Immunogenicity of a bivalent killed thimerosal-free oral cholera vaccine, Euvichol, in an animal model

Purpose: An oral cholera vaccine (OCV), Euvichol, with thimerosal (TM) as preservative, was prequalified by the World Health Organization (WHO) in 2015. In recent years, public health services and regulatory bodies recommended to eliminate TM in vaccines due to theoretical safety concerns. In this study, we examined whether TM-free Euvichol induces comparable immunogenicity to its TM-containing formulation in animal model.

Materials and Methods: To evaluate and compare the immunogenicity of the two variations of OCV, mice were immunized with TM-free or TM-containing Euvichol twice at 2-week interval by intranasal or oral route. One week after the last immunization, mice were challenged with Vibrio cholerae O1 and daily monitored to examine the protective immunity against cholera infection. In addition, serum samples were obtained from mice to measure vibriocidal activity and vaccine-specific IgG, IgM, and IgA antibodies using vibriocidal assay and enzyme-linked immunosorbent assay, respectively.

Results: No significant difference in immunogenicity, including vibriocidal activity and vaccine-specific IgG, IgM, and IgA in serum, was observed between mice groups administered with TM-free and -containing Euvichol, regardless of immunization route. However, intranasally immunized mice elicited higher levels of serum antibodies than those immunized via oral route. Moreover, intranasal immunization completely protected mice against V. cholerae challenge but not oral immunization. There was no significant difference in protection between two Euvichol variations.

Conclusion: These results suggested that TM-free Euvichol could provide comparable immunogenicity to the WHO prequalified Euvichol containing TM as it was later confirmed in a clinical study. The pulmonary mouse cholera model can be considered useful to examine in vivo the potency of OCVs.

Keywords: Thimerosal, Cholera vaccines, Vaccine immunogenicity, Animal models

Introduction

Cholera is a global public health problem, especially in developing countries. Global burden of cholera was estimated 1.4 to 4.0 million cases and 21,000 to 143,000 individuals died annually in the world [1]. Since the disease is generally transmitted by contaminated water and food, access to safe drinking water and sanitation in vulnerable communities is fundamental approach to preventing the disease [2]. Currently, vaccination is the most efficient way to reduce cholera infection in endemic area as short-
to mid-term strategy [3]. Three oral cholera vaccines (OCVs), Dukoral, Shanchol, and Euvichol, have been prequalified by the World Health Organization (WHO) for use in global public health. Among them, Shanchol and Euvichol were manufactured according to the same formulation developed and technology transferred by the International Vaccine Institute (IVI), which contains heat or formalin killed *Vibrio cholerae* O1 Inaba, O1 Ogawa, and O139 with thimerosal (TM) as preservative.

TM, mercury-based preservative, has been widely used in medicinal drugs and vaccines [4]. There is no direct evidence on adverse effect of low doses of TM in vaccines. However, based on data regarding the toxicity of a related substance, methylmercury, the theoretical potential for neurotoxicity caused by low level of organomercurial compounds has been addressed as a concern [5,6]. Therefore, vaccine manufacturers and regulatory agencies in Europe and North America recommended to reduce or eliminate TM in the vaccines for safety in 1999 [7]. The WHO issued guidelines on regulatory expectations related to the elimination, reduction or replacement of TM in vaccines [8] while it continues to recommend the use of vaccines containing TM for global immunization programs, because it determined that the benefits of using such products far outweigh any theoretical risk of toxicity [9].

After the OCV Shanchol, containing TM, received WHO prequalification in 2011, due to the increasing OCV demand and unmet need in the public market, a second technology transfer from IVI to a Korea based company, EuBiologics Co. Ltd., led to the manufacturing of Euvichol with the same formulation as Shanchol. Euvichol received WHO prequalification in 2015. Since then, the OCV stockpile for use by public health agencies like WHO and United Nations Children’s Fund (UNICEF) was dramatically expanded. Here, we investigated in an animal model whether TM-free Euvichol could induce protective immunity and antibody responses against *Vibrio cholerae* comparable to the TM-containing formulation.

### Materials and Methods

#### Bacterial strain and reagents

*V. cholerae* O1 El Tor Inaba strain, T19479 was used for vibriocidal assay and bacterial challenge study. TM-containing Euvichol (Lot. No. 14001) and TM-free Euvichol (Lot. No. TF-15002) were kindly provided by EuBiologics Co. Ltd., Chuncheon, Korea

#### Immunization

All experiments were performed with approval of institutional animal care and use committees of the International Vaccine Institute (IACUC approval No. 2016-003). Seven-week-old female BALB/c mice were purchased from Koatech (Pyeongtaek, Korea). All mice were acclimated for 1 week before use. Immunization and bacterial challenge studies were carried out as previously described [10]. Briefly, mice (n=10-12 per group) were stratified into five groups: non-immunized, immunized via oral or intranasal route with either of the vaccines (TM-containing or -free Euvichol). Mice were administered with 150 µL, equivalent to 10% of vaccine dose, of Euvichol via oral route or 15 µL, equivalent to 1% of vaccine dose, via intranasal route on days 0 and 14. For bacterial challenge, mice were infected intranasally with 2.5×10⁷ colony-forming unit (CFU) of *V. cholerae* strain T19479 at 1 week after the last immunization. Blood samples were collected from non-immunized and immunized mice groups on day 21 and serum samples were separated following blood clotting at room temperature (RT) for 2 hours.

#### Enzyme-linked immunosorbent assay

Anti-*V. cholerae* IgG, IgM, and IgA against *V. cholerae* in serum were measured by enzyme-linked immunosorbent assay as previously described [11]. Briefly, 96-well plate (Nunc, Cat. 469454) were coated with 100 µL of diluted Euvichol (1:667) in phosphate buffered saline (PBS) at 4°C overnight. After blocking with 300 µL of 1% bovine serum albumin (BSA) in PBS, serum samples were serially diluted with PBS containing 1% BSA from an initial 1:50 dilution and incubated for 1 hour at RT. The plates were washed with PBS containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated anti-mouse IgG (γ chain specific, 1:5,000), anti-mouse IgM (μ chain specific, 1:5,000), or anti-mouse IgA (α chain specific, 1:5,000) at RT for 1 hour. After washing the plates, the wells were incubated with 100 µL of TMB substrate reagents for 20 minutes and 50 µL of 2 N H₂SO₄ to stop the reactions. Optical density was read at 450 nm using microplate reader. Endpoint titers were expressed as the reciprocal log₂ of the highest dilution that gave an absorbance value of >0.1. The antibody titers below 50 were regarded as 25 for statistical analysis.

#### Vibriocidal assay

Vibriocidal assay was performed as previously described [12]. Briefly, complements in mouse serum was inactivated by heating at 56°C for 30 minutes before use. Serum sample were 2-fold diluted and incubated with 100 µL of TMB substrate reagents for 20 minutes before use. Inactivated serum samples were incubated with 100 µL of *V. cholerae* at 37°C for 30 minutes and 100 µL of 2 N H₂SO₄ to stop the reactions. Optical density was read at 450 nm using microplate reader. Endpoint titers were expressed as the reciprocal log₂ of the highest dilution that gave a 50% reduction in absorbance value of >0.1. The titer below 25 were regarded as 15 for statistical analysis.

---

**Eun Young Lee et al • Effect of thimerosal on immune responses to Euvichol**

https://doi.org/10.7774/cevr.2018.7.2.104  http://www.ecevr.org/
serially diluted with 0.85% saline from an initial 1:8 dilution and 25 μL of diluted serum was added into 96-well plate (Cat. 269620, Nunc, Roskilde, Denmark). *V. cholerae* was cultivated in brain heart infusion (BHI) media at 37°C for 1.5-2 hours until reaching mid-log phase. The cultured bacteria was washed and resuspended in saline containing 1 × 10⁶ CFU/mL and 5% guinea pig complements (Rockland, Gilbertsville, PA, USA). Then, 25 μL of mixture of bacteria and complements was added to 96-well plate containing diluted serum. After incubation at 37°C for 1 hour, 150 μL of BHI media per well was added and incubated at 37°C for additional 4 hours. Optical density was read at 600 nm to measure bacterial growth using microplate reader. The vibriocidal titer was defined as the reciprocal log of highest dilution that completely inhibited bacterial growth. The vibriocidal antibody titers below 8 were regarded as 4 for statistical analysis.

### Statistics

For comparison of vaccine-induced antibody responses, one-way ANOVA was carried out, followed by Tukey’s multiple comparison, with significance determined at p < 0.05 using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

### Results

**Intranasal immunization with TM-free and -containing Euvichol protects mice against *V. cholerae* challenge**

To examine whether immunization with TM-free and -containing Euvichol could protect mice against *V. cholerae* challenge, mice were immunized with either of the two Euvichol by oral or intranasal route on days 0 and 14. One week after the last immunization, mice were intranasally challenged with a lethal dose (2.5 × 10⁷ CFU) of *V. cholerae* O1 Inaba. As shown in Fig. 1A, all non-immunized mice succumbed within 24 hours after challenge. Oral immunization with Euvichol, regardless of the presence of TM, could not provide protection to mice against *V. cholerae* challenge. However, all mice intranasally immunized with TM-containing and -free Euvichol were protected against bacterial challenge until 10 days post infection. Approximately, 5% of body weight was decreased at 1 day after *V. cholerae* challenge, but mice fully recovered their weight loss from the next day (Fig. 1B). These results suggest that TM-free Euvichol induce protective immunity against *V. cholerae* comparable to the TM-containing formulation in cholera pulmonary infection model.

#### Table 1. Serum vibriocidal titers against *Vibrio cholerae* O1 Inaba

| Immunization route | TM-free Euvichol | TM-containing Euvichol | p-value<sup>a</sup> |
|--------------------|------------------|------------------------|---------------------|
|                    | Mean titer (log<sub>2</sub>) | 95% CI | Mean titer (log<sub>2</sub>) | 95% CI |
| Intranasal          | 12.56            | 11.28-13.84            | 12.28              | 10.73-13.84 | 0.900          |
| Oral               | 6.93             | 5.00-8.86              | 4.90               | 2.66-7.14  | 0.302          |

<sup>a</sup>The p-value were calculated using one-way ANOVA with Tukey’s multiple comparison test.

Fig. 1. Intranasal immunization with thimerosal (TM)-free and -containing Euvichol protects mice against a challenge with *Vibrio cholerae* O1 Inaba. Four groups of mice (n = 10-12) were administered intranasally or orally with TM-containing or -free Euvichol on day 0 and day 14 against a control group of non-immunized mice. Survival rate (A) and percentage weight change (B) were daily monitored following challenge with a lethal dose of *V. cholerae* O1 Inaba. i.n., intranasal; TM(+), thimerosal-containing; TM(-), thimerosal free.

http://www.ecevr.org/ https://doi.org/10.7774/cevr.2018.7.2.104
Intranasal immunization with Euvichol elicited strong systemic antibody responses against *V. cholerae*

Serum vibriocidal antibody is widely used to evaluate immunogenicity of cholera vaccine in clinical trials as surrogate marker [13]. To assess the systemic antibody responses induced by immunization with Euvichol, we measured vibriocidal activity in serum obtained from mice administered with TM-containing or -free Euvichol (Table 1, Fig. 2). Sera from mice intranasally immunized with both variations of Euvichol showed significantly higher vibriocidal titers against *V. cholerae* O1 Inaba than those from orally immunized mice (TM-free, intranasal 12.56 vs. oral 6.93, p=0.001; TM-containing, intranasal 12.28 vs. oral 4.90, p=0.001). When analyzing separately the orally and the intranasally immunized groups, there was no significant difference in vibriocidal titers against *V. cholerae* O1 Inaba between TM-free and -containing Euvichol in both oral (12.56 vs. 12.28, p=0.900) and intranasal (6.93 vs. 4.90, p=0.302) immunization groups, respectively. In addition, antibody responses to *V. cholerae* were also assessed following immunization by oral or intranasal route (Table 2, Fig. 3). When mice were immunized with the TM-free formulation, vaccine-specific IgG, IgM, and IgA levels were significantly higher in intranasally immunized mice compared to orally immunized mice, (intranasal 17.26 vs. oral 8.27, p=0.001 for IgG; intranasal 14.70 vs. oral 11.15, p=0.001 for IgM; intranasal 9.60 vs. oral 5.40, p=0.001 for IgA). Similar trends in antibody responses between oral and intranasal route groups were observed when mice were immunized with TM-containing formulation. Interestingly, TM-free Euvichol generated statistically significant increase of serum IgG compared to TM-containing Euvichol in oral immunization group (8.27 vs. 6.57, p=0.011) but not in intranasal immunization group (17.26 vs. 16.28, p=0.286). There was no significant difference in the level of IgM and IgA between the two vaccine variations in either intranasal (14.70 vs. 13.97, p=0.100 for IgM; 9.60 vs. 9.40, p=0.900 for IgA) or oral immunization group (11.15 vs. 10.42, p=0.078 for IgM; 5.40 vs. 5.02, p=0.678 for IgA). Altogether, these data suggest that TM-free Euvichol could induce similar antibody responses to TM-containing Euvichol.

**Table 2.** Euvichol-specific serum IgG, IgM, IgA titers (log2)

| Immunization route | Isotype | TM-free Euvichol | TM-containing Euvichol | p-value<sup>a</sup> |
|--------------------|---------|------------------|------------------------|-------------------|
| | | Mean titer (log2) | 95% CI | Mean titer (log2) | 95% CI | |
| Intranasal | IgG | 17.26 | 16.78-17.74 | 16.28 | 15.74-16.81 | 0.286 |
| | IgM | 14.70 | 14.11-15.29 | 13.97 | 13.56-14.38 | 0.100 |
| | IgA | 9.60 | 8.95-10.25 | 9.40 | 8.93-9.87 | 0.900 |
| Oral | IgG | 8.27 | 7.36-9.17 | 6.57 | 5.45-7.67 | 0.011 |
| | IgM | 11.15 | 10.75-11.55 | 10.42 | 9.91-10.93 | 0.078 |
| | IgA | 5.40 | 4.83-5.97 | 5.02 | 4.45-5.59 | 0.678 |

<sup>a</sup>The p-values were calculated using one-way ANOVA with Tukey’s multiple comparison test.

**Fig. 2.** Intranasal immunization with thimerosal-free and -containing Euvichol induces vibriocidal antibody response to *Vibrio cholerae*. Four groups of mice (n=10-12) were administered intranasally or orally with TM-containing or -free Euvichol on day 0 and day 14 against a control group of non-immunized mice. Serum samples were collected from non-immunized and immunized mice groups on day 21 and vibriocidal activities were measured. Data represent mean values ± standard error of mean from triplicate assays. *p<0.05 indicates statistically significant difference between two experimental groups by use of one-way ANOVA with Tukey’s multiple comparison test. n.s, not significant; i.n., intranasal; TM(+), thimerosal-containing; TM(-), thimerosal free.
**Discussion**

TM has long been used in vaccines, especially in multi-dose formulation as preservative. Although there is no direct evidence of harmful effect caused by TM on human, public health sector and vaccine manufacturers are trying to remove it from products due to theoretical safety concerns based primarily on data regarding the toxicity of a related substance, methylmercury including potential risk for neurotoxicity [5,8]. In the present study, we evaluated the immunogenicity of TM-free OCV, Euvichol, in a murine pulmonary cholera model. Intranasal immunization with both TM-free and -containing Euvichol completely protected mice against *V. cholerae* challenge and induced strong systemic immune responses including vibriocidal antibody and vaccine-specific IgG, IgM, and IgA in serum. However, oral immunization could not provide protective immunity and antibody responses were less pronounced compared to intranasal immunization in mice model. These results are consistent with our previous study in mice intranasally administered with OCV Dukoral, which is comprised of whole-cell inactivated *V. cholerae* O1 and recombinant cholera toxin B subunit, where immunized mice were completely protected against cholera-induced pneumonia [10]. Given the fact that intranasal administration elicited significantly strong systemic antibody responses but not oral immunization, antibacterial antibodies may play a role in protection against *V. cholerae* infection. Local immunity may also contribute to protection, as observed in our previous study [10], although this was not observed in this study. In addition, the presence of TM in the vaccine did not affect cholera vaccine-induced immune responses in mice.

Serum vibriocidal antibody has long been considered as the best surrogate marker for protection against cholera [14,15]. Intranasal administration, but not oral immunization, with OCV elicited strong vibriocidal antibody response against *V. cholerae* O1 Inaba in the present study. This result is consistent with previous reports [10,16]. Euvichol is composed of inactivated *V. cholerae* O1 Inaba, O1 Ogawa, and O139 and therefore, vibriocidal activities against all of three strains have been evaluated in clinical studies. However, in this animal study we based the comparison of immunogenicity between the two variations of Euvichol on O1 Inaba-specific vibriocidal antibody in consideration of the cross-reactivity between Inaba and Ogawa strain [17], and of the poor responses against O139 strain in previous studies [18,19]. Vaccine-specific serum IgG, IgM, and IgA antibodies are considered to mediate...
protection against cholera infection in this model. This is supported from previous studies where LPS-specific serum IgG and IgA were highly increased in cholera patients and vaccinated individuals [20-22]. In addition, serum IgM and secretory IgA against V. cholerae LPS generated in patients play a role in conferring protective immunity against cholera [23,24].

Since V. cholerae does not induce natural infection in animals except for human, there is no accurate model to reflect the immune reaction occurred in clinical settings. Several animal models have been developed to evaluate cholera vaccine in pre-clinical stage. Infant mouse model has been widely used, but immaturity of sucking mice’s immune systems and microflora does not allow to evaluate active immunity against bacterial challenge [25]. Germ-free mouse model could be used, because V. cholerae can colonize in the gut [26]. However, the cost for maintenance of germ-free animal is expensive and immunity is not fully developed due to lack of intestinal microbiota. In addition, antibiotic-resistant adult mice model has been developed, but the animal should be pre-treated with streptomycin to remove gut microflora, and streptomycin-resistant V. cholerae can only be used to examine bacterial colonization [16]. Overall, it is difficult to assess protection against natural infection with wild-type V. cholerae in this model. The mouse pulmonary cholera model used in this study seems appropriate for evaluation of immunogenicity of cholera vaccines in several aspects: use of adult mice is inexpensive and convenient to manipulate; well-characterized in immune system; protection against wild-type V. cholerae can be assessed. Nevertheless, there are limitations to evaluate the immunogenicity of cholera vaccines in correlation with the human. For instance, cholera infection in human is mainly by oral route and colonizes the small intestine, but, in the present study, only immunization by intranasal route showed successful protection against V. cholerae and fully induced antibody responses.

In summary, this study showed that the TM-free variation of the OCV Euvichol is highly immunogenic and induce similar immune responses compared to TM-containing Euvichol in mice. Following this study, in September 2016, the TM-free Euvichol obtained WHO prequalification. Subsequently, clinical data became available supporting the equivalence for safety and immunogenicity between the two Euvichol variations [27]. In conclusion, the pulmonary mouse cholera model represents a good candidate to examine in vivo the potency of cholera vaccines.

References

1. Ali M, Nelson AR, Lopez AL, Sack DA. Updated global burden of cholera in endemic countries. PLoS Negl Trop Dis 2015;9:e0003832.
2. Clemens JD, Nair GB, Ahmed T, Qadri F, Holmgren J. Cholera. Lancet 2017;390:1539-49.
3. Deen J, von Seidlein L, Luquero FJ, et al. The scenario approach for countries considering the addition of oral cholera vaccination in cholera preparedness and control plans. Lancet Infect Dis 2016;16:125-9.
4. Baker JP. Mercury, vaccines, and autism: one controversy, three histories. Am J Public Health 2008;98:244-53.
5. Geier MR, Geier DA. Neurodevelopmental disorders after thimerosal-containing vaccines: a brief communication. Exp Biol Med (Maywood) 2003;228:660-4.
6. Parker SK, Schwartz B, Todd J, Pickering LK. Thimerosal-containing vaccines and autistic spectrum disorder: a critical review of published original data. Pediatrics 2004;114:793-804.
7. Ball LK, Ball R, Pratt RD. An assessment of thimerosal use in childhood vaccines. Pediatrics 2001;107:1147-54.
8. World Health Organization. Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines. WHO Technical Report Series, No. 926. Annex 4. Geneva: World Health Organization; 2004.
9. World Health Organization. Vaccines and biologicals: recommendations from the strategic advisory group of experts, weekly epidemiologic record. WHO Weekly Epidemiological Record. Vol. 37. Geneva: World Health Organization; 2002.
10. Kang SS, Yang JS, Kim KW, et al. Anti-bacterial and antitoxic immunity induced by a killed whole-cell-cholera...
toxin B subunit cholera vaccine is essential for protection against lethal bacterial infection in mouse pulmonary cholera model. Mucosal Immunol 2013;6:826-37.

11. Yang JS, Kang SS, Yun CH, Han SH. Evaluation of anticoagulants for serologic assays of cholera vaccination. Clin Vaccine Immunol 2014;21:854-8.

12. Yang JS, Kim HJ, Yun CH, et al. A semi-automated vibrioidal assay for improved measurement of cholera vaccine-induced immune responses. J Microbiol Methods 2007;71:141-6.

13. Clemens JD, Stanton BF, Chakraborty J, et al. B subunit-whole cell and whole cell-only oral vaccines against cholera: studies on reactogenicity and immunogenicity. J Infect Dis 1987;155:79-85.

14. Clemens JD, van Loon F, Sack DA, et al. Field trial of oral cholera vaccines in Bangladesh: serum vibrioidal and antitoxic antibodies as markers of the risk of cholera. J Infect Dis 1991;163:1235-42.

15. Mosley WH, Ahmad S, Benenson AS, Ahmed A. The relationship of vibrioidal antibody titre to susceptibility to cholera in family contacts of cholera patients. Bull World Health Organ 1968;38:777-85.

16. Nygren E, Li BL, Holmgren J, Attridge SR. Establishment of an adult mouse model for direct evaluation of the efficacy of vaccines against Vibrio cholerae. Infect Immun 2009;77:3475-84.

17. Villeneuve S, Boutonnier A, Mulard LA, Fournier JM. Immunochemochemical characterization of an Ogawa-Inaba common antigenic determinant of Vibrio cholerae O1. Microbiology 1999;145(Pt 9):2477-84.

18. Baik YO, Choi SK, Olveda RM, et al. A randomized, non-inferiority trial comparing two bivalent killed, whole cell, oral cholera vaccines (Euvichol vs Shanchol) in the Philippines. Vaccine 2015;33:6360-5.

19. Saha A, Chowdhury MI, Khanam F, et al. Safety and immunogenicity study of a killed bivalent (O1 and O139) whole-cell oral cholera vaccine Shanchol, in Bangladeshi adults and children as young as 1 year of age. Vaccine 2011;29:8285-92.

20. Holmgren J, Svennerholm AM. Bacterial enteric infections and vaccine development. Gastroenterol Clin North Am 1992;21:283-302.

21. Qadri F, Ryan ET, Faruque AS, et al. Antigen-specific immunoglobulin A antibodies secreted from circulating B cells are an effective marker for recent local immune responses in patients with cholera: comparison to antibody-secreting cell responses and other immunological markers. Infect Immun 2003;71:4808-14.

22. Robbins JB, Schneerson R, Szu SC. Perspective: hypothesis: serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. J Infect Dis 1995;171:1387-98.

23. Aktar A, Rahman MA, Afrin S, et al. Plasma and memory B cell responses targeting O-specific polysaccharide (OSP) are associated with protection against Vibrio cholerae O1 infection among household contacts of cholera patients in Bangladesh. PLoS Negl Trop Dis 2018;12:e0006399.

24. Nandy RK, Albert MJ, Ghose AC. Serum antibacterial and antitoxin responses in clinical cholera caused by Vibrio cholerae O139 Bengal and evaluation of their importance in protection. Vaccine 1996;14:1137-42.

25. Klose KE. The suckling mouse model of cholera. Trends Microbiol 2000;8:189-91.

26. Butterton JR, Ryan ET, Shahin RA, Calderwood SB. Development of a germfree mouse model of Vibrio cholerae infection. Infect Immun 1996;64:4373-7.

27. Russo P, Ligsay AD, Olveda R, et al. A randomized, observer-blinded, equivalence trial comparing two variations of Euvichol(R), a bivalent killed whole-cell oral cholera vaccine, in healthy adults and children in the Philippines. Vaccine 2018;36:4317-24.