Preliminary study on the immunogenicity of a newly developed GCC Tdap vaccine and its protection efficacy against *Bordetella pertussis* in a murine intranasal challenge model

**Purpose:** Active reduced dose tetanus-diphtheria-acellular pertussis (Tdap) vaccination for adolescents and adults is necessary because waning immunity after primary diphtheria-tetanus-pertussis vaccination is related to the recent emergence of pertussis. This study was conducted to compare the immunogenicity and protection efficacy against *Bordetella pertussis* between a new GCC Tdap vaccine and a commercially available Tdap vaccine in a murine model.

**Materials and Methods:** BALB/c mice were immunized with two doses of diphtheria-tetanus-acellular pertussis (DTaP) vaccine for priming and a subsequent Tdap booster vaccination. According to the type of booster vaccine, mice were divided into four groups: commercially available Tdap vaccine in group 1 and GCC Tdap vaccines of different combinations of pertussis antigens in groups 2 to 4. Humoral and cell-mediated immune responses and protection efficacy using a murine intranasal challenge model after booster vaccination were compared among the four groups.

**Results:** Every group showed significant increases in antibody titers against pertussis antigens such as pertussis toxin, filamentous hemagglutinin, and pertactin after booster vaccination. Spleen cells showed both Th1 and Th2 cell-mediated immune responses stimulated by pertussis antigens in all groups without any significant difference. In the intranasal *B. pertussis* infection model, bacteria were eradicated in all groups five days after challenge infection.

**Conclusion:** This preliminary study did not show significantly different immunogenicity or protection efficacy of the new GCC Tdap vaccines compared to the commercially available Tdap vaccine, although a more extensive study is necessary to assess the differing efficacies of the new GCC Tdap vaccines.

**Keywords:** Diphtheria-tetanus-acellular pertussis vaccine, Immunogenicity, Efficacy, Mice, Republic of Korea

**Introduction**

Pertussis is caused by *Bordetella pertussis*, which exclusively infects humans, and is a highly contagious respiratory disease. Pertussis had decreased with the introduction of the diphtheria-tetanus-pertussis (DTP) vaccine, although it has since re-emerged in the 1990s, even in countries where the DTP vaccination coverage rate is greater than...
90% [1-3]. Infants who have not received the primary series of DTP vaccination are particularly vulnerable to pertussis infection [1-3], and adolescent and adult patients with mild or no symptoms act as a source of transmission. This re-emergence of pertussis is assumed to be caused by waning immunity after primary DTP vaccination during childhood, increase in the diagnoses of pertussis patients with the improvement of diagnostic tests, increased attention to pertussis in the community, and the emergence of vaccine-resistant B. pertussis strains. To overcome these epidemiological changes in pertussis, reduced dose tetanus-diphtheria-acellular pertussis (Tdap) vaccination for adolescents was introduced, and new vaccines against the emerging vaccine-resistant strains are necessary [4-7].

In Korea, the number of pertussis patients has been increasing since the late 2000s: nine patients in 2008, 66 in 2009, 27 in 2010, and 97 in 2011, as well as local outbreaks reported in 2012 [8,9]. Hence, the use of Tdap boosting vaccinations for adolescents and adults should be emphasized in Korea. However, no domestic pharmaceutical company produced Tdap vaccines until recently, which raises the concern of possible vaccine shortages. The Green Cross Corporation (GCC, Yon-gin, Korea), a Korean domestic pharmaceutical company, has developed a new Tdap vaccine, the GCC Tdap vaccine. This study was conducted to compare the immunogenicity among a commercially available Tdap vaccine (Boostrix, GlaxoSmith-Kline, Rixensart, Belgium) and the GCC Tdap vaccine containing different combinations of pertussis antigens. Furthermore, protective efficacy against B. pertussis in a murine respiratory challenge model was compared between the two vaccines.

Materials and Methods

Animals and immunization
BALB/c female mice (4-week-old) were used in this study and were housed in filter-top cages under standard pathogen-free conditions with food and water available ad libitum. The mice were divided into four groups according to the type of booster vaccination received, and each group included 16 mice. All mice received two doses of primary diphtheria-tetanus-acellular pertussis (DtaP) vaccine with Infanrix (GlaxoSmithKline, Rixensart, Belgium) at three-week intervals. Booster vaccines were given at six weeks after the second dose of primary vaccination. Group 1 was a positive control group, in which Boostrix was given as the booster vaccine. Groups 2 to 4 were study groups, and the doses of pertactin (PRN) differed among the three study groups. Group 2 received a standard GCC Tdap vaccine containing the same three pertussis antigens as in Boostrix: pertussis toxin (PT) 8 μg, filamentous hemagglutinin (FHA) 8 μg, and PRN 2.5 μg. Groups 3 and 4 received a moderate dose GCC Tdap vaccine (PT 8 μg, FHA 8 μg, PRN 4.0 μg) and a high dose GCC Tdap vaccine (PT 8 μg, FHA 8 μg, PRN 8.0 μg), respectively. All vaccines were administered intraperitoneally at one-fourth the human dose. This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) in School of Medicine, The Catholic University of Korea (approval number: CUMC-2013-0029-02).

Assessment of humoral immune responses
Blood samples to evaluate the humoral immune response to vaccination were collected before vaccination, two weeks after the first dose of primary vaccination, one week before booster vaccination, and three weeks after booster vaccination. Blood was withdrawn from the retro-bulbar venous plexuses of 10 mice in each group at each scheduled time. Antibody titers against three pertussis antigens (PT, FHA, and PRN) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Alpha Diagnostic International Inc., San Antonio, TX, USA). At each scheduled time, antibody titers against each antigen were compared among the four study and control groups.

Assessment of cell-mediated immune responses
Cell-mediated immune response (CMI) was determined by measuring cytokine levels secreted by spleen cells after stimulation by pertussis antigens. Four mice in each group were sacrificed and spleens were taken at three weeks after booster vaccination. The removed spleens were homogenized to form a single cell suspension of spleen cells (2.0×10^6 cells/mL in RPMI 1640 medium, Life Technologies, Rockville, MD, USA). Each spleen cell suspension was stimulated with PT (5 μg/mL), FHA (5 μg/mL), PRN (5 μg/mL), and a mixture of the three pertussis antigens and then duplicate cell cultures were incubated in a 37°C, 5% CO₂ environment for 72 hours. After incubation, the supernatants were extracted and stored at −70°C. The stored supernatants were then thawed, and concentrations of Th1 cytokines, such as interferon-γ (IFN-γ) and interleukin (IL)-2, and Th2 cytokines, such as IL-4, IL-5, and IL-10, were measured using commercial ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). The levels of each cytokine were compared among the four groups.
Intranasal infection with *B. pertussis*

Mice were intranasally challenged three weeks after booster vaccination. A *B. pertussis* strain obtained from an adult pertussis patient was grown on a Regan-Lowe agar plate, and the grown *B. pertussis* strains were diluted with phosphate-buffered saline (PBS) to form a suspension of $1.2 \times 10^8$ colony-forming units (CFUs)/mL. Fifty microliters of the suspension were administered into the nostrils of mice using a micropipette under peritoneal anesthesia with Zoletil/xylazine. Three mice from each group were sacrificed at 2 hours, 5 days, and 10 days after intranasal infection, and their lungs were removed. The removed lungs were homogenized in 10 mL of PBS, and the homogenates were diluted to $10^{-1}$, $10^{-3}$, and $10^{-5}$ concentrations. The diluted homogenates were inoculated on Regan-Lowe agar plates, and duplicate cultures were incubated at 36°C for four days. The mean numbers of CFUs in each group at each time were compared.

Statistical analysis

The mean levels of antibody titers against each pertussis antigen were compared among the four groups using a Kruskal Wallis test at each sampling time. The mean cytokine concentrations in the CMIs were compared among the four groups and the mean CFUs in the intranasal infection model were also compared among the four groups at each time using a Kruskal Wallis test. Statistical analysis was performed with SPSS Statistics version 17.0 (SPSS Inc., Chicago, IL, USA), and statistical significance was defined as a two-tailed $p<0.05$.

Results

Humoral immune responses

The mean titers of anti-PT IgG increased to more than 2,500 U/mL, while mean titers of anti-FHA IgG increased to more than 20 U/mL after the first dose of primary DTaP vaccination in all groups. These titers further increased after the second dose of primary DTaP vaccination in all groups, increasing much more sharply with Tdap booster vaccination (Table 1, Fig. 1). The final anti-PT IgG and anti-FHA IgG titers after booster vaccination showed no significant difference among the four groups (Fig. 1).

The mean titers of anti-PRN IgG also increased to greater than 50 U/mL in all groups after the first dose of primary vaccination and then increased sharply with booster vaccination (Table 1, Fig. 1). However, unlike the anti-PT and anti-FHA IgG titers, anti-PRN IgG titers were lower at one week before booster vaccination compared with those at two weeks after the first dose of primary DTaP vaccination in all groups (Table 1).

Th1 CMIs (IFN-γ and IL-2)

The IFN-γ levels after stimulation by PT, FHA, PRN, and mixed antigen were not significantly different among the four groups, and most IFN-γ levels were less than 10 pg/mL (Fig. 2). The IL-2 levels of the four groups were also not significantly different, although most levels were greater than 40 pg/mL (Fig. 2).

| Table 1. Results of humoral immune responses against pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN) |
|---------------------------------------------------------------|
| **Group 1** | **Group 2** | **Group 3** | **Group 4** | **p-value** |
|----------------------|-------------|-------------|-------------|-----------|
| **Anti-PT IgG titer (U/mL)** | | | | |
| Before vaccination | 22 ± 15 | 11 ± 11 | 12 ± 19 | 21 ± 35 | 0.292 |
| Two weeks after first vaccination | 3,525 ± 2,359 | 2,565 ± 1,783 | 5,454 ± 3,233 | 6,455 ± 3,149 | 0.028 |
| One week before booster vaccination | 22,900 ± 14,273 | 1,340 ± 2,996 | 7,520 ± 9,049 | 44,580 ± 99,684 | 0.047 |
| Three weeks after booster vaccination | 1,917,420 ± 1,086,900 | 1,660,900 ± 454,195 | 2,484,250 ± 826,771 | 2,397,000 ± 784,952 | 0.266 |
| **Anti-FHA IgG titer (U/mL)** | | | | |
| Before vaccination | 1 ± 1 | 1 ± 1 | 0 | 0 ± 1 | 0.03 |
| Two weeks after first vaccination | 43 ± 39 | 17 ± 14 | 51 ± 32 | 62 ± 60 | 0.035 |
| One week before booster vaccination | 7,200 ± 2,887 | 3,860 ± 744 | 6,880 ± 3,813 | 6,220 ± 4,860 | 0.29 |
| Three weeks after booster vaccination | 221,640 ± 78,916 | 188,720 ± 53,207 | 207,220 ± 38,014 | 298,760 ± 238,737 | 0.934 |
| **Anti-PRN IgG titer (U/mL)** | | | | |
| Before vaccination | 45 ± 98 | 23 ± 33 | 21 ± 40 | 23 ± 25 | 0.775 |
| Two weeks after first vaccination | 67 ± 34 | 103 ± 79 | 159 ± 163 | 50 ± 42 | 0.119 |
| One week before booster vaccination | 18 ± 41 | 2 ± 4 | 166 ± 164 | 32 ± 72 | 0.016 |
| Three weeks after booster vaccination | 13,356 ± 13,389 | 17,235 ± 10,366 | 14,543 ± 10,596 | 14,510 ± 4,748 | 0.548 |

Values are presented as means ± standard deviations.
Th2 CMIs (IL-4, -5, and -10)

After stimulation by PT, FHA, PRN, and mixed antigen, most IL-4 levels were less than 10 pg/mL, most IL-5 levels were between 10 and 30 pg/mL, and IL-10 levels were near 10 pg/mL (Fig. 2). Although the IL-5 levels in groups 1 and 3 were higher than those of the other groups, there was no significant difference in the IL-4, IL-5, or IL-10 levels among the four groups (Fig. 2).

Protection efficacy against *B. pertussis* in the intranasal infection model

The mean CFUs of groups 1 to 4 were 177,000, 233,667, 212,000, and 103,667 CFUs/lung, at two hours after intranasal infection, respectively, and showed no significant difference (*p* = 0.335). At five days after intranasal infection, *B. pertussis* was completely eradicated in all groups (Fig. 3).
Discussion

Although humans are the only natural host of *B. pertussis*, murine models have been accepted as a useful animal study model evaluating pertussis vaccine immunogenicity [10-14]. However, animal studies have some limitations arising from the differences between animals and humans in vaccination route, the number and dose of vaccinations, the intervals between vaccination and blood sampling, and intrinsic immune systems. In the era that used killed whole cell pertussis vaccine, the intracerebral murine challenge model (Kendrick test) was principally used to determine vaccine potency [15,16], although the protective immune mechanisms in the intracerebral challenge model were not clearly defined [17-20]. Because of the high incidence of side effects with the whole cell pertussis vaccine, acellular pertussis (aP) vaccines have been used in most countries since the mid-1990s, and murine respiratory challenge models and humoral and cell-mediated immunity tests have been performed to determine aP vaccine potency and efficacy [10,11,21,22]. However, seed bacteria and purification as well as the detoxification methods of the pertussis antigens in each aP vaccine are different among the DTaP vaccines, and the protective immune mechanisms against pertussis have not been fully defined. Therefore, a generally accepted animal study model to assess aP vaccines has not been established. In addition, human studies to determine the clinical efficacy of Tdap vaccine have also not been standardized, although the incidence of pertussis has been increasing, especially in adolescents and adults [18]. Nevertheless, measurement of humoral and cellular immune responses against *B. pertussis* infection has been widely used to assess DTaP and Tdap vaccines because humoral immune responses and CMIs have shown a complementary role in protecting against pertussis [10,12,23]. Therefore, this study compared the new GCC Tdap vaccine and a commercially available Tdap vaccine with regard to immunogenicity based on antibody titers against pertussis antigens included in Tdap vaccines (humoral immune responses) and cytokine assays in splenocytes stimulated by pertussis antigens (CMI), as well as their protection efficacy based on an intranasal infection model. In addition, we determined an appropriate combination of pertussis antigens by comparing three study groups with different combinations of pertussis antigens.

Natural pertussis infection and diphtheria-tetanus-whole cell pertussis (DTwP) vaccination tend to activate Th1 CMI against pertussis [24], and repeat exposures to natural pertussis infection enhance the Th1 CMI [25]. However, DTaP vaccination tends to activate Th2 CMI [26,27]. In the case of primary DTwP and subsequent Tdap booster vaccination, both Th1 and Th2 CMIs were activated [28], whereas Th2 CMI was activated in the case of primary DTaP and subsequent DTaP booster vaccination [29,30]. This indicates that different CMI may be evoked according to the type of primary vaccination. Reynolds et al. [31] reported that Tdap booster vaccination following two doses of primary DTwP vaccination in mice and humans did not activate Th2 CMI, but did activate Th1 CMI, a result that was thought to be principally caused by the primary DTwP vaccination. In the present study where the Tdap booster vaccination followed two doses of primary DTaP vaccination, both Th1 and Th2 CMIs were activated despite the injection of aP. However, there was no significant difference in either Th1 or Th2 cytokine level among the four groups. Two doses of primary DTaP vaccination and a relatively short interval between the primary DTaP and booster Tdap vaccination may have reduced the effects of different combinations of pertussis antigens for Tdap booster vaccination in groups 2 to 4. Therefore, the interval between the primary and booster vaccination should be extended, and selective stimulation of T cells rather than whole splenocytes should be evaluated for comparison of CMI in future studies.

Reynolds et al. [31] also reported increases in antibody titers against pertussis antigens including PT, FHA, PRN, and fimbriae after Tdap booster vaccination in mice, and the degree of increase in anti-PT IgG titers was associated with pro-
tection against pertussis in an aerosol challenge model. The present study also demonstrated significant increases in antibody titers against pertussis antigens after Tdap booster vaccination, and the antibody titers were not significantly different among the positive control group and three study groups. In particular, anti-PRN IgG titers significantly increased with Tdap booster vaccination, although they had decreased since primary DTaP vaccination. These boosting effects of the Tdap vaccine, which includes lower doses of pertussis antigens compared with the DTaP vaccine, may be triggered by memory T cells established after primary DTaP vaccination. However, anti-PRN IgG titers were not significantly different among the study groups receiving different doses of PRN.

In the intranasal infection model, infected bacterial counts were not significantly different in the four groups, and B. pertussis was completely eradicated five days after intranasal infection in all of the four groups. From a previous study, the unvaccinated mice showed an increase of intrapulmonary bacterial count 10 days after intranasal infection, and then decreased [32]. The intrapulmonary bacterial count in DTP-vaccinated mice decreased without the early increasing phase after intranasal infection; however, a complete eradication of the intrapulmonary bacteria within eight days after infection was very rarely reported [32-35]. Considering the early eradication of infected B. pertussis in the intranasal infection and the lack of significance of different antibody titers against pertussis antigens after Tdap booster vaccination in groups receiving different combinations of antigens in the present study, primary DTaP vaccination may interfere with the Tdap booster vaccination effect. Accordingly, future studies should be performed with one dose of primary DTaP vaccination and a more prolonged interval between primary and booster vaccination.

This study has some limitations. First, as mentioned above, two doses of primary DTaP vaccination provided such a prolonged protective immunity that complete protection against intranasal B. pertussis infection was achieved after booster vaccination in all of the four groups, and therefore we could not differentiate the effects of the three study vaccines. In addition, an appropriate positive control group using universal mitogen-stimulating splenocytes and a negative control group using no antigen were not established when assessing CMI. By assessment of the humoral immune responses and bacterial eradication in the intranasal challenge model, negative control groups including exclusively unvaccinated mice and booster-vaccinated mice without primary DTaP vaccination were not established, and therefore the immunogenicity of the new Tdap vaccine could not be properly determined. Future studies including appropriate negative control groups should be performed.

In conclusion, the immunogenicity and protection efficacy against B. pertussis of the newly developed GCC Tdap vaccine were not significantly different from those of a commercially available Tdap vaccine in a murine model. However, antibody titer levels after booster vaccination were much higher than those after the first DTaP vaccination, and there was no significant difference in antibody titer levels among groups receiving different doses of included pertussis antigens. These results are assumed to be due to the two doses of primary DTaP vaccination and the relatively short interval between primary and booster vaccination. Therefore, future studies should include one dose of primary vaccination and a longer interval between primary and booster vaccination. In addition, selective separation of T cells from the spleen cell suspension should be performed in order to determine the exact cytokine levels after stimulation by pertussis antigens, reflecting the CMI against B. pertussis.

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