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Protection against nontypeable *Haemophilus influenzae* challenges by mucosal vaccination with a detoxified lipooligosaccharide conjugate in two chinchilla models

Wenzhou Hong^{1,2}, Daxin Peng^{1,3}, Maritza Rivera¹, and Xin-Xing Gu^{1,*}

¹Vaccine Research Section, National Institute on Deafness and Other Communication Disorders, Rockville, Maryland 20850

Abstract

Otitis media (OM) can occur following onset of upper respiratory tract infections. Inhibition of bacterial colonization in nasopharynx (NP) by mucosal vaccination may prevent OM by reducing bacterial invasion of the middle ears (MEs). In this study, 80 chinchillas were intranasally (i.n.) immunized with a detoxified lipooligosaccharide (dLOS)-tetanus toxoid conjugate vaccine of nontypeable *Haemophilus influenzae* (NTHi) mixed with cholera toxin (CT) or CT alone. All vaccinated animals responded with elevated levels of mucosal and serum anti-LOS antibodies. Two weeks after the last immunization, 40 chinchillas were challenged i.n. with NTHi to evaluate NP colonization and ME infection while the rest of the animals were challenged transbullarily (T.B.) to examine the development of OM. Compared to the control group, the vaccination inhibited not only bacterial colonization in NP and transmission to MEs in the i.n. challenge group but also bacterial colonization in NP and transmission to unchallenged ears in the T.B. challenge group. Though no difference was found in the challenged ears of either group right after the T.B. challenge, an early clearance of NTHi from NP and unchallenged ears as well as less severity of OM in the unchallenged ears were observed in vaccinated animals. Current results along with our previous data indicate that mucosal vaccination is capable of inhibiting NTHi NP colonization and preventing OM occurrence in chinchillas; the i.n. challenge model is preferable for testing the mucosal vaccines while the T.B. challenge model is superior for testing the systemic vaccines.

Keywords

Mucosal immunization; Nontypeable *Haemophilus influenzae*; Chinchilla models; Lipooligosaccharide conjugate vaccine

1. Introduction

Otitis media (OM) is most common disease in young children [1]. Although the major bacterial pathogens, *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHi) and

*Corresponding author: 5 Research Court, Room 2A31, Rockville, MD 20850, Phone: (301) 402-2456, Fax: (301) 435-4040, guxx@nidcd.nih.gov.

²Current address: Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, 9200 W. Wisconsin Avenue, Milwaukee, WI 53226.

³Current address: School of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, China.

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Moraxella catarrhalis, have not changed over the past decades, it has been found that there is a shift in the distribution of these organisms. Because of immunization with pneumococcal polysaccharide and conjugate vaccines, the proportion of OM caused by the vaccine covered pneumococcal strains has been decreasing while NTHi and *M. catarrhalis* are becoming more frequent [2,3]. Though there are no licensed NTHi and *M. catarrhalis* vaccines available, a recent report showed that a pneumococcal polysaccharide vaccine conjugated to NTHi protein D prevented both pneumococcal and NTHi OM with an overall 33.6% reduction [4]. Currently, antibiotics is still the most common approach for treatment of the disease, and such treatment can encourage the emergence of new drug-resistant bacterial strains [5]. There is a need to develop effective vaccines as well as immunization strategies against OM caused by all of these bacterial species, especially for NTHi that causes a high percentage of chronic or recurrent OM in children [6].

Several NTHi vaccine candidates are under development including outer membrane proteins and lipooligosaccharide (LOS)-based conjugate vaccines [7–10]. Most of these vaccine candidates were administered by parental route and induced systemic antibodies while some of them showed protection against bacterial challenge in animal models. Since NTHi is a mucosal pathogen that needs to colonize in the nasopharynx prior to the onset of AOM, inhibition or reduction of pathogenic microorganisms colonizing in upper respiratory tract by mucosal immunity is believed to be an alternative. It may be even more effective in overcoming or preventing the occurrence of OM [11,12]. Several *in vitro* studies indicated that adherence of NTHi to human nasopharyngeal epithelial cells was inhibited by mucosal NTHi-specific S-IgA [13,14]. In murine models, intranasal immunization with outer membrane proteins or LOS based conjugates of NTHi can induce protective immunity including antibody production in the nasopharynx or middle ears (MEs) and enhancement of bacterial clearance from the upper respiratory tract [15–18]. However, there is a lack of information as to whether the mucosal immunization would generate immunity against NTHi NP colonization and subsequently prevent OM in a chinchilla model of OM. Previous studies have documented that the chinchilla model is a state-of-the-art model which mimics OM in humans and has been used for evaluating the efficacy of vaccine candidates by systemic vaccination against pneumococcal or NTHi-caused OM [19–22]. In the present study, we investigated the roles of mucosal immunization with a detoxified LOS-tetanus toxoid (dLOS-TT) conjugate [23] in the inhibition of bacterial colonization in the nasopharynx as well as for infection in MEs in chinchillas. Two challenge models, intranasal and transbullar infections, were used for evaluation and comparison of the protective properties elicited by the mucosal vaccination.

2. Materials and methods

Conjugate vaccine

NTHi strain 9274, a clinical isolate from ME fluids of a patient with OM, was provided by Dr. M. A. Apicella, University of Iowa. The bacterial growth, purification of LOS from strain 9274, detoxification of the LOS, conjugation of dLOS to tetanus toxin (TT), and characterization of dLOS-TT were described previously [24]. The composition of dLOS-TT was 638 ug/ml of dLOS and 901 ug/ml of TT with a molar ratio of dLOS to TT of 35: 1.

Immunization

80 outbred, healthy chinchillas (weighing between 250 and 350 g) with no evidence of ear infection by otoscopic examination were purchased from Moulton Chinchilla Ranch, Rochester, Minn., and housed in individual cages. The animals underwent procedures in accordance with the National Institutes of Health guidelines under Animal Study Proposal 1074–2. Animals were randomly assigned to two groups. A vaccine group was immunized intranasally (i.n.) with 50 µl of phosphate-buffer saline (PBS) containing 25 µg of dLOS-TT

(dLOS content) plus 2 µg of cholera toxin (CT) as an adjuvant (List Biological Laboratories, Campbell). A control group was immunized with 50 µl of PBS containing 2 µg of CT. The immunization was performed 4 times at one-week intervals. Each dose was inoculated into the nostrils (25 µl each side) under anesthesia with an intramuscular injection of a mixture of ketamine (1.0 mg/kg of body weight) and Xylazine (0.5 mg/kg of body weight) in PBS.

Nasopharyngeal (NP) lavage and blood collection

NP lavage samples were collected before the immunization, 1 week post-immunization, and days 3, 7, 14, 21 and 28 post-challenge as described previously [25,26]. Briefly, under anesthesia, the chinchillas were laid on their sides while 15–20 drops of saline were placed in the upper nostril with a 1 ml syringe filled with 0.5 ml normal saline. NP lavages were withdrawn from the other nostril using 3 ml transfer pipette when the fluid appeared. For serum antibody testing, 1.5 ml of blood was collected from the transverse venous sinus of each chinchilla before immunization and 1 week after the last immunization.

Intranasal and intrabullar challenges

To potentially impair the Eustachian tubes for assistance with bacterial spread, all 80 chinchillas were inoculated i.n. with 5×10^6 50% tissue culture infection dose of adenovirus type 1 (ATCC, Manassas, VA) at the 7th day after the final immunization [20]. For preparation of bacteria, strain 9274 was recovered from Greave's solution stocks by transferring a loop of thawed organisms to a chocolate agar plate and incubated 16 h at 37°C with 5% CO₂. Then 3~5 colonies were streaked in a new plate and incubated an additional 4–5 h. Bacteria were harvested and suspended in Dulbecco's PBS with MgCl₂ and CaCl₂ (Invitrogen, Carlsbad, CA) containing 0.1% gelatin (DPBSG) on ice for the next challenges.

For the intranasal challenge, 20 chinchillas from each group were inoculated i.n. with 0.1 ml ($\sim 1.3 \times 10^8$ CFU) of bacteria at the 14th day post-immunization. For the intrabullar (T.B.) challenge, the other 40 chinchillas were injected transbullarly (right ear) with 0.05 ml (~ 530 CFU) of bacteria at the 14th day post-immunization. Both challenges were performed under anesthesia. Actual challenge doses were confirmed by colony counting.

Otoscope examination

A double-blinded otoscopic examination was performed and images of the tympanic membranes (TM) were taken by video otoscopy (Video VetScope System, MedRx, Seminole, FL) daily for 28 days post challenge. A diagnosis of AOM was rated on a scale of 0 to 4 as described previously [8]. A diagnosis of labyrinthitis (or inner ear infection) was made when chinchillas exhibited head titling or circling behavior [27].

Bacterial counts from nasopharynx and ME effusions

On days 3, 7, 14, 21 and 28 post-challenge, nasal lavages were collected from four chinchillas of each group, and then the animals were euthanized by CO₂. Their superior bullae were opened, and MEs were washed with 1.0 ml of DPBSG. An aliquot of nasal lavages or ME effusions/washes and 0.1 ml of their 10-fold serial dilutions (nasal lavages from 1:10 and ME washes from original) were plated on chocolate agar for a quantitative count of NTHi. The detectable levels were 100 CFU/ml in the nasal lavages and 10 CFU/ml in the ME lavages.

LOS ELISA

Serum and nasal lavage antibodies against LOS were determined by ELISA [8]. Briefly, 96-well plates were coated with 9274 LOS (10 µg/ml) overnight, followed by blocking with 1.5% bovine serum albumin (BSA). Three-fold serially diluted samples from 1:10 were added into the plates and incubated for 2 h. To detect IgG, a rabbit anti-chinchilla IgG serum (1:3,000)

[28] was added and incubated for 1 h, followed by 1 h incubation with an alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO). To detect IgA, a mouse monoclonal antibody to chinchilla IgA (1:2,000, IgG, Pasteur Merieux Connaught, Ont., Canada) was added and incubated for 1 h, followed by 1 h incubation with an AP-conjugated goat anti-mouse IgG (Sigma). After the AP substrate (Sigma) was added for 1 h, the reactions were read with a microplate auto reader to determine the values at OD_{405nm}. The ELISA titer units were calculated as the increased-folds compared to pre-samples of nasal lavage or serum.

Whole cell ELISA

Nasal lavages collected at 1 week after the final immunization were used to detect the cross-reactivity of mucosal antibodies against homologous and heterologous NTHi strains by whole cell ELISA. Briefly, overnight cultured strains 9274, 1479, 2019, 3198, 5657 and 7502 were suspended in PBS to an OD value of 65% transmission at 540 nm. The 96-well plates were coated with 70 μ l of the suspension and evaporated over night at 37°C [16]. Other steps were the same as the LOS ELISA, except that 3% BSA-PBS was used for blocking and a 1:10 diluted sample for IgA and a 1:30 diluted one for IgG detection. Each sample represents a pool of 5 chinchillas from the vaccine or the CT group.

Statistical analysis

Antibody levels were expressed as the geometric mean (GM) ELISA titers (reciprocal) of n (numbers of animals or ears) independent observations $\pm$ one standard deviation (SD). The ME bacterial densities were expressed as the GM CFU of n independent observations $\pm$ one SD. The otoscopic examination outcome was expressed as the mean of n independent observations $\pm$ SD at each time point. Differences between the vaccine and the control groups were determined using the unpaired Student t test (two tailed). For multiple time point comparison, two-way ANOVA was used to analyze the difference. Fisher's exact test (two tailed) was employed to compare the proportions of infected animals found between the vaccine and the control groups.

3. Results

Intranasal immunization elicits both mucosal and systemic antibodies against NTHi

Intranasal immunization with a dLOS-TT elicited a significant rise of LOS-specific IgA and IgG in chinchilla nasal lavages and sera while the CT adjuvant did not (Table 1) (vaccine vs CT cohort, IgA, $p < 0.001$; IgG, $p < 0.01$). The nasal lavages were further tested for their binding reactivity to whole cells of homologous and heterologous strains by whole cell ELISA. The results indicated that the mucosal IgA of the nasal lavages bound not only to the homologous strain 9274 but also to four of five major heterologous strains, 1479, 3198, 5657 and 7502, while the mucosal IgG also bound to above strains except 2019 (Fig. 1; $p < 0.05$ or < 0.01).

Intranasal immunization inhibits NTHi colonization and infection in nasopharynx and MEs in an intranasal challenge model

After the intranasal challenge, bacteria were detected in nasopharynx and MEs of both groups (Table 2A). However, two of 4 vaccinated chinchillas appeared culture-negative in nasal lavages on day 14 post-challenge. All the vaccinated animals were negative on day 28 while the controls were consistently culture-positive. The overall culture-positive rate in the vaccine group was significantly lower than that of the controls (11/20 vs 20/20, $p < 0.01$). In ME fluids, only 2 of 4 vaccinated chinchillas were culture-positive on day 3 and none of them on day 28. In contrast, all the controls were culture-positive on days 3, 7, and 14, 3 of 4 on day 21 and 2 of 4 on day 28. The culture-positive rates of the vaccine group were significantly lower than that of the controls (6/20 vs 16/20 by animals, $p < 0.01$ or 6/40 vs 29/40 by ears, $p < 0.001$).

Time courses of NTHi counts in both nasal lavage and ME fluids revealed that the bacterial counts obtained from the vaccine group were significantly lower than that from the controls during the entire course (Fig. 2A and 2B, $p < 0.001$). There was at least a 10 to 100-fold reduction at each time point. For the nasal lavages, the bacterial counts were significantly lower at each time point ($p < 0.01$) while for the ME fluids, the bacterial counts were significantly lower on days 3 through 21 ($p < 0.05$ or $p < 0.01$) but not on day 28.

In addition, an otoscopic examination found that the intranasal challenge resulted in slight congestion along the edge of tympanic membrane (TM) with a maximal scale of 1, frequently found in control animals. Though clinical signs of AOM were not observed in either group, ME fluids or inflammation was subsequently confirmed by opening the bullas and collecting ME fluids on days 3, 7, 14, 21 and 28 post-challenge. The total number of ME fluids observed in the vaccine group was significantly lower than that of the controls (20% vs 75% by animals, $p < 0.01$; 15% vs 62.5% by ears, $p < 0.001$).

Intranasal immunization inhibits NTHi colonization in nasopharynx and MEs

It protects the unchallenged ear and inner ear infections in an intrabullar challenge model. After the intrabullar challenge, bacteria were detected in nasopharynx and MEs of both groups (Table 2B). However, the vaccinated chinchillas appeared to be culture-negative in nasal lavages on day 14 through 28 while the controls were culture-positive all the times except one on day 28. The culture-positive rate of the vaccine group was lower than that of the controls (8/20 vs 19/20, $p < 0.001$). In ME fluids of the challenged ears, the vaccinated chinchillas appeared culture-negative on day 14 while the controls did not become culture-negative until day 21. The culture-positive rate of the challenged ears in the vaccine group was lower than that in the controls (12/20 vs 15/20, $p = 0.05$). For the unchallenged ears, 3 of 4 vaccinated chinchillas were culture-positive only on days 3 and 7 (Table 2B). In contrast, all 4 unchallenged ears in the controls were culture-positive on days 3 and 7, 2 of 4 on day 14 and 3 of 4 on day 21. The culture-positive rate of the unchallenged ears in the vaccine group was lower than that in the controls (6/20 vs 13/20, $p < 0.05$).

Time courses of bacterial counts with the nasal lavage samples revealed a 10-fold reduction by day 7 in the vaccine group compared to the controls (Fig. 2C). From day 14, no bacterium was detectable in the vaccine group, however, at least 4 log units of bacteria were found in the controls till day 28 ($p < 0.01$). The difference of bacterial counts between the vaccine and control groups was significant during the entire course ($p < 0.01$). Similar time courses of bacterial counts were found with unchallenged ears except that there were no bacteria detectable in the vaccine group on day 28 (Fig. 2D). The bacterial counts in unchallenged ears of the vaccinated animals were lower than those of the controls ($p < 0.01$). In challenged ears, there was also significant reduction of bacterial counts in the vaccine group during the entire course (Fig. 2E, $p < 0.01$).

In addition, an otoscopic examination found that the intrabullar challenge resulted in clinically significant signs of AOM in the challenge ears from day 1 post-challenge (Fig. 3A). Mean indexes of the ME pathology between the vaccine and control groups were similar up to day 14 (3.08 ± 0.67 vs 3.58 ± 0.51). On day 21, there was a significant reduction of the signs found in the vaccine group when compared with those in the controls (1.75 ± 1.04 vs 3.13 ± 0.83 , $p < 0.05$). On day 28, all the vaccinated chinchillas were fully recovered except one with a rating of 2 while the controls showed a mean index of 2. For the unchallenged ears, the severity of the signs of ME pathology in the vaccinated chinchillas was much less than those of the controls during the entire course (Fig. 3B). The mean indexes of OM in the vaccine group were lower than those of the controls at each time point, $p < 0.01$, respectively.

Time courses of the pathological changes of MEs post-transbullar challenge were represented in a pair of chinchillas at Fig. 4. Though there was no difference for the challenged ears between the two groups through day 14 post-challenge, there was accelerated recovery found in the vaccinated chinchillas on days 21 and 28. Furthermore, the vaccinated animals showed much less pathological changes in their unchallenged ears than those in the control animals. Finally, the incidence of inner ear infection as manifested by head tilt or dysequilibrium was 10% in the vaccine group and 55% in the controls (2/20 vs 11/20; $p < 0.01$).

4. Discussion

Previous studies have shown that a dLOS-TT conjugate vaccine, generated from NTHi strain 9274, is able to elicit protection against an NTHi challenge in a chinchilla OM model by systemic immunization [8]. The vaccine also induced mucosal immunity against an NTHi challenge in a mouse clearance model by mucosal immunization [16]. In this study, we further investigated if the vaccine would induce mucosal immunity in chinchillas. We found that high titers of LOS-specific mucosal IgA and IgG were detected after a 4-dose mucosal immunization. With respect to the serum antibody levels, there was a difference between two mucosal animal models. In the murine model, the titers of serum anti-LOS IgA and IgG were 12 and 32-fold higher, respectively, than those of the controls. In chinchillas, the titers of serum anti-LOS IgA and IgG increased less than 10-fold when compared to those of the controls. It is unclear whether the different antibody profiles between murine and chinchilla are due to diversity of immunity in these animal species. However, both chinchilla mucosal and serum antibodies are capable of binding to heterologous strains *in vitro*, and the cross strain protection *in vivo* needs to be confirmed.

To further evaluate the efficacy of the vaccine, two chinchilla challenge models were used: a bacterial clearance model by intranasal inoculation and an OM model by intrabulla injection. Based on the natural infection pathway of microorganisms during OM, a challenge regime through nasopharynx was performed first. [21]. The vaccinated chinchillas showed a significant reduction of NTHi colonization with shortening carriage time in nasopharynx compared with a control group. The vaccinated chinchillas also presented a lower rate of ME infection and less bacterial counts in MEs, indicating a potential correlation between the lower bacterial carriage in nasopharynx and the lower rate of ME infection. These data suggested that the intranasal vaccination inhibited bacterial colonization in the upper respiratory tract and/or enhanced bacterial clearance from the ME infection site, which might result in a reduction of the OM occurrence in chinchillas. In the OM model, all chinchillas revealed similar bacterial counts and pathological symptoms in the right challenged ears, suggesting a poor efficacy of mucosal vaccination in resolving direct infection/challenge in the MEs. However, the mucosal vaccination provided strong evidence of inhibition of NTHi colonization in nasopharynx, as well as prevention of NTHi transmission and OM occurrence in the unchallenged MEs of the vaccinated chinchillas. Our previous study showed that high levels of serum antibodies elicited by the systemic vaccination with a similar vaccine resulted in protection in chinchillas from acute OM of both MEs [8]. These two studies suggest that high levels of serum antibodies are necessary [29,30] to inhibit bacteria loaded directly into the ME while mucosal antibodies along with low levels of serum antibodies are sufficient to inhibit NTHi colonization in nasopharynx. They also prevent NTHi spreading to MEs, which potentially eliminates subsequent OM. Taking account of the fact that OM always starts from an upper airway infection, mucosal immunity should play an important role in prevention of OM in humans.

In conclusion, intranasal immunization with a dLOS-TT conjugate elicited LOS-specific mucosal and systemic antibodies (IgA & IgG) in chinchillas and provided protection against NTHi in both nasopharyngeal colonization and OM development. Importantly, the chinchilla

nasopharyngeal colonization and infection model from intranasal challenge would be preferable for testing mucosal vaccine candidates. The chinchilla OM model from intrabulla challenge would be preferable for testing vaccines through systemic administration [8]. Data in this study support dLOS-TT as a good vaccine component and mucosal immunization might be a desirable strategy for preventing OM caused by NTHi.

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Fig. 1.

Binding activities of mucosal antibodies elicited by dLOS-TT plus CT or CT alone to NTHi strains in the whole cell ELISA. Mucosal IgA and IgG (nasal lavages) bound to homologous strain 9274 and heterologous strains 1479, 3198, 5657 and 7502 but not to 2019. *: $p < 0.05$; **: $p < 0.01$.

Fig. 2.

Time courses of bacterial counts (log 10) recovered from chinchilla lavage samples of nasopharynx (A) and middle ears (B) after an intranasal challenge; lavage samples of nasopharynx (C) and middle ears (D, E) after a transbullar challenge. Four chinchillas from vaccine or CT group were used at each time point and a total of 80 animals used for the entire course. Each CFU symbol represents an individual chinchilla. The ME counts (B) were bacterial CFUs from both ears of each animal. The bars indicated GM of each group. Chinchillas with culture-negative in bacterial counts were shown under the detecting level lines (CFU < 100/ml in the nasal lavages and CFU < 10/ml in the ME lavages). *: $p < 0.05$; **: $p < 0.01$.

Fig. 3.

Indexes of otoscopic examination of chinchillas from different groups up to 28 days post transbullula challenge. The curve on each day is the mean index of each group, rated on a scale of 0 (normal ear) to 4 (severe otitis media with effusions), as determined by otoscopy (A, challenged middle ear; B, unchallenged middle ear). The mean index from both immunization groups, the dLOS-TT and the CT group, was obtained post challenge as follows: 20 chinchillas up to day 3, 16 up to day 7, 12 up to day 14, 8 up to day 21 and 4 up to day 28, were scored for each group, respectively.

Fig. 4.

Tympanic membrane (TM) changes of a representative pair of chinchillas from a transbullar challenge. A double-blinded otoscopic examination was performed daily and the results were expressed as indexes (I: 0~4). Before challenge (day 0), all middle ears were rated as 0 for normal conditions. Overall, the vaccinated chinchillas showed less pathological TM changes in the challenged ears on days 21 and 28, and in the unchallenged ears during the entire course when compared with those of the control chinchillas.

Table 1

Chinchilla antibody responses to NTHi 9274 LOS elicited by intranasal immunization with dLOS-TT conjugate vaccine.

Immunogen ^a	Isotype	GM antibody ELISA units ($\pm$ SD range) ^b	
		Nasal Lavage	Serum
dLOS-TT + CT	IgA	143 (17 ~ 1066) ^c	6 (1 ~ 32) ^d
	IgG	134 (23 ~ 790) ^c	9 (3 ~ 26) ^c
CT	IgA	1 (0 ~ 2)	0 (0 ~ 1)
	IgG	1 (0 ~ 2)	1

^a Chinchillas from each group were given an intranasal immunization on days 0, 7, 14, 21 and 28 with dLOS-TT+ CT, or CT alone. Samples were collected one week after the last immunization. dLOS: detoxified lipooligosaccharide. TT: tetanus toxoid. CT: cholera toxin.

^b LOS antibody levels were measured by ELISA using strain 9274 LOS as a coating antigen, and expressed as the reciprocal of geometric mean (GM) and GM $\pm$ one standard deviation (SD) range within brackets.

^c $p < 0.001$, compared to CT group.

^d $p < 0.01$, compared to CT group.

Table 2
Bacterial culture-positive ratios of nasal and middle ear lavages from intranasal (A) or transbullar (B) challenged chinchillas

(A)	Days post challenge ^a	Nasal lavage		Middle ear lavage			
		dLOS-TT + CT	CT	dLOS-TT + CT	CT	ear ^c	ear ^c
		animal ^b	animal ^b	animal ^b	animal ^b	animal ^b	animal ^b
	3	4/4	4/4	2/4	2/8	4/4	8/8
	7	4/4	4/4	2/4	2/8	4/4	8/8
	14	2/4	4/4	1/4	1/8	4/4	6/8
	21	1/4	4/4	1/4	1/8	3/4	4/8
	28	0/4	4/4	0/4	0/8	2/4	3/8
Total		11/20 ^d	20/20	6/20 ^d	6/40 ^e	16/20	29/40

(B)	Days post challenge ^a	Nasal lavage		Middle ear lavage ^b			
		dLOS-TT + CT	CT	Challenged ear	Unchallenged ear	CT	CT
		animal ^b	animal ^b	dLOS-TT + CT	dLOS-TT + CT	CT	CT
	3	4/4	4/4	4/4	3/4	4/4	4/4
	7	4/4	4/4	4/4	3/4	4/4	4/4
	14	0/4	4/4	3/4	0/4	4/4	2/4
	21	0/4	4/4	1/4	0/4	3/4	3/4
	28	0/4	3/4	0/4	0/4	0/4	0/4
Total		8/20 ^e	19/20	12/20 ^f	15/20	6/20 ^g	13/20

^aFour chinchillas from each group were sacrificed at 3, 7, 14, 21 and 28 days post challenge. Nasal and middle ear lavages were collected and bacterial growth was detected by plating the lavage samples on chocolate agar plates overnight.

^bRatio as number of positive animals/number of total animals.

^cRatio as number of positive ears/number of total ears.

^dp < 0.01

^ep < 0.001

^fp = 0.05

^gp < 0.05, compared to CT group, respectively.