Evaluation of respiratory model employing conventional NIH mice to access the immunity induced by cellular and acellular pertussis vaccines

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The increasing number of pertussis cases reported on the last twenty years and the existence of new acellular vaccines reinforce the need of research for experimental models to assure the quality of available pertussis vaccines. In this study, allotments of whole-cell and acellular pertussis vaccines were tested through the Intranasal Challenge Model (INM) using conventional NIH mice. The results have been compared to those achieved by the "Gold standard" Intracerebral Challenge Model (ICM). In contrast to ICM, INM results did not show intralaboratorial variations. Statistical analysis by Anova and Ancova tests revealed that the INM presented reproducibility and allowed identification and separation of different products, including three-component and four-component accellular pertussis vaccines. INM revealed differences between pertussis vaccines. INM provides lower distress to the mice allowing the reduction of mice number including the possibility of using conventional mice (less expensive) under non-aseptic environment. Thus, INM may be used as an alternative method of verifying the consistence of allotment production, including acellular pertussis vaccines.

Key words: pertussis - vaccine - intranasal challenge model - NIH conventional mice

Pertussis continues to be a significant cause of morbidity and mortality among nonimmunized young infants worldwide. Reported cases of pertussis represent only a fraction of the actual number of infections. Possible explanations for failure to diagnose pertussis include the heterogeneity in pertussis disease expression and low physician awareness and index suspicion, including poorly performed laboratorial tests or their lack of availability (Guiso 2001, Cherry et al. 2005).

Due to the side effects of whole-cell pertussis vaccines (Pw) and the difficulties to produce them in a reproducible manner, researches were undertaken in order to develop new pertussis vaccines as effective but better tolerated. Although the implementation of childhood pertussis immunization programs has significantly reduced the occurrence of the disease in children, there is a change on transmission instead child to child transmission. Waning vaccine-induced immunity permits the disease to affect adolescents and adults, who in turn transmit pertussis to unimmunized and incompletely immunized infants, showing changes in disease epidemiology. For this reason Pw were quickly replaced by acellular pertussis vaccines (Pa) and boosters were introduced for adolescents and adults (Forsyth et al. 2005, Greenberg & Caro 2005, Tan & Plotkin 2005). Due to this change in pertussis vaccine it is now important to evaluate the reproducibility of the new vaccines.

Besides the ethical question on doing clinical studies employing nonimmunized infants with new formulas proposed every year, the increasing number of reported pertussis cases reinforces the need to attempt new methodologies to assure the immunogenic activity for vaccines against *Bordetella pertussis* (Eude et al. 1999, Guiso et al. 1999, Canthaboo et al. 2000a,b). These facts emphasize the need of searching for experimental models to efficiently demonstrate relation on field, bringing information about the mechanisms and agents involved in human disease (Mills et al. 1998).

The recently proposed Intranasal Model (INM) is a test relatively simple, with accuracy, and with high reproducibility. In contrast to Intracerebral Model (ICM), this respiratory model presents the possibility to evaluate acellular vaccines available in the market as news formulations in terms of the antigen composition and their concentration (WHO 1998). It can also be used on the evaluation of changes in the production process, vaccine stocks and methodology. Other use for this method would be the comparison among the *B. pertussis* strains used in the vaccine formulation and the bacterial isolates from the population, to verify the lot-to-lot consistency, prelicensing assays and to bring light to the better knowledge of the components involved in *B. pertussis* infection and immune response (Corbel et al. 1999a,b, Andre et al. 2000, Vandebriel et al. 2003). The humoral and cellular immunity plays an important role for complete elimination of the infection in mice in INM but not in ICM potency test (Atkinson et al. 2000, Coutte et al. 2003). Previous reports also demonstrated a high correlation between the

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lung clearance in mice and the efficacy of the vaccine in children (Mills et al. 1993, 1998, Watanabe et al. 2002a,b). Systemic and local reactions (infection of respiratory tract, pneumonia, and leucocytosis including the reversions of the disease) in INM test approaches to what happen in human pertussis disease.

During the last two decades of the XX century, the number of reported cases increased in all age groups, including adolescents and adults, indicating resurgence of the disease in developed countries. Since mass immunization in Brazil began only in the 1980s, one cannot rule out the endemicity and the possibility of pertussis re-emergence in the near future. Therefore, it is important that public health services closely monitor the epidemiological situation of pertussis in order, if necessary, to rapidly update the current immunization strategy (Luz et al. 2003). Thus research for less expensive and simple potency tests for pertussis vaccines would be helpful. In the present study, we examined the INM using conventional NIH mice for its potential utility in assessment of the immunity induced by cellular and acellular vaccines combinations.

MATERIALS AND METHODS

Animals - Conventional NIH Mice, both sexes, 12 to 16 g, from Cecal-Fiocruz (Centro de Criação de Animais de Laboratório) were used in these experiments. All the procedures using laboratory animals were taken following SOP: Boas Práticas em Experimentação Animal no. 65.3340.022, INCQS (2005). The study protocol, number P0133-02, was approved by the Fiocruz Ethic Commitee for Animal Experiments.

Bacterial reference strain and culture medium - B. pertussis 18323 (ATCC 9797 Type strain) from American Type Culture Collection was obtained from the Laboratory of References Microorganisms-INCQS. Bacterial strain was consecutively cultured on sheep blood Bordet-Gengou (BG) medium added 400 mg/l cycloheximide (McGinnis 1980) for 48 h, 24 h, and 18 h and then used in both ICM and INM experiments (WHO 1990).

Vaccines - The reference pertussis vaccine used on this study for both INM and ICM methods was Lot 10 – freeze-dried non-adsorbed preparation – from Federal and Drugs Administration – FDA/US. The vaccines samples used in the INM included pertussis whole cell (Pw) – adsorbed preparation – lot produced in Butantan Institute (strain: 137 – AGG I, II, III, IV, V and VI from NIH/US) – SP, Brazil and Acellular pertussis vaccines (Pa) – Europe. Pa lots were divided into Pa3 and Pa4 due to preparation differences per dose (Pa3 – 25 µg of Pertussis toxin, 25 µg of FHA, and 8 µg of Pertactin; Pa4 – 10 µg of Pertussis toxin, 5 µg of FHA, 3 µg of Pertactin, and 5 µg of Fimbriae). Experiments included negative control groups of mice inoculated with saline solution 0.85%. All vaccines tested were previously approved by the national authority – INCQS-Fiocruz and Brazilian National Immunization Program.

Evaluation of the immunity induced by cellular and acellular vaccines combinations ICM - The Kendrick test was performed as described by WHO (1990). In brief, groups of NIH mice from local sources were immunized intraperitoneally with serial dilutions of the test and reference vaccine preparation. The intracerebral challenge was carried out two weeks after immunization. The number of protected mice was recorded up to two weeks after challenge. The potency of the test vaccine was calculated by means of probitos assay against the reference preparation.

INM-NIH conventional mice - Experiments were carried out in triplicate and based in methods previously described by Guiso et al. (1999) and Guiso (2001) using conventional mice. Similarly to the metodology described for the Kendrick test, NIH mice groups, 12 mice each group were inoculated intraperitoneally with 0.5 ml from the ¼ dilution of the test and reference vaccines (Mills et al. 1998, Xing et al. 1999, Andre et al. 2000, Canthaboo et al. 2000b, Watanabe et al. 2002a,b). A booster dose was given two weeks later. Two weeks after the booster dose, the animals were anesthetized by ether inhalation and 25 µl of *B. pertussis* suspension (10^8 CFU/ml) was instilled into one nostril of each mouse. Infected mice were killed by cervical dislocation – 3 animals per cage – 1 day, 7 days and 15 days post-challenge. Groups of 12 mice inoculated with saline solution were also included as control in all performed experiments.

After ablation, the right lung of each animal was homogenized in 1 ml sterile 0.85% NaCl saline solution, 10-fold serial dilutions of homogenates were plated on BG and incubated at 36ºC for five days for determination of viable counts (Colony Forming Units - CFU). The mean number and standard deviation of the log of CFU/mice was calculated for each time point.

Statistical analysis - Each lot of reference, Pw, Pa3, Pa4 vaccines was tested at least four times. Analysis of variance (Anova) and covariance (Ancova) were carried out using the Statistica for Windows – Release 4.0 Program – ©Statsoft Inc. 1993 to compare means of experiments. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In mice, the ICM test shows only local reactions, mainly a fatal cerebral edema, which is the base for the dose-response curve to access the potency of Pw vaccine (Canthaboo et al. 2000b). The current potency ICM test for Pw vaccines has considerable disadvantages as it uses a severe challenge procedure and the results tend to show significant intra and interlaboratory variation. The results from the evaluation of potency of different lots of Pw vaccines by ICM are represented in Fig. 1. Interlaboratorial differences were observed among different lots of Pw analyzed mainly in lots G, L, M, Q, R, and V. Evaluation by the Kendrick test also showed significant intralaboratory variation. The minimum requirements (≥ 8 UI/ml) were reached in all lots tested. The statistical analysis showed similarities between data (UI/ml) obtained by the ICM-local test (mean: 13.27 ± 3.82) and ICM-producer test (mean: 14.29 ± 4.63) (Anova; p 0.84).

In the INM the number of animals and the distress is much lower than ICM challenge (Xing et al. 1999). In at-

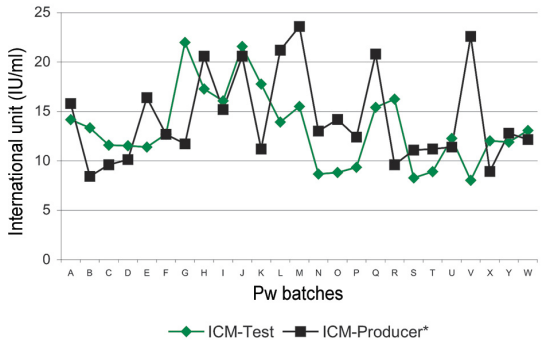

Fig. 1: graph of the results of intracerebral challenge (ICM) conventional mice of Pw vaccines. Each point represents the value of the potency of each tested lot (UI/ml) able to protect the mice. The values were defined in face of reference vaccine. Assays performed in groups (A to W) of 60 mice. *Results given by Butantan Institute, São Paulo.

tempt to turn this respiratory model less expensive and easily handled in non-aseptic environmental conditions, we evaluated the use of conventional NIH mice in INM challenges. The INM model may be used to compare the immunity induced by different vaccines but cannot quantify their potency for this moment.

Here in, we also would like to emphasize that INM-conventional NIH mice test procedures required the following conditions: (i) selection of *B. pertussis* strain and growth conditions for enhancement of the expression of adhesins and toxins; (ii) use of fresh bacterial suspensions since frozen *B. pertussis* cells were unable to infect the respiratory tract of mice; (iii) use of cycloheximide for inhibition of contamination of culture medium.

Results of statistical analysis (Table I) indicate similarities in number of viable bacteria (CFU) obtained from lungs of infected mice within unimmunized control groups (Ancova; p 0.1919) obtained by INM.

The number of viable bacteria obtained from lungs of infected mice immunized with reference vaccine LOT 10/FDA in INM is represented in Table II. Statistical differences (Anova; p 0.0000) were found among CFUs obtained from mice lungs at 1, 7, and 15 day(s) post infection: (4.03Log CFU > 2.95Log CFU > 0.12Log CFU, respectively). Comparative analysis among mice groups (R1 to R8) revealed similar results (Ancova; p 0.05) demonstrating the reproducibility of the INM-conventional NIH mice. As shown in Tables I and II, the mean number of viable bacteria in the mice control group (4.48Log CFU) was similar to mice group inoculated with reference vaccine (4.03Log CFU) at the first day after INM-conventional NIH mice challenge. In contrast to the control mice group (4.46Log CFU), a lower number of viable bacteria in the lungs of vaccinated mice were observed in the 7th day after challenge (2.95Log CFU). Increased differences were detected at the 15th day after challenge with 0.12Log CFU and 3.83Log CFU in vaccinated and control group, respectively. These results showed the ability of the INM-conventional NIH mice to verify the efficacy of the reference pertussis vaccine to promote lung clearance.

The mean number of viable bacteria in Pw 1 to Pw 9 mice groups inoculated with cellular pertussis vaccine is presented in Table III. Statistical analysis revealed similarities among results of assays performed with Pw, indicating the reproducibility of the assays (Ancova, p 0.2342). The number of viable bacteria determined at the first day after infection with *B. pertussis* of Pw mice groups was 4.30Log CFU. These results were similar to those obtained with the control mice group (Table I; 4.48Log CFU) and mice group immunized with the reference vaccine (Table II; 4.03Log CFU). A decrease in number of viable bacteria was observed at the 7th and 15th days after infection, represented by 3.23Log CFU and 1.56Log CFU, respectively. Data reinforce the ability of the INM using conventional

TABLE I

Number of viable (CFU) *Bordetella pertussis* cells obtained from mice control group 1, 7, and 15 days after infection

Mice control groups[a]	Log CFU		
	1st day	7th day	15th day
C1	3.92	4.34	2.33
C2	4.24	4.75	4.44
C3	5.50	4.30	4.12
C4	4.33	4.11	4.21
C5	4.32	4.26	4.21
C6	4.13	4.21	3.80
C7	4.19	4.78	3.79
C8	4.81	4.56	3.63
C9	5.22	4.60	4.13
C10	4.19	4.77	3.70
C Mean	4.48 ± 0.51	4.46 ± 0.23	3.8 ± 0.59

CFU: Colony Forming Units; experiments were done in triplicate; *a*: mice inoculated with saline solution; ANCOVA, p 0.1919; ANOVA among the groups of days after challenge 1 and 7 and 15, p 0.006868; between days 1 and 7, p 0.9246; between days 7 and 15, p 0.0060.

TABLE II

Number of viable (CFU) *Bordetella pertussis* cells obtained from mice immunized with reference vaccine 1, 7, and 15 days after infection

Reference vaccine mice groups	Log CFU		
	1st day	7th day	15th day
R1	3.67	2,80	0,00
R2	4.18	3,00	0,00
R3	3,25	3,48	0,00
R4	3,84	3,49	0,00
R5	4.70	2,34	0,00
R6	4.23	2,64	0,00
R7	4.77	3,45	1,00
R8	3,60	2,47	0,00
R Mean	4,03 ± 0.53	2,95 ± 0.46	0.12 ± 0.35

CFU: Colony Forming Units; experiments were done in triplicate; Reference vaccine (Lot 10/FDA) prepared at 10 IU/ml; ANCOVA, p 0.5424; ANOVA days 1 and 7 and 15, p 0.00000.

NIH mice to verify the efficacy of Pw vaccine to promote lung clearance.

Table III reveals the number of viable bacteria (4.30Log CFU) determined at the first day after infection with *B. pertussis* of Pw mice groups. These results were similar to those obtained with reference vaccine mice groups (4.03Log CFU). A decrease in number of viable bacteria was observed at the 7th and 15th days after infection, represented by 3.23Log CFU and 1.56Log CFU for Pw group and 2.95Log CFU and 0.12Log CFU for reference group, respectively (Anova test p 0.0999 Table VI).

Table IV shows the mean number of viable bacteria in infected mice groups immunized with Pa3 vaccine. A decrease in the mean number of viable bacteria determined at the 1st, 7th, and 15th days after infection with *B. pertussis* of Pa3 mice groups was 4.27Log CFU, 3.71Log CFU, and 1.67Log CFU, respectively.

Table V present data of INM-conventional NIH mice immunized with component Pa4 vaccine. The mean number of viable bacterial cells determined at the 1st, 7th, and 15th days after infection was 4.34Log CFU, 4.28Log CFU, and 3.42Log CFU, respectively. These results were similar to those obtained with the mice control group.

TABLE III

Number of viable (CFU) *Bordetella pertussis* cells obtained from mice immunized with cellular pertussis vaccine (Pw) 1, 7, and 15 days after infection

	Log CFU		
Pw mice groups	1st day	7th day	15th day
Pw 1	3.62	1.67	1.80
Pw 2	4.79	3.78	2.77
Pw 3	4.20	3.84	0.00
Pw 4	4.76	3.88	0.00
Pw 5	4.20	3.84	3.77
Pw 6	4.30	2.91	2.47
Pw 7	3.95	2.85	0.00
Pw 8	4.25	2.91	1.00
Pw 9	4.65	3.40	2.30
Pw Mean	4.30 ± 0.38	3.23 ± 0.73	1.56 ± 1.38

CFU: Colony Forming Units; experiments were done in triplicate; ANCOVA, *p* 0.2342; ANOVA, days 1 and 7 and 15, p 0.0000087.

TABLE IV

Number of viable (CFU) *Bordetella pertussis* cells obtained from lungs of mice immunized with three component acellular pertussis vaccine (P_A3) 1, 7, and 15 days after infection

	Log CFU		
P_A3 mice groups	1st day	7th day	15th day
P_A3-1	3.49	3.66	0.00
P_A3-2	4.09	3.61	3.05
P_A3-3	5.14	3.38	2.00
P_A3-4	4.16	4.72	2.15
P_A3-5	4.48	3.19	1.15
P_A3 Mean	4.27 ± 0.59	3.71 ± 0.21	1.67 ± 1.15

CFU: Colony Forming Units; experiments were done in triplicate; ANCOVA, *p* 0.2779.

Mean number of viable (CFU) *B. pertussis* cells obtained from lungs of NIH conventional mice inoculated with saline, reference vaccine, Pw, Pa3, and Pa4 is represented in Fig. 2. Similar numbers of viable bacterial cells were observed at 1st day after infection for all products tested. Significant differences observed at 7th and 15th day after infection yielded differentiation among products tested (Table VI).

Statistical analysis by Ancova test indicated the consistency ($p > 0.05$) of INM-conventional NIH mice for each product (saline control, p 0.1919 – reference vaccine, p 0.5424 – Pw, p 0.2342 and Pa3, p 0.2779).

Significant differences were observed among INM-conventional NIH mice results for Pa4 when compared with other products (Anova; reference vaccine, *p* 0; Pw, *p* 0.02; Pa3, *p* 0.02) (Table VI). The performance of Pa4 vaccine at INM method brings it near to saline control group (Anova; p 0.26) (Fig. 2). Although significant, the difference between the data obtained with Pa3 and Pa4 component vaccines could be partially explained by differences in antigen concentration and the fact that the antigens are not similarly adsorbed on the adjuvant. For this reason it will be of importance to repeat experiments with INM to compare both vaccines by immunizing both vaccines with non diluted vaccines.

Comparative analysis revealed equivalent performance of INM-conventional NIH mice and the "gold standard" ICM method for determining the ability of cellular vaccine to protect mice against *B. pertussis* infection (unshown data).

TABLE V

Number of viable (CFU) of *Bordetella pertussis* cells obtained from lungs of mice immunized with four component acellular pertussis vaccine (P_A4) 1, 7 and 15 days after infection

	Log CFU		
P_A4 mice groups	1st day	7th day	15th day
P_A4-1	4,14	4,49	3.00
P_A4-2	3,62	3.69	2,82
P_A4-3	5.08	4,79	4,08
P_A4-4	4.54	4.18	3,78
P_A4 Mean	4.34 ± 0.61	4.28 ± 0.48	3.42 ± 0,60

CFU: Colony Forming Units; experiments were done in triplicate; ANCOVA, *p* 0.00042

TABLE VI

Statistical analysis by of the Intranasal model results among mice groups inoculated with saline, reference vaccine, cellular vaccine (Pw), three component acellular vaccine (P_A3) and four component acellular vaccines (P_A4)

	P values			
Products	Pw	P_A3	P_A4	Saline
Saline	0.0001	0.0002	0.2600	-
Reference vaccine	0.0999	0.0021	0.0000	0.0000
Pw	-	0.7916	0.0286	0.0001
P_A3	-	-	0.0294	0.0002

Experiments were done in triplicate; ANOVA (p 0.05)

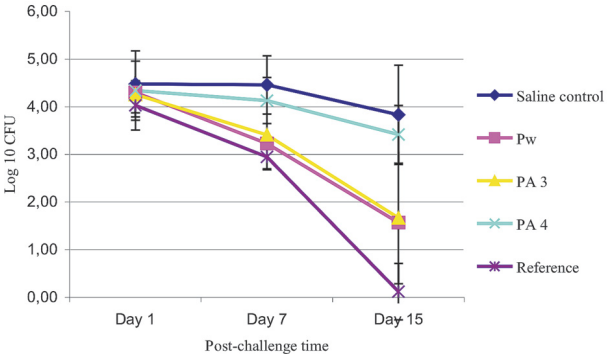

Fig. 2: graph of the mean of viable bacteria (Log CFU) in mice groups inoculated with saline solution, reference vaccine, cellular vaccine (Pw), three component acellular vaccine (P_A3) and four component acellular vaccine (P_A4) at the intranasal challenge (INM) conventional mice.

The potency test using conventional NIH mice was also able to demonstrate a higher performance of cellular vaccines when compared with Pa4 acellular composition. The INM-conventional NIH mice differentiated Pa3 and Pa4 acellular pertussis compositions. Pa3 showed higher performance in face of Pa4. Data indicate the ability of INM-conventional NIH mice to demonstrate differences in antigen concentrations of pertussis compositions.

Thus the results indicate the INM – conventional NIH mice as an alternative method for evaluation of immunity induced by both cellular and acellular pertussis vaccines. Notwithstanding the results obtained on this and other studies, there is still a need of reference vaccines (whole-cell and acellular, according to its composition) established by this method and with proved efficiency on field trials (Mills et al. 1998, Andre et al. 2000). Some conclusions concerning the differences between pertussis vaccines cannot be reached without repeating some experiments in a different way and comparing with the epidemiology in the field.

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