BACTERIOLOGICAL PROFILES OF SKIN FLORA ON EARLY CADAVER

By

CHONG CHI KWAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Master of Science

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Post-mortem microbiology (PMM) has gained significant importance in the forensic department nowadays to determine the actual cause of death of adults and infants, especially for deaths caused by infection. PMM is of utmost importance when the cadaver does not show any apparent signs of infection. An infective that leads to death is usually suspected in sudden death. Therefore, the determination of infective cause can help in further management of pathogenic transmission and is thus able to prevent the occurrence of outbreaks. However, the current challenge in PMM is the difficulty in interpreting culture results, for example, differentiating the true pathogen from the contaminants, especially when there is a mixed growth in the samples. Mixed growth or contamination from skin flora may induce false positive results, leading to uncertainty and failure in identifying true pathogen. Therefore, information on post-mortem skin flora can be helpful in providing references for interpreting microbiological investigations in post-mortem cases. Early cadavers (n=39), which have an estimated post-mortem (ePMI) of less than 24 hours, under sudden unexpected death cases, were included in this study. Two body sites, namely the neck and femoral, of each cadaver were chosen as the sites to obtain skin samples. Blood samples from these two sites were routinely obtained for post-mortem microbiological culture. Cotton swabs were moistened with normal saline and were then rubbed vigorously and rotated to ensure that homogeneity in the skin areas were achieved by each swab. The swabs were then kept in 200µl of phosphate-buffered saline (PBS) for storage and transportation. DNA was extracted from each skin swab and high-throughput 16S rRNA sequencing was performed on the extracted DNA using the Illumina Miseq system to assess bacterial diversity and abundance. The sequence outputs were analysed by using the LotuS pipeline while the α-diversity and β-diversity were analysed by using the Quantitative Insights into Microbial Ecology (QIIME) software. The samples were mostly predominated by phylum *Proteobacteria* (61.20%), followed by *Firmicutes* (28.10%), *Actinobacteria* (10.00%) and *Bacteroidetes* (0.60%). In addition, the top 10 dominant genera from the samples were *Ochrobactrum* (24.70%), *Staphylococcus* (20.60%), other members of *Enterobacteriaceae* (17.70%), *Corynebacterium* (6.00%), *Enhydrobacter* (5.30%), *Acinetobacter* (4.30%), *Klebsiella* (3.40%), *Pseudomonas* (2.70%),

Stenotrophomonas (2.10%) and *Phycicoccus* (2.00%) accordingly. Moreover, there were no statistical difference between the bacterial communities of the neck and femoral sites, except for genus *Corynebacterium* which showed significant difference in abundance between both body sites. Nevertheless, the results showed significant difference between the abundance of some bacterial genera found on the cadavers with ePMI less than 5 hours (ePMI<5H) and the cadavers with ePMI more than 5 hours (ePMI>5H). The findings of this study provides data on the known skin flora that presents on early cadavers, thus may help in determining the actual cause of death due to pathogenic infections.

Key words: Post-mortem microbiology; contamination; skin flora; post-mortem interval

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PROFIL BAKTERIOLOGI FLORA KULIT KE ATAS KADAVER AWAL

Oleh

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Ujian mikrobiologi bagi spesimen post-mortem atau dikenali sebagai post-mortem microbiology (PMM) telah mendapat perhatian oleh jabatan forensik bagi menentukan sebab sebenar kematian dalam kalangan orang dewasa dan bayi, terutama bagi kematian yang disebabkan oleh jangkitan. PMM adalah paling penting ketika kadaver tidak menunjukkan sebarang tanda jangkitan yang ketara. Jangkitan yang membawa kepada kematian biasanya disyaki dalam kes kematia tiba-tiba. Oleh itu, penentuan penyebab jangkitan membantu dalam pengurusan dan kawalan jangkitan dan berupaya mengelak berlakunya wabak. Walau bagaimanapun, cabaran PMM adalah kesukaran dalam mengintrepretasi keputusan kultur dan membezakan patogen sebenar daripada kontaminan, terutama ketika terdapatnya pertumbuhan campuran dalam sampel. Pertumbuhan campuran atau kontaminasi dari flora kulit boleh menyebabkan keputusan positif palsu, seterusnya membawa kepada ketidakpastian dan kegagalan dalam pengenalpastian patogen sebenar. Oleh yang demikian, maklumat mengenai flora kulit dari ujian post-mortem dapat membantu dalam memberikan rujukan bagi menginterpretasi investigasi mikrobiologi dalam kes post-mortem. Kajian ini melibatkan kadaver awal (n=39) daripada kes kematian tiba-tiba, dengan anggaran selang post-mortem (ePMI) kurang daripada 24 jam. Dua bahagian tubuh, iaitu leher dan femoral, daripada setiap kadaver telah dipilih sebagai tempat bagi mendapatkan sampel kulit. Manakala, sampel darah daripada kedua-dua tempat tersebut secara rutin telah diperoleh bagi kultur mikrobiologi post-mortem. Swab kapas telah dibasahkan dengan salinus normal dan kemudian digosokkan dengan kuat dan diputar bagi memastikan bahawa homogeneiti pada kawasan kulit tercapai bagi setiap swab. Swab tersebut kemudiannya di simpan dalam 200µl salin fosfat tertimbal (PBS) bagi penyimpanan dan pemindahan. DNA telah diekstrak daripada setiap swab kulit dan penjujukan 16S rRNA celusan tinggi telah dijalankan ke atas DNA yang diekstrak menggunakan sistem Illumina Miseq bagi menilai kepelbagaian bakteria dan kelimpahan. Urutan output telah dianalisis menggunakan talian paip LotuS manakala α-diversiti dan β-diversiti telah dianalisis menggunakan Insight Kuantitatif ke dalam perisian Ekologi Mikrobial (QIIME). Sampel kebanyakannya didominasi oleh filum Proteobacteria (61.20%), diikuti oleh Firmicutes (28.10%), Actinobacteria (10.00%) dan Bacteroidetes (0.60%). Di samping itu, 10 genus

dominan daripada sampel ialah Ochrobactrum (24.70%), Staphylococcus (20.60%), penghuni lain Enterobacteriaceae (17.70%), Corynebacterium (6.00%), Enhydrobacter (5.30%), Acinetobacter (4.30%), Klebsiella (3.40%), Pseudomonas (2.70%), Stenotrophomonas (2.10%) dan Phycicoccus (2.00%) mengikut urutan. Di samping itu, tidak terdapat perbezaan secara statistik antara komuniti bakteria bagi bahagian leher dan femoral, kecuali bagi genus Corynebacterium yang menunjukkan perbezaan yang signifikan dari segi kelimpahan antara kedua-dua lokasi tubuh. Walau bagaimanapun, hasil kajian menunjukkan perbezaan yang signifikan antara kelimpahan beberapa genus bakteria yang didapati pada kadaver dengan ePMI kurang daripada 5 jam (ePMI<5H) dan kadaver dengan ePMI lebih daripada 5 jam (ePMI>5H). Hasil kajian ini memberikan maklumat mengenai flora kulit yang wujud pada kadaver awal. Oleh itu, maklumat ini dapat membantu dalam menentukan sebab sebenar kematian yang diakibatkan oleh jangkitan patogen.

Kata Kunci: Mikrobiologi post-mortem; kontaminasi; flora kulit; selang post-mortem

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PMM	Post-mortem Microbiology
PMI	Post-mortem Interval
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
ATCC	American Type Culture Collection
QIIME	Quantitative Insights Into Microbial Ecology
SPSS	Statistical package for the social science
PCoA	Principal coordinate analysis
UPM	Universiti Putra Malaysia
HKL	Hospital Kuala Lumpur
PBS	Phosphate-buffered saline
TBE	Tris Borate EDTA
RTA	Real-time Analysis
SAV	Sequencing Analysis Viewer
OTU	Operational taxonomic units
RDP	Ribosomal Database Project
TTD	Time to detection

CHAPTER 1

INTRODUCTION

1.1 Background

Post-mortem microbiology (PMM) is becoming a trend in the field of forensic pathology. It is useful for investigating the possible cause of death, especially in cases of sudden death in adults and infants. PMM is important for determining the pathogenic agents that could contribute to deaths caused by infection. Identification of unknown pathogenic agents or biological warfare agents that can harm human life is one of the roles of PMM in preventing outbreaks. The 2001 anthrax letter attacks incident in the United States has highlighted the importance of PMM in preventing the occurrence of bioterrorism.

PMM examination is useful for evaluating ante-mortem clinical laboratory reports, as it can compare ante-mortem laboratory findings and post-mortem culture results. The results from a post-mortem culture can be used to confirm an ante-mortem diagnosis. The results can also be used to evaluate the efficacy of ante-mortem antimicrobial therapy (Riedel, 2014). Moreover, drug resistance can also be evaluated through PMM examination (Fernández-Rodríguez et al., 2015). Furthermore, the bacterial community changes as the time since death, the post-mortem interval (PMI), increases, as reported by Pechal et al. (2014). The bacterial community showed significant differences in the taxon richness and relative abundance at both phylum and family levels as the decomposition process proceeded (Pechal et al., 2014). Hence, PMM can be used to estimate the PMI. Determination of the PMI is crucial in forensic science, especially in criminal cases; it can be used as a clue to track down a criminal.

The results of PMM can be categorised into (a) genuine positive, (b) agonal spread, (c) bacterial translocation and (d) contamination (Riedel, 2014). Genuine positive can be referred to as true infection, which means the pathogenic agent that causes the death has been successfully isolated, and the microorganism isolated is usually a single isolate. The result is significant for confirming an infection. On the other hand, false positive results can include situations such as agonal spread, bacterial translocation and contamination. False positive results of post-mortem microbiological cultures may have mixed growth results, which means there are more than two microorganisms isolated (Spagnolo et al., 2019). Both agonal spread and bacterial translocation can represent the result of bacterial invasion of the bloodstream. Agonal spread occurs during the agonal period when systemic circulation is weakening, whereas bacterial translocation occurs when the circulation has ceased and the bacteria invade the bloodstream from the mucosal surface. Although the concept of agonal spread has been proposed, the definition of the concept is not literally verified. The occurrence of agonal spread is rare, and there are only a few historic studies explaining it (Riedel, 2014). Compared to agonal spread, bacterial translocation has been proven by researchers for decades. Bacterial translocation has been proven by substantial evidence, and usually occurs when the body is not being stored at a low temperature (Riedel, 2014). The microorganisms involved in bacterial

translocation are usually gastrointestinal tract commensals. Bacterial translocation can be avoided by storing the body at a low temperature (4°C), at which the PMM culture results will not be affected (Palmiere et al., 2016; Spagnolo et al., 2019).

Contrary to the concepts of agonal spread and bacterial translocation, contamination is the main problem in the field of forensic science in identifying true pathogenic agents. Standards issued by the American Society of Microbiology state that the rate of blood culture contamination should not exceed 3%. However, in practice, most hospital contamination rates are greater than that, with some of them reaching 7% of the contamination rate (Miller et al., 2018; Nannan Panday et al., 2019). Contamination generally happens during sampling. Bacteria are introduced into samples while the samples are being collected and therefore cause false positive results. Improper sampling techniques, the use of unsuitable transport media, anatomical sites for sampling and transient or resident bacterial colonisation of skin prior to death can be the reasons for contamination. Differentiation between true infection and contamination remains a major challenge in PMM examinations. Blood from heart, spleen cultures or peripheral venous sites is commonly obtained for PMM cultures. The positive rate has been reported as between 7% and 69% (Riedel, 2014). The contaminants can be normal skin flora, since bacteria may be introduced into samples while obtaining the blood cultures. For example, coagulase-negative staphylococci are commonly isolated and treated as contaminants because they are generally normal skin flora. On that premise, the patient does not associate with catheters or vascular prostheses.

1.2 Problem Statement

There are approximately 600 post-mortem cases per year in Hospital Kuala Lumpur, and PMM tests are indicated to rule out infections caused by a bacterial pathogen. However, occasionally culture results with isolated mixed growth of bacteria pose challenges in interpreting the PMM examination results. When there is mixed growth in the culture, true infection or contamination is unable to be determined when the source of bacteria is undetermined. Contamination can be due to inappropriate handling of specimens while collecting them. Furthermore, the true pathogen may be neglected, and this has made it more difficult to determine the possible cause of death.

Sampling site and the relevant skin flora are recommended to take into account for the interpretation of PMM examination results (Nordin, 2018). In order to deal with the possible contamination problem, it seems critical to know the skin microbiome of early cadavers. The skin microbiome can not only exclude contaminants, but can also help to determine the PMI. There are a few studies about the skin microbiome from cadavers; however, those studies mostly included cadavers with PMIs of more than 24 hours (Johnson et al., 2016; Metcalf et al., 2013). It is rare to have studies focus on cadavers with a PMI of less than 24 hours.

Therefore, this study aims to determine the commonly found skin flora on early cadavers. The outcome of this study can prevent misinterpretation in certain deaths of skin flora as

pathogens in post-mortem blood culture that may result from the contamination of the needle. It is necessary to know the normal skin flora of the early cadavers in order to help with the contamination problem.

1.3 Research Questions

a. What type of skin flora will be present on early cadavers within 24 hours of time since death?
b. What is the relationship between types of skin flora and anatomical sites as well as post-mortem interval?

1.4 Research Objectives

1.4.1 General Objective

To determine the bacterial profiles of skin flora on early cadavers

1.4.2 Specific Objectives

i. To study the bacterial profiles of skin flora collected from neck and femoral sites on early cadavers.
ii. To compare the differences of bacterial profiles collected from neck and femoral sites
iii. To study the dynamic pattern of the flora profiles in relation to duration of earliest documented time of death to sampling time.

1.5 Hypothesis

There are different microorganisms appearing at different anatomical sites at different times of death.

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