Acta Med. Nagasaki 56: 1−4

Demonstration of pneumococcal capsule under immunoelectron microscopy

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It is challenging to demonstrate the capsule of *Streptococcus pneumoniae* (*S. pneumoniae*) under immunoelectron microscopy because of the thick mucopeptide cell wall hampering proper fixation. A novel rapid freeze fixation method was established to observe the capsule of *S. pneumoniae*. A strain of serotype 3 of *S. pneumoniae* isolate was analyzed after rapid freezing. An ethanol freezing-substitution fixing method was applied and immunohistochemical staining with osmium tetroxide was tested. The capsule was confirmed using the serotype 3 specific polyclonal antibodies labeled with colloidal gold particles. To the best of our knowledge, this is the first report of *S. pneumoniae* capsule by immunoelectron microscope.

ACTA MEDICA NAGASAKIENSIA 56: 1−4, 2011

Keywords: Pneumococcal capsule, rapid freeze-substitution fixation method, colloidal gold, electron microscopy

Introduction

Streptococcus pneumoniae (S. pneumoniae) colonizes in human nasopharynx, which may sequentially invades the lungs, the blood stream, and the central nervous system. This Gram-positive cocci bacterium is frequently isolated from patients with acute respiratory infection, chronic respiratory tract infection (1) and otitis media (2). This bacterium also causes sepsis and meningitis in children (3). The capsule of the *S. pneumoniae* has various roles in the pathogenesis; for example, it inhibits the activation of the complement necessary for phagocytosis by white blood cells (1-4), and restrains the activation of complement (5). Till now 91 different types of capsules have been identified in *S. pneumoniae* (4). However, demonstration of capsule of *S. pneumoniae* using electron microscopy has not been reported yet. This paper reports excellent electron microscopic images of pneumococcal capsule by using quick freezing substitution fixation for the first time.

Materials and Methods

S. pneumoniae serotype 3 isolated from an otorrhea patient at the Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University was used for immunoelectron microscopy. Gram stain smear test showed that the bacterium was typical of pneumococci with a thick capsule (Figure 1). The serotype was identified using Pneumotest-Latex (Statens serum institute, Copenhagen, Denmark), according to the instructions of the manufacturer.

The isolated *S. pneumoniae* was cultured in 7% rabbit blood agar plate and the cultured bacterial colonies were transferred to the middle of a high anoxic copper sheet

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Received September 3, 2010; Accepted April 12, 2011

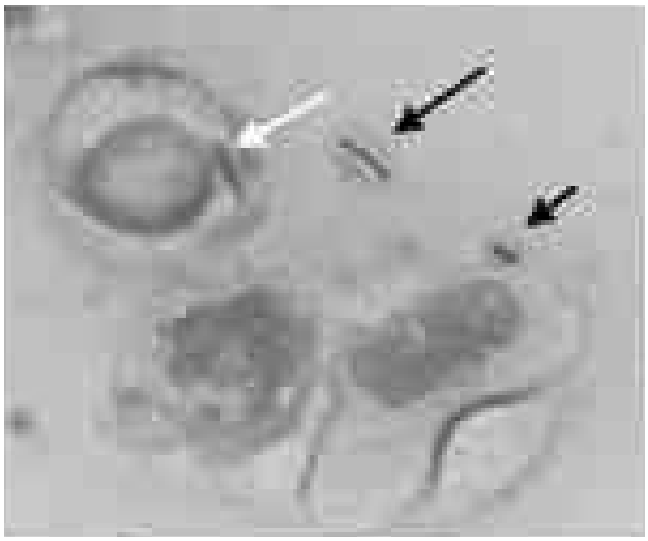

Fig. 1. Pneumococcus isolated from otorrhoea (Gram stained x 1000). Around the bacterial body, capsule was observed. The black short-arrow indicates pneumococcus (a diplococcus). The black long-arrow indicates pneumococcus (a streptococcus). The white arrow indicates phagocytosed pneumococcus in macrophage.

(1mm x 7mm x 0.02mm), bent into the shape of a sandwich as shown in Figure 2. Bacteria on the copper sheet was cooled down to 0℃ for 1min and immediately dipped into the super-cooled liquid ethanol at -120℃, using tweezers and swiveled continuously for 30 seconds to facilitate the rapid freezing. The temperature of the ethanol was maintained by liquid nitrogen (Figure 3).

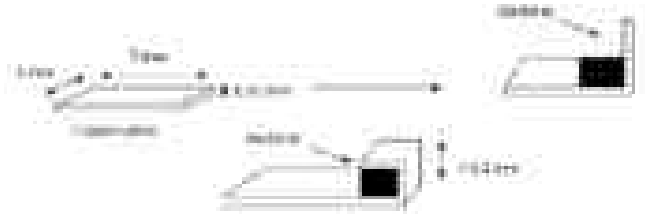

Fig. 2. Schematic representation of pneumococcal colony being inserted into an anoxic copper sheet.

Two different fixatives 0.2% glutaraldehyde ethanol solution and 0.5% osmium tetroxide ethanol solution were used. Each solution was cooled to -80℃ before use. After rapid freezing, the bacteria sandwiched between the copper sheet was submerged into the two fixative solutions separately and left for 48 hours at -80℃. Then the samples were thawed within fixative solutions in a stepwise manner by keeping at -20℃ for 2 hours, at 4℃ for 2 hours and finally at room temperature for 1 hour. After fixing, the bacteria on the copper sheet were rinsed by absolute ethanol. Then a segment of bacteria (approx. 1mm x 1mm x 0.15mm) was

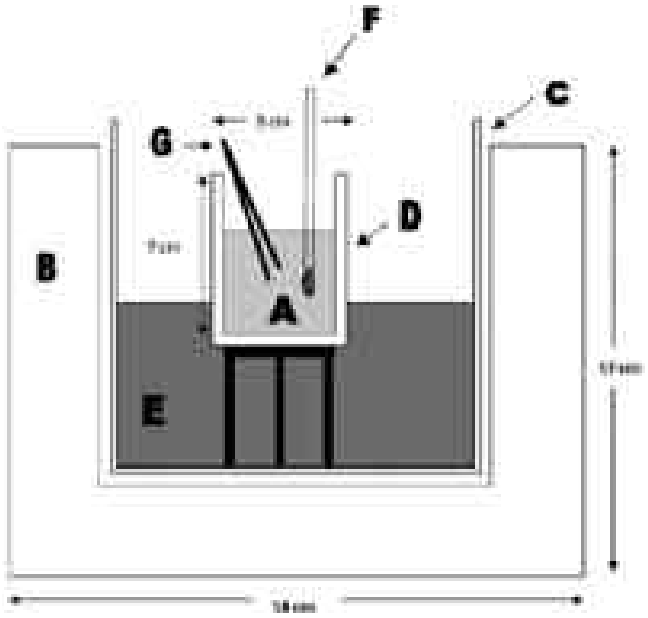

Fig. 3. Schematic representation of rapid freezing system. A: Ethanol, B: Firing styrene, C: A beaker polyethylene jacket, D: A copper beaker, the copper tripod installation stand, E: Liquid nitrogen, F: A thermocouple sensor, G: Tweezers for submerging the bacteria.

stripped and put into absolute ethanol on a shaker (Penetron mark IV) and dehydrated twice for 20 mins. After dehydration, the sections of bacteria were first put into a solution of 50% ethanol and 50% LR white medium (London Resin Co. Ltd, Berkshire, UK) water-soluble resin. The bacteria was subsequently placed into 100% LR white medium water-soluble resin and heated to 47℃ then left to rest for 72 hours.

The bacteria segments were then cut into ultrathin slices (0.5mm x 0.5mm x 0.09mm) using an ultra microtone (FC6, Lica Microsysteme, Wien, Austria). Samples were then treated for 20 mins with 0.02M phosphate buffer (pH7.4) containing 1% BSA (Bovine Serum Albumin) and 0.05% sodium azide and subsequently washed with 0.02M phosphate buffer. The specimen segments were treated with 1:800 diluted rabbit polyclonal antibody against *S. pneumoniae* (pneumococcal type 3 serum: Statens Serum Institute: Copenhagen, Denmark) for 90 mins. After washing with 0.05% Tween 20 for 4 times, the segments were treated with colloid gold particle conjugated goat- anti-rabbit IgG antibody (Anti-rabbit IgG, Goat, Gold EM diameter 15nm; BB International, Golden Gate, UK) for 40 mins. After washing with 0.05% Tween 20 as above, the segment was immersed in distilled water and dried. The tissue segments were treated with 0.5% osmium tetroxide for 5 min and washed with distilled water, prior to double-

stained with 6% uranyl acetate and lead citrate. All specimens for transmission electron microscopy were examined with a JEM-1230 (JEOL Ltd., Tokyo, Japan) electron microscope operated 80 KV accelerating voltages.

Results

For fixation, 0.5% osmium tetroxide ethanol solution achieved better results than 0.2% glutaraldehyde ethanol solution. Using 0.5% osmium tetroxide ethanol solution as fixative, the capsule was observed under the immunoelectron microscope as fine granules surrounding the bacterial cell wall (Figure. 4A). This colloidal gold was reacted with a specific capsule of bacterial body, however the cytoplasm of *S. pneumoniae* was indistinct. In spite of this, the fine structures such as the mucopeptide layer of pneumococcal cell wall and cytoplasmic membrane were intact (Figure. 4A, 4B, 4C). In control, the capsule of *S. pneumoniae* was not reacted with normal rabbit serum (Figure 4B), and several colloidal-gold granular structures were observed in the capsule of *S. pneumoniae* with type 19F capsule specific antibody (Figure. 4C). The capsule was visualized as radiated fibers surrounding the bacterial cell wall (Figure. 4A', 4B', 4C').

Discussion

There is little difficulty for conducting immunoelectron microscopy for Gram-negative bacteria, for which conventional freeze substitution fixation with glutaraldehyde ethanol perfectly works. However, for Gram-positive bacteria, the conventional glutaraldehyde ethanol fixation is improper fixation probably because of the thick mucopeptide cell wall. To overcome this problem, several different methods were attempted. The cytoplasmic membrane of the bacterial body is demonstrated more clearly, when osmium tetroxide instead of glutaraldehyde was used during ethanol freeze-substitution fixation. Fixation process of the sample is known to disturb the post embedding process of immunoelectron microscopy. Especially the choice and level of fixatives are critical as it affects the fine structure of antigens. To retain the fine structures and its antigenecity, 0.1% of glutaraldehyde in 4% para-formaldehyde is conventionally used. However, the thick polypeptide cell wall of Gram-positive bacteria, in contrast to Gram-negative bacteria, is impermeable and difficult to fix with this condition. As a consequence, characteristics of *S. pneumoniae* cell structures are easily altered during the process of dehydration and embedding due to inadequate fixation. To overcome this problem, osmium tetroxide ethanol was utilized as a fixative and the rapid freezing substitution fixation method was applied before conducting immunoelectron microscopy experiment.

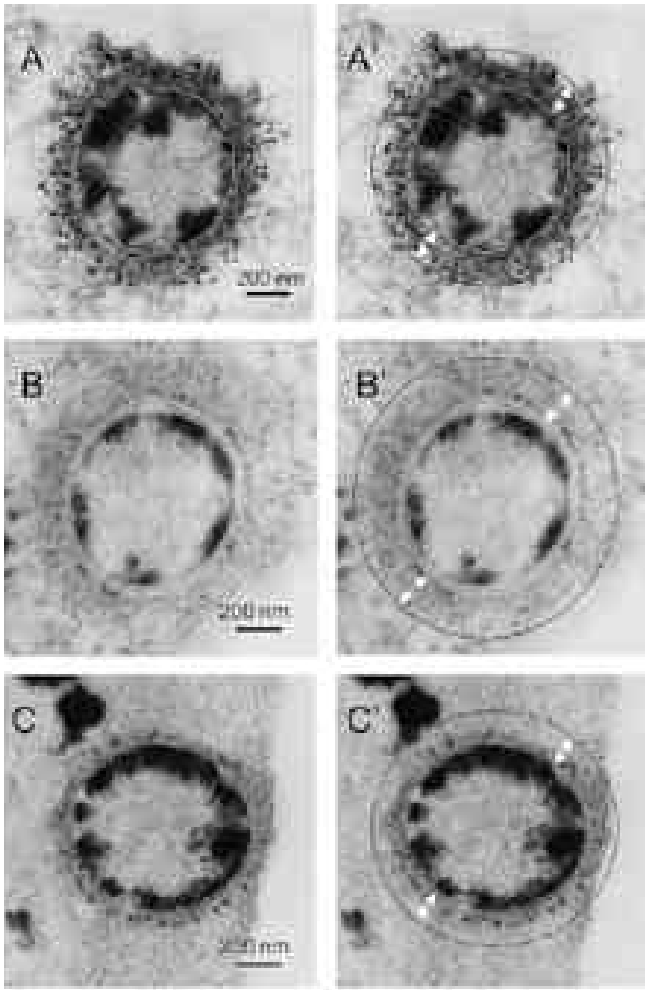

Fig. 4. The transmission immunoelectron microscope photographs show capsule of *Streptococcus pneumoniae (S. pneumoniae)*. Pneumococcal capsule zone is indicated by white-arrows. A: Osmium tetroxide ethanol fixation and immunohistochemistry. Immunoelectron microscopy clearly shows numerous colloidal-gold granular structures. The capsule of *S. pneumoniae* was reacted with type-3 capsule specific antibody. B: The colloidal-gold granular structures are not shown by immunoelectron microscopy. The capsule of *S. pneumoniae* was reacted with normal rabbit serum. The capsule was visualized as radiated fibers surrounding the bacterial cell wall C: The several colloidal-gold granular structures are observed on the capsule of *S. pneumoniae* with type 19F capsule specific antibody by immunoelectron microscopy. The capsule is visualized as radiated fibers surrounding the bacterial cell wall (Figure. 4A', 4B', 4C').

In this manuscript we describe this novel method in obtaining a detailed electron microscopic image of pneumococcal capsule. We demonstrated that the capsule maintained its

antigenecity as evident the pneumococcal type 3 polyclonal antibodies successfully bound to the capsule. The most critical point in this method is the concentration of osmium tetroxide in ethanol. If it is too high, the capsule loses its antigenecity and if too low, the cytoplasmic membrane loses its structure.

As shown in figure 3, the most important condition was this rapid freezing. It requires over a 10^4k/sec to prevent a cryohydrate phenomenon and a warp form to occur when freezing cells where over 60% of components was water. The ultrathin sectioning method by the quick freezing substitution fixation was established a long time ago, but the technique was complicated and there was a big problem in reproducibility (6-13). This method has an advantage that fine structures are preserved which is suitable for immunoelectron microscopy. Lately, the quick freezing-substitution fixing method has been greatly improved together with the vast improvement of apparatus. This technique has varieties such as, metal contact freezing method (8), liquid dipping freezing method (14-15), propane jet-gas freezing method (16-18) and high-pressure method (19). However, there is fear of explosions during the use of propane, and the use of liquefied chlorofluorocarbons. There is also a concern of global warming, because chlorofluorocarbons affect the ozone layer of the atmosphere. Therefore in this new method, we used ethanol, as a refrigerant. The reason using ethanol is the significant difference between its melting (-114.5℃) and boiling (78.3℃) points. By using quick freezing technique, we succeeded in the pneumococcal capsule observation using immunoelectron microscopy. This technique may be used to stain the capsule of other Gram-positive bacteria as well.

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