Rapid Detection of Bacterial DNA in Mastoid Granulation Tissue with Nested-PCR Technique

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Objective: *To detect bacterial DNA in mastoid granulation tissue from patients with chronic suppurative otitis media (CSOM).*
Material and Method: *A two-step polymerase chain reaction (nested polymerase chain reaction) technique was employed. A 16s rRNA universal primer common to all bacteria was used as a bracket primer for the first step PCR reaction. Primers specific to P. aeruginosa and S. aureus were then used as nested primers for the second step PCR. Products of this process were identified by DNA sequencing.*
Results: *Among 15 clinical specimens collected, five showed positive bands specific to the species P. aeruginosa, and 11 showed bands specific to the genus Staphylococcus. DNA sequencing showed 99.7 to 100% accuracy for target organisms in clinical specimens with a positive signal. The average time taken to conduct the PCR procedure was about four hours*
Conclusion: *The nested PCR technique described worked well, even when the size of the mastoid granulation tissue was very small.*

Keywords: *Polymerase chain reaction, Bacteria, Chronic suppurative otitis media*

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Chronic suppurative otitis media (CSOM) is a chronic infection of the middle ear mucosa accompanied by perforation of the tympanic membrane, resulting in chronic ear discharge or otorrhea[1]. Without appropriate treatment, this disease can lead to severe complications such as labyrinthitis, mastoiditis, and brain abscesses[2].

CSOM treatment involves administration of an appropriate antibiotic to kill the causative organism, aural toilet, and surgical closure of the perforation if this site does not close spontaneously[1]. Bacterial culture is the technique currently used to detect and identify the causative organism, but this can take three to seven days to yield a result. This is extremely inconvenient for outpatient-based treatment of the disease. Most otolaryngologists prescribe antibiotics empirically, based on epidemiologic statistics or professional experience. This can lead to inappropriate antibiotic use, resulting in sub-optimal treatment for the patient and the emergence of drug resistance[1,3].

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The most common causes of CSOM worldwide and in Thailand are *Pseudomonas aeruginosa* and *Staphylococcus aureus*[1,3-6]. Standard diagnosis involves culture of pus from the middle ear. However, a study by Yamamoto and Iwanga[7] comparing bacterial culture results between pus from the ear canal and tissue from the middle ear found a 53% difference. This indicates that culture results from ear canal pus can be contaminated, and does not give a true indication of the causative organism.

The above issues led the authors to consider the use of PCR for detection of bacteria in middle ear tissue from CSOM patients. *P. aeruginosa* and *S. aureus* were chosen as the target organisms, and a two-step PCR (nested PCR) technique was developed. 16S rRNA universal primer common to all bacteria was used as a bracket primer for the first step PCR reactions, and primer specific to *P. aeruginosa* and *S. aureus* were used as nested primers for the second step PCR. Our aim was to develop a fast and reliable technique for detection of the causative organisms of CSOM.

Material and Method

Participants

Fifteen Japanese patients who had been diagnosed with chronic otitis media, with and without cholesteatoma, and scheduled for middle ear or mastoid surgery between August 2010 and June 2011 were recruited into the present study. All patients stopped topical or systemic antibiotic treatment two weeks before surgery. Written consent was obtained before surgery.

Specimens

Tissue was collected from the mastoid cavity during mastoid surgery of CSOM patients. Fifteen specimens were collected (each between 0.5 and 1 millimeter in size).

Extraction of bacterial DNA from middle ear mucosa

DNA extraction was performed using a standard method described in the DNeasy Blood and Tissue Handbook[8]. A Qiamp DNA minikit (Qiagen^{R}) was employed. Specimens were treated with lysozyme and 10% SDS for 30 minutes to lyse bacterial cell walls prior to extraction. Reference strains *P. aeruginosa* ATCC 15692 and *S. aureus* strain BAA-1556 (obtained from the Infectious Control Unit of Kyoto University Hospital) were used as positive controls for the PCR.

Extraction of bacterial DNA

Pseudomonas aeruginosa strain ATCC 15692 and *Staphylococcus aureus* strain BAA-1556 separated from patient by Infectious control unit Kyoto university hospital were extracted for DNA by Qiamp DNA minikit (QiagenR) to be used as positive control for PCR reaction.

Detection of bacterial DNA by nested PCR technique

DNA extracted from clinical samples and reference strains of *P. aeruginosa* and *S. aureus*) was amplified by nested PCR. A 16s rRNA universal primer (27f and 1525r) was used as a bracket primer for the first step PCR reaction. Primers specific for *P. aeruginosa* (PA-GS-F and PA-SS-R)[9] and *S. aureus* (Staph-756F and 750R) (Zhang et al)[10,11] were then used as nested primers for the separate second step PCR reaction (Fig. 1, Table 1).

Condition for DNA amplification by nested PCR

A 50 ul PCR mixture was prepared containing 2.5 mM dNTP mix (1 ul), 10x Ex Taq Buffer with $MgCl_2$ 1.5 mM (5 ul), Taq polymerase (5U/ul) (0.25 ul), forward and reverse primer (1 ul of each), and DNA template for the first step PCR reaction (100 ug). Then, 2 ul of product from this reaction was used as DNA template for both the second step PCR reactions.

Temperature cycling:

- First step universal PCR: initial hold at 95°C for two minutes before 30 cycles of denature at 95°C for one minute, annealing at 50°C for one minute, elongation at 72°C for 90 seconds and final extension at 72°C for two minutes.

- Second step specific PCR for *P. aeruginosa*: initial hold at 95°C for two minutes before 30 cycles of denature at 94°C for one minute, annealing at 62°C

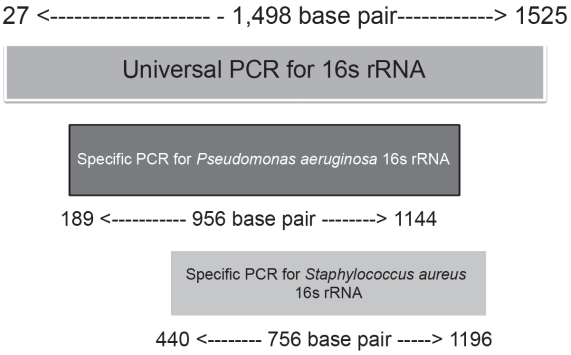

Fig. 1 Two step polymerase chain reaction technique (Nested PCR technique) use in this study.

Table 1. Primer used in this study and their expected product size

Primer	Sequence (5'-3')	Target	Location	Product size
27f	AGAGTTTGATCCTGGCTCAG	16s rRNA	27-47	1498
1525r	AAAGGAGGTGATCCAGCC		1507-1525	
PA-GS-F	GGGGGATCTTCGGACCTCA	*Pseudomonas aeruginosa*	189-206	956
PA-SS-R	TCCTTAGAGTGCCCACCCG		1124-1144	
Staph756F	AACTCTGTTATTAGGGAAGAACA	*Staphylococcus aureus*	440-462	756
Staph750R	CCACCTTCCTCCGGTTTGTCACC		1174-1196	

for one minute, elongation at 72°C for 90 seconds and final extension at 72°C for two minutes.

- Second step specific PCR for *S. aureus*: initial hold at 94°C for five minutes before 30 cycles of denature at 94°C for 30 seconds, annealing at 58°C for 30 seconds, elongation at 72°C for one minute and final extension at 72°C for 10 minutes.

Analysis of PCR product (amplicons):

products from each reaction were separated in 2% agarose gel containing ethidium bromide (100 volts; 30 minutes) and visualized under ultraviolet illumination.

DNA sequencing

Amplicons from the nested PCR technique were sequenced using an automated sequencer. Sequencing results were then compared with the GenBank database by basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Ethical considerations

The present study was approved by the Ethics Committee of the Graduate School of Medicine, Kyoto University. Written informed consent was obtained from all participants.

Results

DNA extraction from middle ear mucosa

DNA was successfully extracted from all 15 clinical specimens. The small size of these specimens left the total concentration of extracted DNA quite low (Table 2). The total concentration of bacterial DNA may have been lower still, since the specimens also contained human DNA. Importantly, the quantity of extracted DNA (100 ug) was enough for PCR reactions in the present study.

Detection of bacterial DNA by nested PCR technique

When the 16S rRNA universal primer was used, it was possible to detect amplified bacterial DNA from the cultured reference strains of *P. aeruginosa* and *S. aureus*. A 1498 bp signal was observed for both of these positive controls (Fig. 2A). However, no amplified bacterial DNA was detected for any of the 15 clinical samples (Fig. 2B).

After the second step nested PCR was performed with primer specific for *P. aeruginosa*, a 956 bp band was detected in five of the 15 clinical specimens (Fig. 3A). Nested PCR with primer specific for *S. aureus* generated a 756 bp band in 11 of the 15 specimens, though the band in specimen 13 was relatively weak (Fig. 3B).

Table 2. Concentration and quality of DNA from samples

Sample ID	ng/uL	A260	260/280	260/230	Constant
Sample 1	10.13	0.203	1.79	0.58	50
Sample 2	6.03	0.121	2.24	0.34	50
Sample 3	5.01	0.100	2.73	0.37	50
Sample 4	7.09	0.142	1.96	0.41	50
Sample 5	18.47	0.369	2.47	1.02	50
Sample 6	5.69	0.114	2.75	0.43	50
Sample 7	2.16	0.043	14.54	0.15	50
Sample 8	1.03	0.021	-0.81	0.11	50
Sample 9	9.92	0.198	1.96	1.00	50
Sample 10	2.52	0.050	1.38	0.60	50
Sample 11	4.43	0.089	1.49	0.65	50
Sample 12	15.59	0.312	1.92	1.60	50
Sample 13	10.58	0.212	1.85	1.00	50
Sample 14	17.83	0.357	1.79	1.03	50
Sample 15	20.29	0.406	1.77	1.32	50
P. aeruginosa positive control	95.26	1.905	2.01	1.78	50
S. aureus positive control	105.63	2.113	1.46	0.63	50

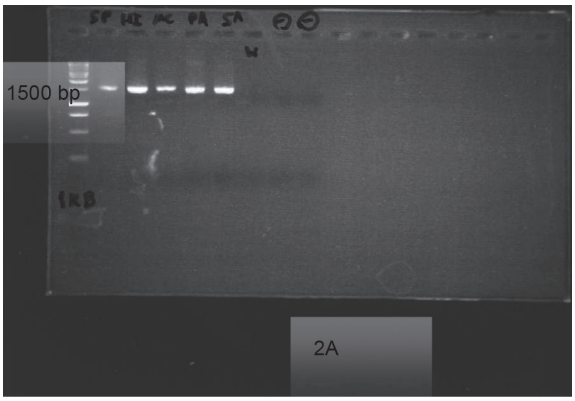

Fig. 2A Universal PCR: positive control show positive signal at 1,500 bp, the desired product size. Lane 1 was 1 kB ladder, lane 2-6 were cultured bacterial DNA used as positive control, lane 7-9 were negative control.

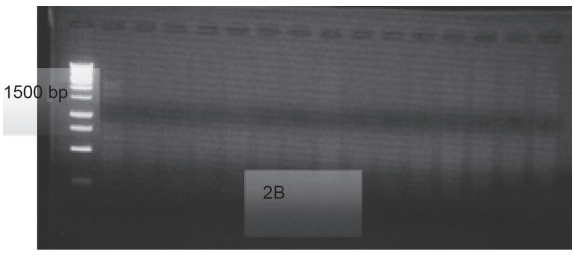

Fig. 2B Universal PCR result from 15 patient sample; there was no positive signal at desired size, it could be interpreted that there were not enough amplicon to generate signal. Lane 1 was 1 kB ladder, lane 2-16 were clinical sample.

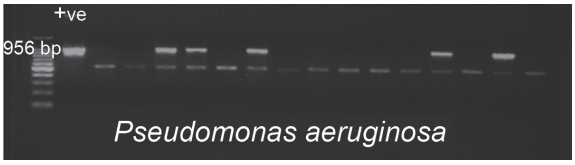

Fig. 3A Specific Nested-PCR for *Pseudomonas aeruginosa*: Positive control and Clinical sample show signal specific for *P. aeruginosa* at 956 bp. Lane 1 was 100 kb ladder, lane 2 was positive control, the rest was clinical specimens.

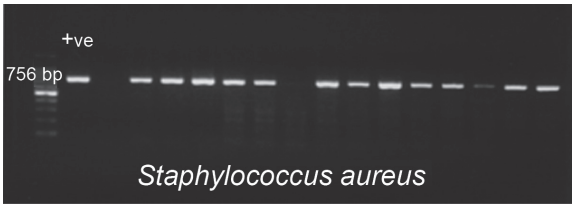

Fig. 3B Specific Nested-PCR for *Staphylococcus aureus*: Positive control and Clinical sample show signal specific for *S. aureus* at 756 bp. Lane 1 was 100 kb ladder, lane 2 was positive control, the rest was clinical specimens.

DNA sequencing

The 956 bp products generated from each of the five *P. aeruginosa* positive clinical samples were excised from the gel, and their DNA sequences were determined. Nucleotide lengths were within 769-869 bp and with database comparison. All amplicons from these clinical specimens were 99.8 to 100% compatible with *P. aeruginosa*.

PCR products from the 11 clinical samples with a 756 bp signal specific to *S. aureus* were also sequenced. Nucleotide lengths were within 621 and 701 bp. When compared with the BLAST database, the authors found the amplicons were 99.7 to 100% compatible with the *Staphylococcus* genus. However, these were not specific to *S. aureus*. Similarly, the DNA sequence from the cultured reference strain of *S. aureus* DNA amplified with the 16s rRNA universal primer was not specific for *S. aureus* (Table 3).

Discussion

For diagnostic purposes, the sensitivity and specificity of PCR is superior to that of conventional culture[12-15]. PCR also generates results more rapidly than bacterial culture. These attributes mean PCR is highly suitable in an outpatient setting. In the present study, diagnosis of CSOM by PCR took less than four hours, a major improvement on the typical three to seven day culture time.

In a previous study by Kuczkowski et al[16], different techniques were compared for the diagnosis of CSOM in 53 patients. The authors found PCR to be much more sensitive than conventional culture for the detection of bacteria. Given that the specimens used in the Kuczkowski study were pus samples however, questions are raised regarding the involvement of the isolated organisms. Bacteria present in middle ear pus may not truly reflect the organisms present in the middle ear. Many studies describe how bacteria from the ear canal's normal flora can contaminate the collected specimen[1,3,4]. When Yamamoto and Iwanga compared culture results between the mastoid cavity and tympanic cavity, they noted a 53% difference[7]. This has raised doubts regarding the suitability of pus from the middle ear for CSOM diagnosis, especially when using PCR, which is so sensitive to contamination. Contamination with bacteria from the normal flora could be responsible for misleading results and inappropriate antibiotic prescription. According to a study by Albert et al, the mastoid granulation of CSOM patients can harbor multiple microbial species[17]. Nevertheless, the procedure the present

Table 3. Summary of DNA sequencing in Positive control and clinical sample

Sample ID	No. of nucleotides sequenced	No. of nucleotides matched	Matched (%)	Identification
Positive control: DNA of *P. aeruginosa*	1433	1433	100.0	*Pseudomonas aeruginosa*
Sample 3	794	794	100.0	*Pseudomonas aeruginosa*
Sample 4	769	769	100.0	*Pseudomonas aeruginosa*
Sample 6	794	794	100.0	*Pseudomonas aeruginosa*
Sample 10	734	734	100.0	*Pseudomonas aeruginosa*
Sample 14	792	792	100.0	*Pseudomonas aeruginosa*
Positive control: DNA of *S. aureus*	1446	1446	100.0	*Staphylococcus aureus*, *Staphylococcus haemolyticus*
Sample 2	692	692	100.0	*Staphylococcus aureus*, *Staphylococcus haemolyticus*
Sample 3	695	695	100.0	*Staphylococcus aureus*, *Staphylococcus haemolyticus*
Sample 4	692	692	100.0	*Staphylococcus aureus*, *Staphylococcus haemolyticus*
Sample 5	696	696	100.0	*Staphylococcus epidermidis*, *Staphylococcus caprae*, *Staphylococcus saccharolyticus*, *Staphylococcus capitis*, *Lysinibacillus sphaericus*, *Bacillus amyloliquefaciens*
Sample 6	621	621	100.0	*Staphylococcus epidermidis*, *Staphylococcus caprae*, *Staphylococcus saccharolyticus*, *Staphylococcus capitis*, *Lysinibacillus sphaericus*, *Bacillus amyloliquefaciens*
Sample 8	683	683	100.0	*Staphylococcus aureus*, *Staphylococcus haemolyticus*
Sample 9	688	688	100.0	*Staphylococcus* sp. (*aureus*, *haemolyticus*, *epidermidis*...)
Sample 10	690	690	100.0	*Staphylococcus* sp. (*aureus*, *haemolyticus*, *epidermidis*...)
Sample 11	701	699	99.7	*Staphylococcus auricularis*
Sample 12	693	693	100.0	*Staphylococcus epidermidis*, *Staphylococcus caprae*, *Staphylococcus saccharolyticus*, *Staphylococcus capitis*, *Lysinibacillus sphaericus*, *Bacillus amyloliquefaciens*
Sample 13	693	693	100.0	*Staphylococcus epidermidis*, *Staphylococcus caprae*, *Staphylococcus saccharolyticus*, *Staphylococcus capitis*, *Lysinibacillus sphaericus*, *Bacillus amyloliquefaciens*

study used to collect tissue specimens was performed in a relatively sterile operative field, and the possibility of contamination from mastoid granulation is likely to be lower than that of middle ear pus.

Since our middle ear and mastoid granulation tissues were very small, the concentration of extracted DNA was insufficient to allow identification of all organisms by 16s rRNA universal primer amplification

and cloning. As shown previously, the nested PCR technique is capable of accurately detecting bacterial DNA even in small quantities of tissue[15]. Here we have demonstrated that *P. aeruginosa* can be detected and, through DNA sequencing, identified to species level with 100% accuracy. This is not the case for *S. aureus*. *S. aureus* can be identified to genus level but cannot be specified. This limitation notwithstanding, clinical application of the nested PCR technique for rapid identification of the causative organisms of CSOM could aid clinical judgment in selection of the most appropriate post-operative antibiotic.

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Potential conflicts of interest

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การตรวจหาดีเอ็นเอของแบคทีเรียในเนื้อเยื่อจากมาสตอยด์ด้วยเทคนิค nested-PCR

ภีม เอี่ยมประไพ, ยาซุฟุมิ มัตซึมูระ, ฮารุคาซุ ฮิราอุมิ, โนริโอะ ยามาโมโต้, ชุนจิ ทาคาคระ, จูอิจิ อิโต้

วัตถุประสงค์: *เพื่อตรวจหาดีเอ็นเอของแบคทีเรียก่อโรคหูชั้นกลางอักเสบเรื้อรังในเนื้อเยื่อจากโพรงมาสตอยด์ของผู้ป่วยหูน้ำหนวกเรื้อรัง*

วัสดุและวิธีการ: *ใช้กระบวนการโพลีเมอเรส เชน รีแอคชัน สองขั้นตอน ซึ่งมีชื่อเรียกเฉพาะว่า nested PCR โดยทำการขยายจำนวนดีเอ็นเอส่วน 16s ribosomal RNA อันเป็นส่วนที่จำเพาะต่อเซลล์โปรคาริโอต แล้วจึงขยายส่วนของดีเอ็นเอ อันจำเพาะต่อเชื้อแบคทีเรียที่ต้องการตรวจหาด้วย polymerase chain reaction อีกครั้ง เชื้อแบคทีเรียเป้าหมายในการศึกษานี้คือ P. aeruginosa และ S. aureus ตัวอย่างที่ผ่านกระบวนการทั้งหมด จะได้รับการตรวจยืนยันโดยการอ่านรหัสเบสบนสารดีเอ็นเอ*

ผลการศึกษา: *ภายในระยะเวลาที่ศึกษา สามารถเก็บเนื้อเยื่อจากโพรงมาสตอยด์ผู้ป่วยหูน้ำหนวกเรื้อรังได้ 15 ชิ้น ในจำนวนนี้ 5 ชิ้น แสดงแถบแสงจำเพาะต่อ P. aeruginosa และ 11 ชิ้น แสดงแถบจำเพาะต่อเชื้อ genus Staphylococcus การอ่านคู่เบสเพื่อตรวจทาน พบว่าวิธีนี้มีความแม่นยำสูงถึงร้อยละ 99.7 ถึง 100 และใช้ระยะเวลาในการตรวจเพียงสี่ชั่วโมง*

สรุป: *เทคนิค nested PCR สามารถตรวจหาดีเอ็นเอของแบคทีเรียได้ดี แม้ในเนื้อเยื่อที่มีขนาดเล็กมาก*