Toxicity

The results of the MWGT overall toxicity assessment on the inactivated *B. pertussis* suspensions are shown in Table 4. This test demonstrated acceptable outcomes for bacterial suspensions inactivated under conditions of F₂ (56°C, 20 minutes and 50 rpm), F₄ (formaldehyde 0.05% w/v, 37°C, 24 hours, 50 rpm), F₈ (formaldehyde 0.15% w/v, 37°C, 24 hours, 50 rpm), F₁₂ (glutaraldehyde 0.05% w/v, 22.5°C, 10 minutes, 50 rpm), F₁₅ (glutaraldehyde 0.075% w/v, 22.5°C, 15 minutes, 50 rpm), and F₁₇ (thimerosal 0.01% w/v, 37°C, 36 hours, 50 rpm).

4.6. Test for Potency

The in vivo potency test was performed on the pooled *B. pertussis* bacterial suspensions F₂, F₄, F₈, F₁₂, F₁₅, and F₁₇. Table 4 shows that the ED₅₀ of these bacterial suspensions were calculated. The potency analysis of the aforementioned suspensions showed their ED₅₀ order as follows: F₁₇ > F₁₂ > F₈ > F₁₅, F₄ > F₂. The bacterial suspension of inactivation condition F₁₇ indicated a higher ED₅₀ (1:333 of a human dose) compared to other inactivation conditions.

Table 2. The Opacity of *B. pertussis* Suspensions in Different Work Stages

Strains	Before Inactivation	Inactivation Status	After Inactivation		
	Opacity (Organism/mL)		Strain 509 (Organism/mL)	Strain 134 (Organism/mL)	Pooled Suspensions (organism/mL)
509	150 × 10 ⁹ /mL	F1	139 × 10 ⁹	138 × 10 ⁹	139 × 10 ⁹
		F2	138 × 10 ⁹	138 × 10 ⁹	138 × 10 ⁹
		F3	133 × 10 ⁹	132 × 10 ⁹	132 × 10 ⁹
		F4	142 × 10 ⁹	144 × 10 ⁹	141 × 10 ⁹
		F5	141 × 10 ⁹	141 × 10 ⁹	141 × 10 ⁹
		F6	141 × 10 ⁹	142 × 10 ⁹	141 × 10 ⁹
		F7	140 × 10 ⁹	140 × 10 ⁹	140 × 10 ⁹
		F8	140 × 10 ⁹	141 × 10 ⁹	141 × 10 ⁹
		F9	138 × 10 ⁹	138 × 10 ⁹	138 × 10 ⁹
		F10	141 × 10 ⁹	142 × 10 ⁹	Ex ^a
		F11	139 × 10 ⁹	138 × 10 ⁹	139 × 10 ⁹
		F12	140 × 10 ⁹	139 × 10 ⁹	139 × 10 ⁹
		F13	139 × 10 ⁹	138 × 10 ⁹	139 × 10 ⁹
		F14	138 × 10 ⁹	139 × 10 ⁹	139 × 10 ⁹
134	150 × 10 ⁹ /mL	F15	138 × 10 ⁹	137 × 10 ⁹	138 × 10 ⁹
		F16	143 × 10 ⁹	142 × 10 ⁹	Ex ^a
		F17	141 × 10 ⁹	141 × 10 ⁹	141 × 10 ⁹
		F18	141 × 10 ⁹	140 × 10 ⁹	141 × 10 ⁹
		F19	139 × 10 ⁹	139 × 10 ⁹	139 × 10 ⁹
		F20	138 × 10 ⁹	139 × 10 ⁹	139 × 10 ⁹
		F21	138 × 10 ⁹	138 × 10 ⁹	138 × 10 ⁹

^aExcluded from study.

5. Discussion

Bacterial suspensions should not be utilized in the manufacturing of vaccines if impurity has been observed at any step in their preparation. From this point of view, bacterial characteristics such as morphology, purity, and appearance of bacterial suspensions should be controlled in bacterial harvest. The opacity of harvested bacterial suspensions should be monitored in order to estimate bacteria count per volume (5). The harvests of *B. pertussis* bacterial strains 509 and 134 showed purity by Gram staining, cultural evaluation, and from the point of appearance.

In this study, the bacterial count in suspensions of *B. pertussis* before and after inactivation and after pooling of inactivated bacterial suspension was measured. Table 2 shows that the average opacity of suspensions was decreased under different treatment conditions from 8% to 12% by heat, 6% to 8% in formaldehyde, 6% to 8% in glutaraldehyde, and 5% to 8% in glutaraldehyde. Various inac-

tivating factors lyse *B. pertussis* cells with different intensities and decrease the opacity of bacteria suspension. These results are in agreement with other reports (19, 28).

The protective role of anti-agglutinogens (1, 2, and 3) is important for fighting whooping cough (5, 29). As is shown in Table 3, the outcomes of this research indicated that the bacterial harvests of strain 509 contained agglutinins 1 and 2, and strain 134 contained agglutinins 1 and 3. A test for agglutinins on bacterial suspensions 509 and 134 after inactivation under various conditions and pooled inactivated bacterial suspensions (Table 3) showed inactivation conditions due to heat, formaldehyde, and glutaraldehyde not degraded agglutinins of both *B. pertussis* strains. There are other reports with similar findings to our results, showing that inactivated agents such as formaldehyde, glutaraldehyde, and heat do not destroy agglutinins of *B. pertussis* during the inactivation process (18, 19, 30). This work also revealed that thimerosal does not change agglutinins of *B.*

Table 3. The Agglutinins Test Results of *B. pertussis* Bacterial Suspensions in Different Steps of the Study

Strains	Before Inactivation	Inactivation Status	After Inactivation		
	Agglutinins		Strain 509	Strain 134	Pooled Suspensions
			Agglutinins		
		F1	1, 2	1, 3	1, 2, 3
		F2	1, 2	1, 3	1, 2, 3
		F3	1, 2	1, 3	1, 2, 3
		F4	1, 2	1, 3	1, 2, 3
509	1, 2	F5	1, 2	1, 3	1, 2, 3
		F6	1, 2	1, 3	1, 2, 3
		F7	1, 2	1, 3	1, 2, 3
		F8	1, 2	1, 3	1, 2, 3
		F9	1, 2	1, 3	1, 2, 3
		F10	1, 2	1, 3	-
		F11	1, 2	1, 3	1, 2, 3
		F12	1, 2	1, 3	1, 2, 3
		F13	1, 2	1, 3	1, 2, 3
		F14	1, 2	1, 3	1, 2, 3
134	1, 3	F15	1, 2	1, 3	1, 2, 3
		F16	1, 2	1, 3	-
		F17	1, 2	1, 3	1, 2, 3
		F18	1, 2	1, 3	1, 2, 3
		F19	1, 2	1, 3	1, 2, 3
		F20	1, 2	1, 3	1, 2, 3
		F21	1, 2	1, 3	1, 2, 3

pertussis during the inactivation conditions of F16 to F21.

The overall toxicity test on inactivated bacterial suspension pools by the MWGT method after the inactivation process showed that various inactivation condition parameters had different influence patterns on the toxicity of bacterial suspensions (Table 4). Previous studies showed that in the MWGT, the early deaths of mice is a sign of the dermonecrotic toxin (DNT) content of the vaccine; the endotoxin content is responsible for the weight loss at 24 hours, and the leucocytosis promoting factor (LPF) content is the reason for the reduced rate of late weight gain (13, 31). Results of this work revealed that inactivated bacterial suspensions F₅, F₁₁, F₁₃, F₁₄, and F₂₁ resulted in early weight loss while F₁, F₃, F₇, F₉, F₁₈, and F₁₉ caused a reduced rate of late weight gain. The bacterial suspensions inactivated under the conditions of F₂, F₄, F₈, F₁₂, F₁₅, and F₁₇ were passed through the toxicity test from the points of weight gain pattern and death of the mice. In all of the other formulations, except to these six selected bacterial suspen-

sions that passed toxicity test, either deaths or undesirable weight gain pattern were observed.

For a long time, manufacturers have used the Kendrick test for potency analysis of *B. pertussis* cellular vaccines (1, 32, 33). The six selected inactive bacterial suspension pools (F₂, F₄, F₈, F₁₂, F₁₅ and F₁₇) that passed the toxicity test were comforted under potency estimation. Table 4 shows the ED₅₀ of these bacterial suspensions calculated. The results presented in this table showed that the ED₅₀ of selected formulations had the following order: F₁₇ > F₁₂ > F₈ > F₁₅, F₄ > F₂, and the bacterial suspension pool F₁₇ indicated a higher ED₅₀ (1:333 of a human dose) compared to other formulations.

Improving the presently accessible wP vaccine is necessary, especially in regard to decreasing toxicity while preserving the vaccine's efficacy. This aim is achievable if attention is given to procedures used in detoxifying all the toxins of *B. pertussis*, without destroying its immunogenicity (12, 34). The results obtained in this study revealed high

Table 4. The in Vivo Toxicity (MWGT) and Potency Test of Different Pooled Inactivated *B. pertussis* Suspensions

Pooled Killed <i>B. pertussis</i> Bacterial Suspensions	Average Weight Gain (g) Per Mouse			Death Number and Days
	Day 3	Day 7	Percent Weight Gain Relative to the Control	
Toxicity (MWGT) Test				
F1	0.8	0.3	26.1	1 (day 4)
F2	0.8	1.1	95.6	-
F3	1.15	0.1	8.7	1 (day 4), 1 (day 5)
F4	0.3	0.8	69.6	-
F5	0.3	0.6	52.2	2 (day 4)
F6	0.8	0.6	52.2	2 (day 5)
F7	0.95	0.3	26.1	1 (day 2), 1 (day 6), 1 (day 7)
F8	0.8	0.75	65.2	-
F9	0.9	-0.3	-26.1	1 (day 4), 1 (day 6)
F11	-0.75	0.95	82.6	1 (day 5)
F12	0.0	0.75	65.2	-
F13	-0.5	0.4	34.8	1 (day 4), 1 (day 6)
F14	-0.45	0.5	43.5	1 (day 3)
F15	0.0	1.25	108.7	-
F17	0.9	0.75	65.2	-
F18	0.8	-0.5	-43.5	2 (day 4), 1 (day 6)
F19	0.2	0.1	8.7	1 (day 2), 1 (day 4), 1 (day 5)
F20	0.25	0.25	21.7	1 (day 3)
F21	-0.25	0.05	4.3	1 (day 4)
Control	1.25	1.15	-	-
Pooled Killed <i>B. pertussis</i> Bacterial Suspensions	Dose (mL)	Challenge Dose (LD ₅₀)		ED ₅₀ (Human dose)
Potency Test				
F2	0.5	100		1:62
F4	0.5	100		1:83
F8	0.5	100		1:100
F12	0.5	100		1:200
F15	0.5	100		1:83
F17	0.5	100		1:333

immunogenicity for an inactivated bacterial suspension pool in F₁₇, which had the smallest bacteria content. It seems that the inactivation condition of F₁₇ is able to detoxify *B. pertussis* toxins without destroying the potency of the vaccine.

It can be concluded that the inactivating condition of F₁₇ successfully inactivates *B. pertussis* suspension. This formulation also showed desirable outcomes in the overall toxicity test and good immunogenicity with low bacteria content. Therefore, less adverse effects and better im-

munogenicity are foreseeable for vaccine preparation using this method.

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Footnotes

Authors' Contribution: Naser Mohammadpour Dounighi, Fereshteh Shahcheraghi, Mehdi Razzaghi-Abyane, Mojtaba Nofeli, and Hossein Zolfagharian planned the study; Naser Mohammadpour Dounighi performed the experimental work; Naser Mohammadpour Dounighi and Mojtaba Nofeli extracted the data and wrote the first draft of the paper; Naser Mohammadpour Dounighi, Fereshteh Shahcheraghi, Mehdi Razzaghi-Abyane, Mojtaba Nofeli, and Hossein Zolfagharian participated in the writing of the final article.

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