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NOTES

Development of a Chinchilla Model To Allow Direct, Continuous, Biophotonic Imaging of Bioluminescent Nontypeable *Haemophilus influenzae* during Experimental Otitis Media

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We transformed a nontypeable *Haemophilus influenzae* clinical isolate with a plasmid containing the *luxCDABE* operon driven by the *H. influenzae* outer membrane protein P2 promoter. Herein, we demonstrate the ability to detect bioluminescence and to monitor infection within the nasopharynges, eustachian tubes, and middle ears of chinchillas after intranasal and transbullar challenges.

Nontypeable *Haemophilus influenzae* (NTHI) is a predominant causative agent of otitis media (OM), a prevalent pediatric disease. Members of our laboratory have studied the pathogenesis of NTHI-induced OM by using an animal model that mimics the disease course in children (1, 6). To further improve our model, we developed a sensitive method to monitor OM by transforming a clinical isolate of NTHI with a plasmid containing the *luxCDABE* gene cluster of *Photorhabdus luminescens* (9, 10). In the presence of oxygen and $FMNH_2$, organisms expressing the *lux* operon emit light that penetrates tissues and can be externally detected in a qualitative and quantitative manner. The imaging of bioluminescent organisms within a live host provides the capacity to monitor gene expression noninvasively as well as to view and document organ-, tissue-, and cell-specific disease progression events over time.

For the construction of luminescent NTHI, the pSB417 plasmid (10) was digested with EcoRI to release the *luxCDABE* cassette. The insert was purified by gel extraction (Brinkmann, Westbury, N.Y.), and the ends were blunted with *Pfu* Turbo Hotstart polymerase (Stratagene, La Jolla, Calif.) and a 10 mM deoxynucleoside triphosphate mix (Invitrogen, Carlsbad, CA). The pRSM2211 plasmid contains the strong promoter for the outer membrane protein P2 driving the expression of green fluorescent protein (3, 8). It was digested with BamHI and EcoRI to excise *gfpmut3*, the gene was gel purified, blunt ended, and treated with alkaline phosphatase (Invitrogen). The vector and the *luxCDABE*-containing fragment were ligated by the use of T4 DNA ligase (Invitrogen), and the ligation products were used to transform *Escherichia coli* DH10B (Invitrogen) by electroporation. Plasmid DNAs were purified from kanamycin-resistant clones (Qiagen, Valencia, Calif.), and a plasmid with the correct restriction map was saved and named pKMLN-1 (Fig. 1). The plasmid pKMLN-1 was used to transform NTHI 1885MEE, a minimally passaged clinical isolate of chronic OM (2, 5). Kanamycin-resistant, luminescent clones were designated NTHI 1885MEE/pKMLN-1.

For confirmation of the plasmid's stability in vitro, NTHI 1885MEE/pKMLN-1 was passaged 15 times on chocolate agar. Additionally, we determined the effects of temperature and/or the growth phase on luminescence. In other *lux* systems, a decline in luminescence corresponds to a decrease in the metabolic activity upon entry into stationary phase (7). NTHI 1885MEE/pKMLN-1 was inoculated into brain heart infusion broth supplemented with 2 μg of both hemin and NAD/ml and was then incubated at 33 rpm in 5% CO_2 at temperatures ranging from 25 to 43°C. The amount of luminescence per bacterium was minimally affected from 25 to 43°C during mid-log-phase growth, but a decrease in the expression of *lux* was consistently observed as the culture entered stationary phase within the temperature range of 31 to 39°C. Since the entry of NTHI into stationary phase is not anticipated during colonization or infection in vivo (4) and because luminescence expression was stable over a broad temperature range in vitro, we found this strain to be suitable for use in vivo.

An adult chinchilla (*Chinchilla lanigera*) was inoculated intranasally and transbullarly with NTHI 1885MEE/pKMLN-1. After the intranasal inoculation of 10^8 CFU, luminescent NTHI cells were visible within the anterior nares and pharynx (Fig. 2A and B, arrows), while the transbullar inoculum of 1,500 CFU was not detectable. Two days later, NTHI reached a load that was readily observed within both bullae (Fig. 2C), demonstrating our ability to detect a bioluminescent signal through the bony process surrounding the middle ear space. Additionally, NTHI cells were observed within

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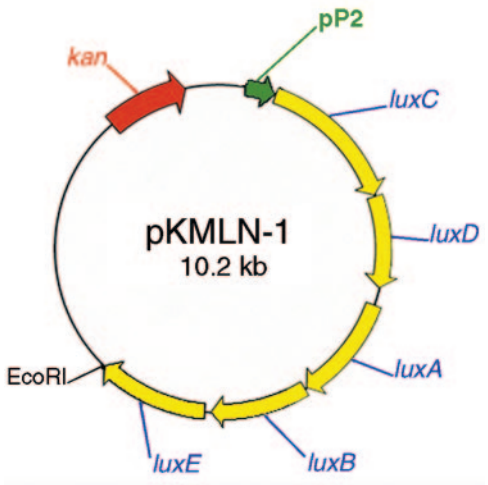

FIG. 1. Schematic diagram of plasmid pKMLN-1. pP2, the promoter for the outer membrane protein P2 (green). The *luxCDABE* operon (yellow) and kanamycin resistance cassette (red) are also shown.

the anterior nares and pharynx (Fig. 2C and D, arrows), and lateral imaging showed the presence of NTHI within a focus of infection in the pharynx as well (Fig. 2E and F), demonstrating that NTHI established a colonization site distal from the intranasal inoculation site. The bioluminescent signal extending between these two foci represents NTHI within the eustachian tube.

On day 4, the luminescent signal decreased within the bullae (Fig. 2G). Within the anterior nares and nasopharynx, however, the luminescent signal increased over that observed 2 days prior (Fig. 2G, H, and J). Lateral images (Fig. 2I and J) confirmed the presence of NTHI within the middle ears, and luminescence was noted emanating through the tympanic membranes (arrows). On day 6, the luminescent signal was maintained within the bullae but was decreased within the pharynx and was not detected within the anterior nares (Fig. 2K to N). Via otoscopy, we noted that the left tympanic membrane was perforated, thus explaining the intense signal observed in Fig. 2M.

On days 8 (Fig. 2O to R) and 10 (Fig. 2S to V), the bioluminescent signals remained stable within the bullae but were lost from within the nasopharynx. Nasopharyngeal (NP) lavage fluids and epitympanic taps collected on each assessment day were plated on chocolate agar and on agar containing 20 μg of kanamycin/ml both to determine the CFU of NTHI per milliliter and to demonstrate the stable luminescence of NTHI recovered from a site of active colonization and infection. For both NP lavage fluids and middle ear tap specimens, the number of luminescent NTHI cells on chocolate agar was equivalent to that on selective medium, indicating that the plasmid pKMLN-1 was stably maintained in vivo.

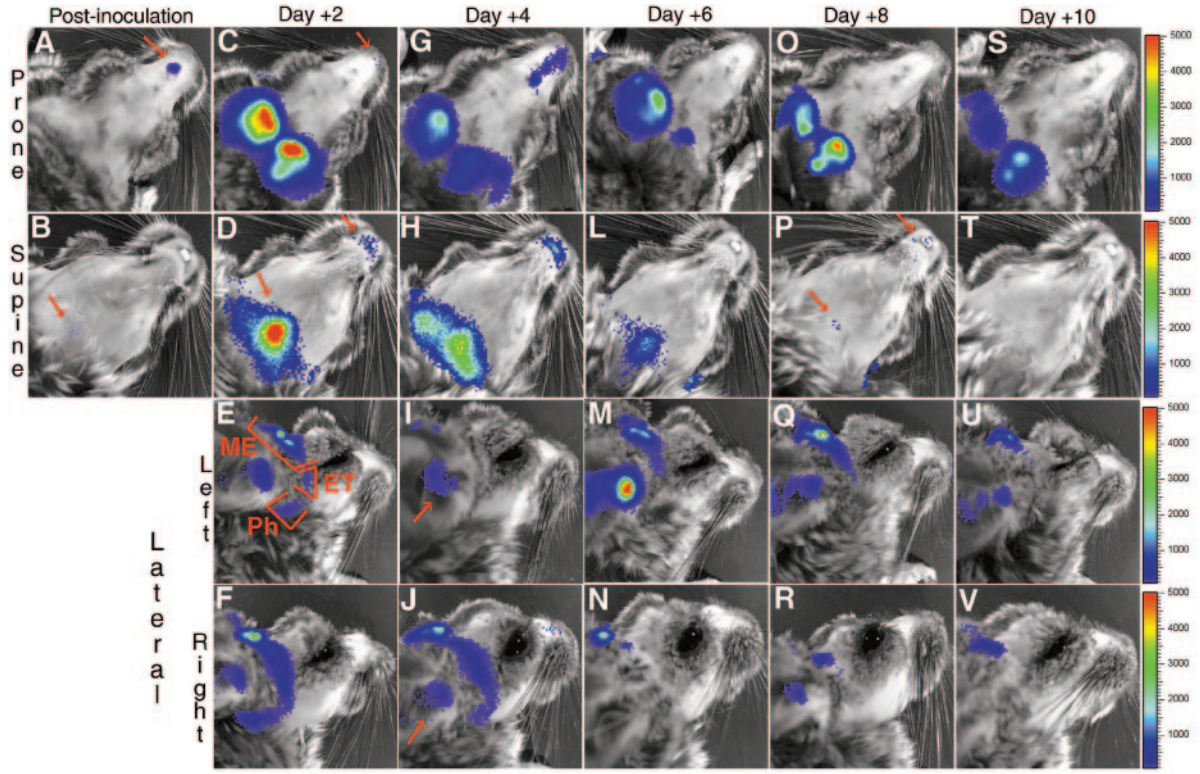

FIG. 2. Detection of bioluminescent NTHI 1885MEE/pKMLN-1 over a 10-day period within the pharynx, eustachian tube, and middle ear of a chinchilla host after intranasal and transbullar inoculation. A lightly anesthetized chinchilla was imaged via a Xenogen IVIS system to detect luminescent NTHI as the animal lay prone, supine, or lateral for 5 min. Ph, pharynx; ET, eustachian tube; ME, middle ear. Imaging and quantification of luminescent signals were maintained on the same photon scale. The color bars indicate the relative signal intensities.

Despite the absence of a signal in the nasopharynx on day 10, nasopharyngeal lavage fluids contained 4.4×10^4 CFU of luminescent NTHI/ml. A slight signal was detected within the nasopharynx on day 8 (Fig. 2P, arrow), when NP lavage fluids contained 4.2×10^5 CFU of NTHI/ml. Therefore, we estimated the limit of detection for this luminescent NTHI construct to be approximately 10^5 CFU of NTHI/ml of NP lavage fluid. Conversely, despite the fact that all middle ear fluids contained between 6.0×10^7 and 8.0×10^8 CFU of NTHI/ml on days 4 to 10, there were clear differences in luminescent signal intensity among these ear fluids, suggesting that in some cases a subpopulation of adherent NTHI (i.e., not recoverable by lavage and perhaps within a biofilm) remained in the bullae.

This system represents a significant improvement in our ability to model OM in a rodent host due to its noninvasive nature. Whereas the sensitivity was lower than that which is obtainable by repeated culturing of NP lavage fluids (1–3, 5), the detection of luminescent NTHI cells within the middle ear cavity appears to be more sensitive than culture. Thereby, we anticipate that in future studies we will be able to track luminescent NTHI in the chinchilla as it colonizes the nasopharynx, ascends a virus-compromised eustachian tube, and establishes an active infection in the middle ear after intranasal inoculation. Likewise, we expect to be able to sensitively monitor whether these steps have been modified or prevented by immunization. The use of this system to assay NTHI gene expression in vivo is reported by Mason et al. (3a).

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