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学 位 論 文 名	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
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論文内容の要旨

1 研究目的

Chronic suppurative otitis media is a persistent infection of the middle ear associated with a perforated tympanic membrane resulting in ear discharge for more than six weeks. Identification of the causative pathogens is essential for treatment strategies. Human cell-controlled identification of the respiratory agent (HIRA-TAN method) is a technique that utilizes the battlefield hypothesis 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. Chronic suppurative otitis media 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 chronic suppurative otitis media diagnosis. However, causative pathogen determination from suppurative specimens of the middle ear is challenging. The current study investigated the utility of HIRA-TAN defining the causative pathogen in chronic suppurative otitis media based on the cycle-threshold difference between the targeted pathogen and the single-copy human gene by quantitative PCR.

2 研究方法

Thirty-nine patients, who had persistent and recurrent mucopurulent otorrhea for more than two months with a perforated tympanic membrane, were recruited to the study and the otorrhea was swab-collected. The samples were processed for bacterial culture and HIRA-TAN which the cycle-threshold cut-off was determined using receiver operating characteristic curves. 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. 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 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. baumannii*, *H. influenza*, *M. catarrhalis*, and *B. fragilis*), a methicillin resistance gene (*mecA*), and a human tumor necrosis factor gene (*TNF*).

3 研究成果

A total of 35.9% of samples were positive for bacterial culture; *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis*, and *M. morganii*. While *B. fragilis*, *A. baumannii*, *M. catarrhalis*, and *E. coli* were not identified by culture though high cycle-threshold values were obtained suggesting the inability of the culture system in detecting some pathogens. Our results indicate that HIRA-TAN is a potential diagnostic tool in suppurative otitis media and warrant more extensive studies. 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. Samples were also subjected to bacterial culture, and positive results were obtained in 14 patients (36%). Cultured bacteria included *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis*, and *M. morganii*. Samples positive for culture showed a high $\Delta Ct_{Pathogen}$. However, some samples negative for culture 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. 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* spp.: 8.29 (90%, 100%). 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.

4 考察

Pathogens that were identified by bacterial culture showed a higher $\Delta Ct_{Pathogen}$ 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 chronic suppurative otitis media is a disease involving all age groups. 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. Early and complete eradication of infection is critical for managing chronic suppurative otitis media. Our results reported that the most frequently isolated bacteria from chronic suppurative otitis media

samples are *P. aeruginosa*, *Proteus* spp., and *Klebsiella* spp. are in line from earlier studies. The difference with other reports may be due to the difference in the study population or geography. Our results are consistent with a previous study that reported only a limited number of bacterial species cause Chronic suppurative otitis media. Accordingly, many hospital laboratories screen only a small number of species during routine tests. Considering the number of candidate species and consistency of our results with the previous reports, HIRA-TAN may be an attractive approach for identifying the causative pathogen of chronic suppurative otitis media. *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. Furthermore, the culture-sterile middle ear effusion has been suggested to be viable and metabolically active. 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 chronic suppurative otitis media, the 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.

5 結論

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 $\Delta Ct_{pathogen}$ 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 a larger cohort study that investigates the utility of HIRA-TAN for Chronic suppurative otitis media.

論文審査の結果の要旨

慢性中耳炎は中耳に長期にわたって慢性炎症が存在し、耳漏の消長を繰り返す疾患である。基本的に細菌感染に起因することが多いが、主に経耳管あるいは経鼓膜の感染から、鼓室内あるいは乳突洞、乳突蜂巣に頑固な感染を繰り返し、保存的治療を行う場合には長期にわたって抗菌薬の投与を行うために菌交代現象が起きたり、薬剤に対する耐性が生じたりする問題がある。更に、慢性中耳炎が持続すると、耳小骨連鎖の障害、内耳への炎症波及により聴覚障害を起こす。

慢性中耳炎の抗菌治療は、起因菌を同定した上での、エビデンスに基づいた抗菌薬の適正使用が求められている。中耳、外耳道には常在菌があるため感染部位の検体から起因菌と常在菌の区別が難しく、経験的抗菌治療を行っている検体（耳漏）では細菌数が減少し、また検体の採取不備などの理由で、培養による起因菌の同定は難しい。そのため、申請者 ALIA 氏は、指導教員らが開発した起因菌と常在菌を区別する迅速診断法(HIRA-TAN 法)の応用して、上記問題が解決される可

能性を検討した。HIRA-TAN 法は、ヒト細胞と病原体との細胞数比を Real-time PCR 結果で表現し、起因菌と定着菌を鑑別することができるとされている。

本研究で ALIA 氏は、慢性中耳炎患者から採取した検体から起因菌と常在菌を判別することを目的とし、慢性中耳炎患者の検体（耳漏）を採取して、HIRA-TAN 法による起因菌の同定を行なった。そして、培養法による細菌同定の結果を比較研究を行い、臨床現場で迅速に起因菌を同定する病原菌診断法として応用できる可能性を提唱した。一方で、使用した検体数が少ないため、更なる検証が必要であると結論に至った。ALIA 氏は、学位論文で実験結果を客観的に分析したことも評価すべき点である。論文は、現在投稿中である。ALIA 氏は、学位論文に関する審査委員の指摘に対して丁寧に対応しており、最終的にかなり改善された論文を提出した。

以上の理由で、全審査委員が一致して、学位論文として相応しいものと判断した。

最終試験の結果の要旨

慢性中耳炎は鼓膜穿孔部からの排膿を繰り返しやすい病態であり、治療は局所の清掃と適切な抗菌薬投与である。しかしながら通常の細菌培養検査や、一部で施行可能な PCR による細菌検出では必要かつ十分な起炎菌同定が困難である。申請者はインドネシアの非都市部の患者由来の耳漏検体を HIRA-TAN method を用いて、起炎菌が同定できる可能性を示したものであり、日常臨床において耳漏中起炎菌の同定が可能となればその意義は大きい。一方、検体を収集した個々の患者の治療歴が不明であり、前治療が検出細菌に及ぼした影響がある。また、検出細菌の偏りがあるものの、今回の HIRA-TAN 法の研究結果に及ぼすバイアスは少ない。特に改定は必要ないものと判断する。

最終試験では、申請者に対して、本研究の着想に至った背景と仮説、その仮説に答えるために研究に用いた材料・方法の妥当性と限界、得られた結果と解釈、及び考察について発表、口頭試問を実施した。それらに関する質問に対して適切に答え、研究全般及び関連情報について十分に理解していることが確認された。

本学位論文は、目的と結果が明瞭であることから、学位研究としてふさわしく、合格と判断する。

最後に、提出論文のオリジナリティ検査結果について、最初の審議で二重投稿の疑義が生じたが、プレプリントとしてインターネット上に掲載されていたものであることが判明され、問題はない。

以上から、諮問の結果は合格と判定した。