

## 慢性中耳炎患者検体中にみられる病原体を、起炎病原体と定着病原体とに判別する分子技術とその応用

(論文の区分 博士課程)

著者名 DINA ALIA

担当指導教員氏名 萩原弘一 教授

自治医科大学大学院医学研究科  
地域医療学系  
内科系総合医学・総合医学

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## Introduction

Chronic suppurative otitis media (CSOM) is a persistent infection of the middle ear associated with a perforated tympanic membrane resulting in ear discharge for more than six weeks [1]. The morbidity in children is high in many countries including Indonesia. Affected children often lose hearing capability resulting in poor academic performances [2]. Early diagnosis, eradication of infection, and prevention of recurrence are warranted for effective control.

Identification of the causative pathogens is essential for treatment strategies. However, accurate identification is challenging owing to colonizing bacterial contamination and bacterial culture difficulty from suppurative specimens. Consequently, antibiotics misuse and disease recurrence are frequent [3, 4] and additional techniques that can exclude the colonizing pathogens is required to define the causative pathogens. Human cell-controlled identification of the respiratory agent (HIRA-TAN method) is a technique that utilizes the battlefield hypothesis [5] for accurate pathogen identification. According to the battlefield hypothesis, the ratio of causative pathogen cells to human cells in a purulent sample is considered as an indicator for the discrimination of the causative pathogen from the colonizing organisms. A pathogen with a high ratio indicates a higher number of pathogen cells to inflammatory cells, which suggests that it is likely to be the causative pathogen. HIRA-TAN has been successfully used to differentiate the causative pathogen from commensal organisms in the untreated Community-Acquired Pneumonia (CAP) and progressive course pneumonia [6, 7].

CSOM is a chronic inflammation where the number of pathogen cells and human inflammatory cells are in a balance – neither the pathogen overwhelms the inflammatory cells, nor the inflammatory cells dominate the pathogens. Therefore, the ratio of pathogen cells to human cells would be in a specific range, and the HIRA-TAN approach might be

applicable for CSOM diagnosis. However, the technique should be validated before adopting it to the clinic. In the current study, we examined the utility of the HIRA-TAN method for pathogen identification that cause CSOM.

## **Methods**

### **Ethics approval and consent to participate**

The study was approved by both institutional review boards of Dr. Zainoel Abidin Hospital and Universitas Syiah Kuala (approval number: 06/KE/FK/2016), and Jichi Medical University (approval number: 17-123). The signed informed consent was obtained from the study participants. Informed consent was sought from the parents of all children participating in this study.

### **Specimen collection**

Samples were collected at the Dr. Zainoel Abidin Hospital between December 2016 and January 2017. The study recruited patients who had persistent and recurrent mucopurulent otorrhea for more than two months with a perforated tympanic membrane(s). Children <16 years old were seated in examination chair accompanied by the parents. The ear canal was cleansed, and the otorrhea was swab-collected under otoscopic guidance. Audiometry was performed to confirm hearing impairment. The samples were processed for bacterial culture and HIRA-TAN assay.

### **Bacterial culture**

**The sample collection.** Samples were collected from the patient diagnosed as CSOM at ENT outpatient clinic Zainoel Abidin hospital. The ear was inspected by using a sterile cone otoscope. The external auditory canal was cleansed from the dry crust and discharge on the

concha to minimize the contaminants. The speculum was placed in the external auditory canal under otoscopic guidance. The sterile swab stick was gently introduced into the external auditory meatus. With gentle rotation, the otorrhea was swabbed in front of the perforated tympanic membrane. The sample collection was placed in the Stuart transport medium and transported to the laboratory within 2 hours.

**Bacterial culture process.** The direct gram smear was performed at microbiology laboratory Zainoel Abidin Hospital directly after the sample arrived from the outpatient clinic.

Inspection of the specimen and cell counting was performed to scrutinize the specimen quality. The samples were inoculated with streaking techniques onto blood agar, MacConkey, and chocolate agar plates.

**Bacteria isolation and identification.** The medium plates were incubated in the incubator on  $35 \pm 2$  °C for 24 hours. The colony morphology from the culture plates was evaluated to identify isolates. The colony morphology, Gram stain, and the biochemical identification test were performed to distinguish the species.

### **Primer and probe design**

Primers and fluorescent probes were designed for common pathogens reported such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Escherichia coli*, *Klebsiella* spp., *Bacteroides* spp., and *Proteus* spp. [8, 9] based on the genome sequences obtained from the National Centre for Biotechnology Information (NCBI) nucleotide database. A pair of primer and a fluorescent-labeled probe reflect a target detection. Sixteen targets were able to differentiate five genera (*Pseudomonas* spp., *Enterobacter* spp., *Staphylococcus* spp., *Streptococcus* spp., and *Proteus* spp.), nine species (*P. aeruginosa*, *E. coli*, *S. aureus*, *S. pneumoniae*, *K. pneumoniae*, *A.*

*baumanii*, *H. influenza*, *M. catarrhalis*, and *B. fragilis*), a methicillin resistance gene (*mecA*), and a human tumor necrosis factor gene (*TNF*).

### **Multiplex TaqMan assay**

DNA extraction: Genomic DNA extraction was performed by Genomic DNA Mini Kit (Blood/Cultured Cell) according to the instructions of the manufacturer (Geneaid, Taipei, Taiwan). Briefly, sample was suspended in 150 µl, GT Buffer. After adding 200 µl GB Buffer, sample was vigorously shaken in a 1.5 ml microcentrifuge tube. Incubation of mixture at 60 °C for 15 minutes were performed prior DNA elution with 100 µl deionized distilled water.

Real-time PCR: The reaction for 16 different targets was performed in four separated tubes (Table 1 and 2). The final solution contained 1 µL of sample (20 ng), 250 nM of each primer, 250 nM of each fluorescence-labeled probe, 12.5 µL Takara Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and deionized distilled water up to 25 µl. The real-time PCR reaction was performed using a SmartCycler II (Cepheid Inc., Sunnyvale, USA) with continuous monitoring of fluorescence. Temperature and time parameters were 30 s of an initial denaturation prior 30 cycles of 95 °C for 8 s, 61 °C for 25 s, and 72 °C for 20 s.

**Table 1. Primer and probes of the target genes**

No	Target	Gene (Accession number)		Oligonucleotide	Amplicon (bp)
1	<i>Homo sapiens</i>	TNF (NC_000006.12)	F*	5'-GTGGAGCTGAGAGATAACCAGC-3'	153
			B	5'-GACCTTGGTCTGGTAGGAGACG-3'	
			P	FAM-CTGTACCTCATCTACTCCCAGGTC-BHQ1	
2	<i>Pseudomonas sp.</i>	16S rRNA (AY486350.1)	F	5'-GTGAGTAATGCCTAGGAATCTGC-3'	135
			B	5'-CTAATCCGACCTAGGCTCATCTG-3'	
			P	ALEXA532-AGTGGGGGATCTTCGGACCTC-BHQ1	
3	<i>Enterobacter sp.</i>	tusB (AH002539.2)	F	5'-CGTCAGACTTACGGTTAAGCAC-3'	193
			B	5'-GTACCAGCTGGTTAACTGTTGC-3'	
			P	ALEXA594-AGCCAGATGGCCTGGTGATG-BHQ2	
4	<i>Staphylococcus sp.</i>	Tuf (NC_007168.1)	F	5'-CTCAATCACTGGTCGTGGTACTG-3'	163
			B	5'-GTCACCAGCTTCAGCGTAGTC-3'	
			P	ALEXA647-CGTGTTGAACGTGGTCAAATCA-BHQ2	
5	<i>Pseudomonas aeruginosa</i>	23S rRNA (AJ549386.1)	F	5'-GTTGTCCAAGTTTAAGGTGGTAGG-3'	97
			B	5'-CCACTTCGTCATCTAAAAGACGAC-3'	
			P	FAM-TTCAAGGCCGAGAGCTGATGAC-BHQ1	
6	<i>Klebsiella pneumoniae</i>	gapA (M66869.1)	F	5'-CATCGAGATCGTTGCAATCAACG-3'	81
			B	5'-CGACCGTGAGTGGAGTCATAC-3'	
			P	ALEXA532-AGACGCAGAGTACATGGCTTACATG-BHQ1	
7	<i>Streptococcus pneumoniae</i>	Pneumolysin (NC_003098.1)	F	5'-CAAGGTAAGGAAGTCTTGACTCC-3'	193
			B	5'-GCTTACGCACTAGTGGCAAATCGG-3'	
			P	ALEXA594-AGGGAATGTTCGTAATCTCTCTGTC-BHQ2	
8	<i>Staphylococcus aureus</i>	Thermonuclease (NC_007795)	F	5'-GTCCTGAAGCAAGTGCATTTACG-3'	282
			B	5'-GACCTGAATCAGCGTTGTCTTC-3'	
			P	ALEXA647-CGAAGCTTTAGTTCGTCAAGGCTTG-BHQ2	
9	<i>Acinetobacter baumannii</i>	ompA (AY485227.1)	F	5'-GAACTATACAGCTCTTGCTGGC-3'	87
			B	5'-CTCTTGTGGTTGTGGAGCAAC-3'	
			P	FAM-GTTCTTGGTGGTCACTTGAAGC-BHQ1	

10	<i>E.coli</i>	phoA (M29670.1)	F	5'-CGAAGAGGATTCACAAGAACATACC-3'	88
			B	5'-CATTAAGTCTGGTTGCTAACAGC-3'	
			P	ALEXA532-TCAGTTGCGTATTGCGGCGTATG-BHQ1	
11	<i>Streptococcus sp.</i>	Tuf (AY267003.1)	F	5'-CACTGGACGTGGTACAGTTGCTTC-3'	188
			B	5'-GAACACCACGAAGAAGGACACCTAC-3'	
			P	ALEXA594-CAACTTGACGAAGGTCTTGCTGG-BHQ2	
12	<i>Hemophilus influenza</i>	16S rRNA (Z22806.1)	F	5'-GACATCCTAAGAAGAGCTCAGAG-3'	266
			B	5'-CTTCCCTCTGTATACGCCATTG-3'	
			P	ALEXA647-CCTTCGGGAACCTTAGAGACAG-BHQ2	
13	<i>Moraxella catarrhalis</i>	CopB (U69982.1)	F	5'-GTGCGTGTTGACCGTTTTGAC-3'	134
			B	5'-GTGGCATAGATTAGGTTACCGCTG-3'	
			P	FAM-CCGACATCAACCCAAGCTTTG-BHQ1	
14	<i>Bacteroides fragilis</i>	16S rRNA (AP006841.1)	F	5'-GACTGCAACTGACACTGATGCTC-3'	316
			B	5'-CAACCATGCAGCACCTTCACAG-3'	
			P	ALEXA532-AGATACCCTGGTAGTCCACACAG-BHQ1	
15	<i>Proteus sp.</i>	16S rRNA (NC_010554.1)	F	5'-CTCTTCGGACCTTGCACTATC-3'	127
			B	5'-CGTGTCTCAGTCCCAGTGTG-3'	
			P	ALEXA594-CGACGATCTCTAGCTGGTCTG-BHQ2	
16	<i>Staphylococcus aureus (MRSA)</i>	mecA (AY786579.1)	F	5'-CGGTAACATTGATCGCAACGTTC-3'	108
			B	5'-CTTTGGTCTTTCTGCATTCTG-3'	
			P	ALEXA647-TGGAAGTTAGATTGGGATCATAGCG-BHQ2	

\*F: Forward primer, B: Backward primer, P: Taqman probe, BHQ: Black whole quencher™



**Table 2. Multiplex PCR reactions**

Reaction	FAM	Cy3	Texas Red	Cy5
I	<i>Homo sapiens</i> (TNF)	<i>Pseudomonas sp.</i> (16S rRNA)	<i>Enterobacter sp.</i> (tusB)	<i>Staphylococcus sp.</i> (Tuf)
II	<i>P. aeruginosa</i> (23S rRNA)	<i>K. pneumoniae</i> (gapA)	<i>S. pneumoniae</i> (Pneumolysin)	<i>S. aureus</i> (Thermonuclease)
III	<i>A. baumannii</i> (ompA)	<i>E. coli</i> (phoA)	<i>Streptococcus sp.</i> (Tuf)	<i>H. influenzae</i> (16S rRNA)
IV	<i>M. catarrhalis</i> (CopB)	<i>B. fragilis</i> (16S rRNA)	<i>Proteus sp.</i> (16S rRNA)	Methicillin-resistant <i>S. aureus</i> ;MRSA (mecA)

PCR was performed in four separate multiplex reactions. The SmartCycler II (Cepheid Inc., Sunnyvale, USA) is able to simultaneously detect four fluorescent channels. We used four fluorophores – FAM for the FAM channel, ALEXA532 for the Cy3 channel, ALEXA594 for the Texas Red channel, and ALEXA647 for the Cy5 channel. Temperature and time parameters were 30 s of an initial denaturation prior 30 cycles of 95 °C for 8 s, 61 °C for 25 s, and 72 °C for 20 s.

### HIRA-TAN method

The copy number of a pathogen-specific gene represents the number of bacterial cells and a human-specific gene represents the number of human cells. Thus, the number ratio of pathogen cells to human cells,  $\Delta Ct_{Pathogen}$  was determined using the following equation.

$$\begin{aligned}\Delta Ct_{Pathogen} &= -(Ct_{Pathogen} - Ct_{Human}) \\ &= \log \left( \frac{\text{Number of pathogen cells}}{\text{Number of human cells}} \right)\end{aligned}$$

Where,  $Ct_{Pathogen}$  is the cycle-threshold for the pathogen-specific gene and  $Ct_{Human}$  is the cycle-threshold for the human-specific gene. Based on the battlefield hypothesis, pathogens with a  $\Delta Ct_{Pathogen}$  over the cut-off value overpower the human inflammatory cells in numbers thus the likely causative pathogens can be deduced. In the current study, the TNF gene was used as a human-specific gene and the  $\Delta Ct_{Pathogen}$  cut-off value was determined by a receiver operating characteristic (ROC) curve analysis.

### **ROC (Receiver Operating Characteristic) curve analysis**

Receiver-operating characteristic (ROC) curve analysis was performed by designating 1 as positive and 0 as negative in the culture result column along with the delta Ct value for each sample. The data were tabulated in the spreadsheet program. The EZR version 1.36 on R 3.3.1 and R commander 2.3-0 [10] were executed for performing ROC curve analysis. The culture was used as the Response and Ct values as the Predictor. The area under curve value with the 95% confidence interval, sensitivity vs (1-specificity) graph, and sensitivity/specificity were graphed.

### **Semi-quantitative determination of pathogen and human cell numbers**

Cycle-threshold (Ct) values were obtained from 4-fold serial dilutions of genomic DNA. The genomic DNA used in the study was derived from *Streptococcus pneumoniae* (Klein) Chester ATCC BAA-255D-5, *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC 700699D-5, *Haemophilus influenzae* (Lehmann and Neumann) Winslow et al. ATCC 51907D, *Moraxella catarrhalis* (Frosch and Kolle) Bovre ATCC 25240D-5, *Acinetobacter baumannii* ATCC BAA-1605D-5, *Bacteroides fragilis* (Veillon and Zuber) Castellani and Chalmers ATCC 25285D-5, and *Proteus mirabilis* Hauser ATCC 12453D, which were purchased from the American Type Culture Collection (Rockville, MD, USA); *Pseudomonas aeruginosa* (106052G), *Klebsiella pneumoniae* subsp. *pneumoniae* (14940G), and *Escherichia coli* (12713G), which were purchased from Biological Resource Centre, NITE (Chiba, Japan); and human genomic DNA, which was purchased from Promega (Madison, WI, USA). The Ct values were plotted against the log of the copy number of genomic DNA, and a linear regression equation was obtained.

## Results

### Clinical characteristics

Thirty-nine patients (mean age = 32 years) (1.7–62) were enrolled in the study. Twenty-nine were adults (> 20-year-old), and 10 were children. The symptoms reported were ear discharge (100%), hearing problems (69.2%), ear pain (61.5%), ear itching (64.1%), and fever (33.3%). Adults (24/29 patients) had a significantly higher rate of hearing impairment than children (3/10 patients; Table 3).

**Table 3. Clinical characteristics**

Signs & symptoms	Children (%)	Adult (%)
Ear discharge	10 (100)	29 (100)
Ear pain	4 (40)	20 (68.9)
Itching	7 (70)	18 (62)
Fever	3 (30)	10 (34.4)
Hearing impairment*	3 (30)	24 (82.7)

\* Fisher's exact test,  $p < 0.005$

### BACTERIAL CULTURE

The ear canal was cleansed prior to sample swab collection to decrease the contamination. The otorrhea was collected in front of the ruptured tympanic membrane. The swab was placed in Stuart transport medium for culture within 2 hours. The microscopic examination of gram stain was performed at microbiology laboratory Zainoel Abidin Hospital. The culture yielded normal flora in 3 (7.7 %) samples, while no colonies grew in 22 (56 %) samples. The positive results were obtained in 14 patients (36%), where the detected bacteria were *P. aeruginosa* 6 (15%), *K. pneumoniae* 5 (13%), *P. mirabilis* 2 (5 %) and *M. morganii* 1 (2.5 %). (Black dots in Figure 1; Table 4).

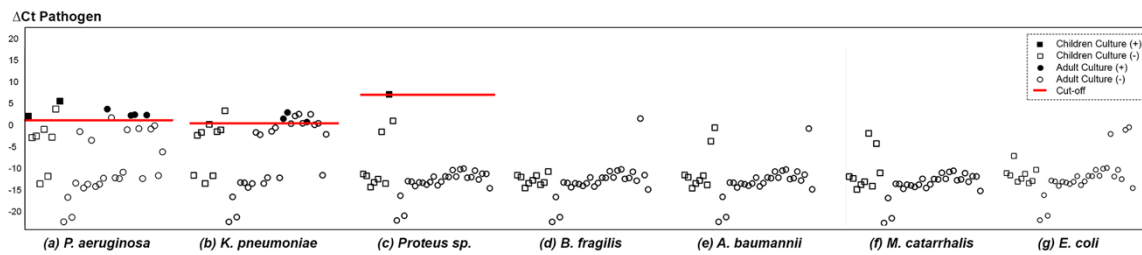
**Table 4. Culture Result**

SAMPLE	CULTURE	SAMPLE	CULTURE
1	No growth	21	<i>Morganella morganii</i>
2	Normal flora	22	<i>Klebsiella pneumoniae</i>
3	No growth	23	<i>Klebsiella pneumoniae</i>
4	No growth	24	No growth
5	Normal flora	25	No growth
6	No growth	26	<i>Pseudomonas aeruginosa</i>
7	No growth	27	<i>Pseudomonas aeruginosa</i>
8	No growth	28	No growth
9	No growth	29	<i>Klebsiella pneumoniae</i>
10	No growth	30	<i>Pseudomonas aeruginosa</i>
11	<i>Pseudomonas aeruginosa</i>	31	No growth
12	No growth	32	No growth
13	No growth	33	No growth
14	No growth	34	<i>Klebsiella pneumoniae</i>
15	<i>Pseudomonas aeruginosa</i>	35	<i>P. mirabilis</i>
16	No growth	36	<i>P. mirabilis</i>
17	Normal flora	37	No growth
18	No growth	38	<i>Pseudomonas aeruginosa</i>
19	No growth	39	No growth
20	<i>Klebsiella pneumoniae</i>		

## HIRA-TAN

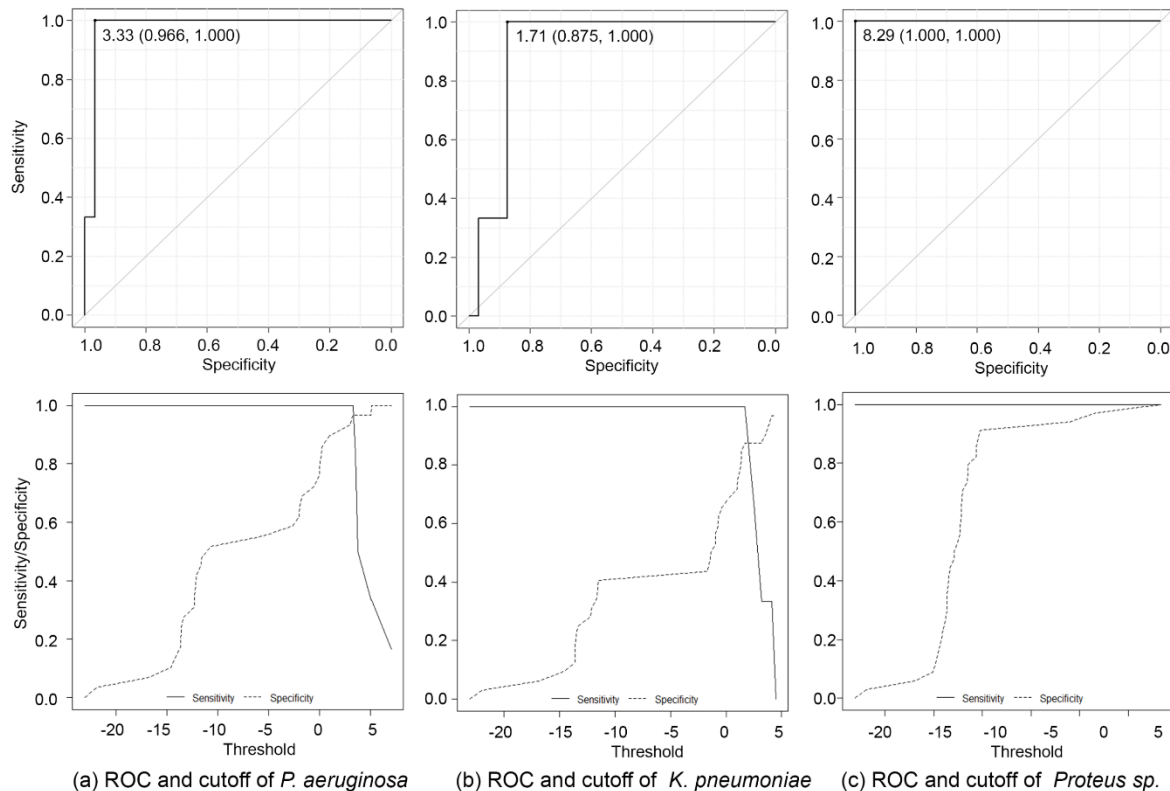
For determining whether HIRA-TAN can be used to identify the causative pathogen, we performed a real-time PCR analysis for all 16 targets (15 targets were for pathogen genes and one for the human gene) and plotted the  $\Delta Ct_{Pathogen}$  values (Figure 1). Samples positive for culture (black dots) showed a high  $\Delta Ct_{Pathogen}$ . However, some samples negative for culture (white dots) showed similar  $\Delta Ct_{Pathogen}$  values. These samples were considered as false negative culture results or samples in which most of the bacteria are not alive. We performed the ROC curve analysis in four pathogens that were detected by culture in at least one sample for determining the  $\Delta Ct_{Pathogen}$  cut-off for identifying the causative pathogen (Figure 1, red lines). The  $\Delta Ct_{Pathogen}$  cut-off, sensitivity, and specificity values were as follows: *P. aeruginosa*: 3.33 (90%, 100%), *K. pneumoniae*: 1.71 (85%, 100%), and *Proteus sp.*: 8.29

(90%, 100%) (Figure 2). The  $\Delta Ct_{Pathogen}$  cut-off for *B. fragilis*, *A. baumannii*, *M. catarrhalis*, and *E. coli* could not be determined, as they were not detected by culture. The results indicated that HIRA-TAN picked up pathogens that are likely to be the causative pathogen among the culture positives.



**Figure 1. HIRA-TAN test results**

The  $\Delta Ct_{Pathogen}$  of bacterial detection by the HIRA-TAN method. The red line indicates the cut-offs. (a) *P. aeruginosa*, (b) *K. pneumoniae*, (c) *Proteus sp.*, (d) *B. fragilis*, (e) *A. baumannii*, (f) *M. catarrhalis*, and (g) *E. coli*. Bacteria a–c were isolated from bacterial cultures, and the cut-off values were calculated using ROC analysis, while d–g were not isolated, and the ROC cut-off values were not calculated. Ct above 30 was eliminated.

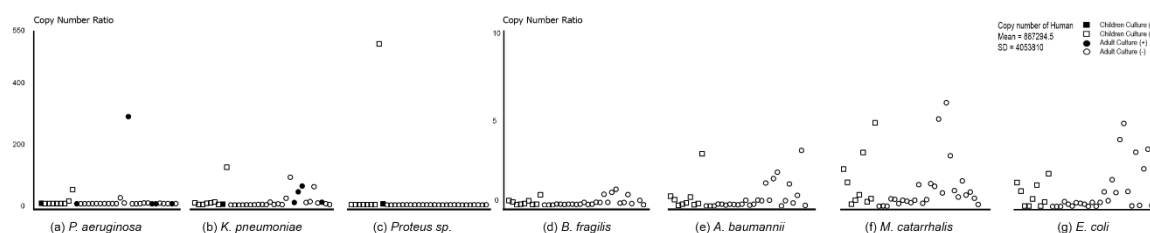


### Figure 2. The $\Delta C_t$ pathogen cut-offs generated from ROC curve analysis.

Plot of sensitivity and specificity against  $\Delta C_t$  pathogen values for identifying the threshold value or cut-off value. The  $\Delta C_t$  pathogen cut-off values with sensitivity and specificity values of (a) *P. aeruginosa* were 3.33 (90%, 100%), (b) *K. pneumoniae* were 1.71 (85%, 100%), and (c) *Proteus sp.* were 8.29 (90%, 100%).  $C_t$  above 30 was eliminated.

### HIRA-TAN using copy number

Real-time PCR was able to determine the copy number of the targets in the samples semi-quantitatively. Therefore, HIRA-TAN may be performed to determine copy numbers both human and pathogen genome. Using the human genomic DNA and the genomic DNA from 10 pathogens, we plotted a standard curve and performed HIRA-TAN analysis (Figure 3). The results were not conclusive—culture-positive samples were not well discriminated from the culture-negative samples.



### Figure 3. Copy number ratio of pathogens to human

Copy number ratio of each pathogen to human genomic DNA, showing that some samples had 287.87 times *P. aeruginosa*, 123.79 times *K. pneumoniae*, and 530.03 times *Proteus sp* genomic DNA. The copy number ratios of *B. fragilis*, *A. baumannii*, *M. catarrhalis*, and *E. coli* were below 10.

## Discussion

Pathogens that were identified by bacterial culture showed a higher  $\Delta C_{tPathogen}$  value, suggesting that HIRA-TAN may be used for causative pathogen identification. The patient age in the study population showed a broad distribution, consistent with the reports that CSOM is a disease involving all age groups [8, 9]. The rate of hearing impairment was higher

in adults than in children. Repeated episodes of infection and inadequate treatment during childhood have been associated with acquired hearing loss [11, 12] Early and complete eradication of infection is critical for managing CSOM.

Our results reported that the most frequently isolated bacteria from CSOM samples are *P. aeruginosa*, *Proteus* spp., and *Klebsiella* spp. are in line from earlier studies [13, 14].

The difference with other reports may be due to the difference in the study population or geography [15]. Our results are consistent with a previous study that reported only a limited number of bacterial species cause CSOM. Accordingly, many hospital laboratories screen only a small number of species during routine tests[16]. Considering the number of the candidate species and consistency of our results with the previous reports, HIRA-TAN may be an attractive approach for identifying the causative pathogen of CSOM.

Figure 3 showed the copy gene number ratio of each pathogen to human genomic DNA which reported that some samples had 287.87 times *P. aeruginosa*, 123.79 times *K. pneumonia*, and 530.03 times *Proteus sp* genomic DNA. In addition, the copy gene number ratios of *B. fragilis*, *A. baumannii*, *M. catarrhalis*, and *E. coli* were below 10. For example, the copy gene number of *P. aeruginosa* was 343558 and human was 1193 in sample 19.

Then, the copy gene number of the pathogen to human was 287.9 and the pathogen cells outnumbered the human cells. Since the CSOM is a chronic state, then the copy gene number ratio can inform the clinicians of the current situation on the disease progression.

*B. fragilis*, *A. baumannii*, *M. catarrhalis*, and *E. coli* showed high  $\Delta Ct_{Pathogen}$  values.

Nevertheless, they were not detected by bacterial culture, and the ROC curve analysis could not be performed. The results of PCR indicate that numerous bacteria are present in the specimen. It may be possible that many bacteria were nonviable. It is known that DNA from nonviable bacteria does not persist for more than one day in middle ear effusions [17].

Furthermore, the culture-sterile middle ear effusion has been suggested to be viable and

metabolically active [18]. Therefore, a positive HIRA-TAN result likely indicates viable but not culturable bacteria in the sample, which may be included as therapeutic targets. For improving pathogen identification in CSOM, HIRA-TAN method may be alternative in addition to culture. Further investigation is required to be implemented and the current study is a reasonable beginning.

### **Limitations**

- We recognized that the sample was small thus statistical analysis may be inadequate requiring larger cohort to verify the sensitivity and specificity.
- The culture-positive samples were not well discriminated from the culture-negative samples requiring further investigation of the non-viability issue.
- The pathogens above the  $[\Delta Ct]_{\text{Pathogen}}$  cut-off and those that were culture-negative might also be causative pathogens and requires further verification.

### **Conclusion**

HIRA-TAN test results were consistent with the bacterial culture results and suggested a list of pathogens that may be considered as therapeutic targets. A high  $\Delta Ct_{\text{Pathogen}}$  in culture-negative samples may indicate viable but non-culturable bacteria. These bacteria are ignored when determining the therapeutic regimen, but their pathogenic role requires reconsideration. Our results warrant larger cohort study that investigates the utility of HIRA-TAN for CSOM.

### **Abbreviations**

CSOM : chronic suppurative otitis media; HIRA-TAN: human cell-controlled identification of the respiratory agent from “TAN” (sputum in Japanese); CAP : community-acquired



pneumonia ; MRSA : methicillin-resistant *S. aureus*; NCBI : national centre for  
biotechnology information Ct : cycle threshold ; ROC : receiver operating characteristic

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