GSC Biological and Pharmaceutical Sciences, 2019, 06(03), 061–068

Available online at GSC Online Press Directory

GSC Biological and Pharmaceutical Sciences

e-ISSN: 2581-3250, CODEN (USA): GBPSC2

Journal homepage: https://www.gsconlinepress.com/journals/gscbps

(RESEARCH ARTICLE)

Rectal Firmicutes, Actinobacteria and Gammaproteobacteria phyla fitness belong to post mortem intervals in the terrestrial environment death victims evaluation in Malang, East Java, Indonesia

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Publication history: Received on 04 January 2019; revised on 22 January 2019; accepted on 25 January 2019

Article DOI: https://doi.org/10.30574/gscbps.2019.6.3.0001

Abstract

The increasing of urbanization will lead the increasing violence-related death, like traffic accident, murdered and suicide which contribute to 20 leading causes of death. Post mortem interval (PMI) estimation is important information for suspicious death. Microbes have important role for decomposition of bodies. By evaluate the certain bacteria will know the post mortem interval because the community of bacteria have certain condition regarding to the composition. The aim of this study is to figure out the community of Firmicutes, Actinobacteria and Gammaproteobacteria from rectal swab post mortem interval of the terrestrial environment in Malang, East Java, Indonesia. We used Wistar carcass as a model for human decomposition. Identification of bacterial phyla using Real Time PCR (qPCR) method after cultured in nutrient broth solution. The results showed that Firmicutes has dominant composition in fresh stage (0-24 hours) and in advance decomposition at 120 hours after death. Actinobacteria was dominant in the early decomposition, observed from the 48 to 72 hours after death. While Gammaproteobacteria was dominant in the end of fresh stage and early decomposition stage when bloating time occur.

Keywords: Post Mortem Interval (PMI); Actinobacteria; Firmicutes; Gammaproteobacteria

1. Introduction

The trend of urbanization, especially in Asia and Africa, has led to the violence development problems that are concentrated in the urban centers of developing countries [1]. Cases of violence-related deaths, like traffic accidents, murder and suicide, are expected can contribute to increased compared to other causes of death and are among the 20 leading causes of death in the world by 2030 [2].

Significantly post-mortem interval (PMI) estimation very useful to investigate suspicious deaths by helping to indicate when a death occurred, so as to limit the number of suspects and deny the suspect's false alibi when the crime that caused the death took place [3]. Traditionally, PMI estimation depend on physical changes that occur after death, such as algor mortis, livor mortis, rigor mortis and supravital activity [4, 5]. However, this method cannot be used to the advanced post-mortem period, because it only provides a general estimate of PMI. In recent years, additional techniques have been developed for estimating PMI such as thanatochemistry [6], DNA / RNA degradation [7, 8] and forensic entomology [9, 10].

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Finley *et al*., [11] revealed that insects have always been used as important indicators of PMI in terrestrial environments. However, insect invasion of the new carcass occurs after a few hours to a few days after death, causing an incorrect PMI estimate. Whereas microbial community structures succession is a continuous process and not a separate processes [11]. Microbial forensics succeed the world highlight after named bioterrorism and can be helpful in investigation for efficient reason. Deoxyribose Nucleic Acid (DNA) bacteria play a role in forensic science, because bacterial DNA is more resistant to environmental factors than human DNA, and also lasts longer on the surface than human DNA.

Microbes play an important role in the complex decomposition of cadavers. The decomposition process on the ground shows a change in the role of the bacterial community [12]. The existence of changes and number of certain bacterial phyla that are consistent during the decomposition stage shows that the bacterial community can be used to estimate the time of death. However, various studies that have been carried out on microbes that play a role in the decomposition stage, in general, unknown definitely anaerobic bacteria species in the gastrointestinal tract that are involved in the process.

Polymerase Chain Reaction (PCR) is a molecular technique that is simple to understand, use, fast process [13], very sensitive by producing large amounts of DNA fragments or certain genes and can be done using DNA sources from various tissues and organisms, including peripheral blood, skin, hair, saliva, and microbes a limited number of [14]. PCR use in microbial study that play a role in decomposition has several advantages because culture techniques have limitations in their implementation. Estimated almost 99% of bacterial species found in nature cannot be cultured in the conventional way [15]. Most microbes that are inside and outside the human body are difficult to be cultured, increasingly culture-based research is not able to accurately assess bacterial species.

Dominance patterns of Firmicutes, Actinobacteria and Gammaproteobacteria gastrointestinal tract bacteria study on the fresh stage, initial decomposition and advance decomposition at the rectum using PCR molecular technique, potential to estimate the post mortem interval of death victims in terrestrial environments. This study expected to be useful as a scientific review and provide additional information to support the use of microbes as potential agents to estimate post mortem interval death victims in terrestrial environments and provide evidence of case investigations crime and forensic purposes, also increased the potential use of swab-based sampling techniques by police officers in future forensic investigations.

2. Material and methods

2.1. Sample collection

Wistar carcass (*Rattus norvegicus* strain wistar) adult male (n = 3) weighing 200-220 g, aged 30-100 days as models of human decomposition. Its individually placed in a plastic box (0.5 × 0.3 × 0.25 m) coated with a thick layer of sterile sawdust and wrapped in gauze to allow normal air circulation but not entered by the insect. The boxes were placed at least 25 cm apart on the ground in a herbaceous plants area at the same time and left to rot. All specimens were observed during the process. The study was approved by the Medical Ethics Committee of Medical Faculty Brawijaya University (approval code: 21/EC/KEPK-S2/02/2018), and the methods were carried out in accordance with the approved guidelines.

Rectum gently rubbed for 60 s using sterile swab cotton that has been moistened with sterile phosphate buffers. The tip of the swab cotton used for sampling was cut and placed in a 1.5 mL microcentrifuge tube containing 1 mL of sterile phosphate buffer. Sampling was carried out at 2 minutes, 24 hours, 48 hours, 72 hours and 120 hours post-mortem. The swab samples were inoculated in nutrient broth and incubated for 24 hours at 37^{o}C for enrichment of microbial culture, which was used to isolate and identify DNA by the qPCR method.

2.2. Deoxyribose nucleic acid (DNA) extraction

The genomic DNA was extracted using Jena Bioscience Bacteria DNA Preparation Kit (Jena, German), as described in procedure handbook. DNA purity was measured by absorbance at 260 nm using BioDrop Spectrophotometer (Thermo Scientific). DNA was stored at −20 °C until further analysis.

2.3. Amplification with real-time PCR

RealMOD™ Green W^2 qPCR mix prepared to the number of running samples. The master mix were mixed in one 2 ml microcentrifuge tube and homogenized with vortex, taken and inserted into each well then DNA, primary and DNase / RNase free water templates are added to each well . Furthermore, it is homogenized on MixMate PCR 96, inserted in real-time PCR machine, then run. The qPCR amplification reaction contained an initial activation step of 10 min

at 95 °C, followed by 40 cycles of 20 s at 95 °C (denaturation), 40 s at 52 °C (annealing), 1 min at 72 °C (elongation), and a final extension for 5 min at 72 °C. Primers used to identify bacterial phyla [16], as follows:

2.3.1. Firmicutes

Forward: 5' TGA AAC TYA AAG GAA TTG ACG- 3'

Reverse: 5' ACC ATG CAC CAC CTG TC- 3'

2.3.2. Actinobacteria

Forward: 5'- TAC GGC CGC AAG GCT A- 3'

Reverse: 5'- TCR TCC CCA CCT TCC TCC G -3'

2.3.3. Gammaproteobacteria

Forward: 5'- TCG TCA GCT CGT GTY GTG A- 3'

Reverse: 5'- CGT AAG GGC CAT GAT G-3'

3. Results and discussion

This study took place outdoors at the Medical Faculty of Brawijaya University, Malang, East Java, Indonesia in January 2018. Rat carcasses were used in this experiment in lieu of human remains due to their similarity to human tissue, as well as due to practical constraints. Three rats (*Rattus norvegicus* strain wistar*)* were humanely euthanized. Carcasses were placed on the boxes in a herbaceous plants area. Air temperatures were recorded daily. The study was completed when rectum specimens was not enable to sampling. In this study, 60 samples were identified consisting of 30 gram positive isolates and 30 gram negative isolates. Temperature during February 2018 around location ranges from 24-28˚C with a high relative humidity level, but was warmer than the average in the previous month because it is at the end of the rainy season (Table 1). Rat decomposition occurs long enough, decomposition process usually ends on the third day, but until the fifth day rectum still enable to sampling.

Table 1 Temperature and precipitation for sampling

Date	Temperature in °C	Precipitation in %
Feb 19th	27	56
Feb 20th	28	56
Feb 21st	27	56
Feb 22nd	23	52
Feb 23rd	23	56

The fresh stage (day 0) was the decomposition stage without discoloration except livor mortis or lividitiy, which is a discoloration due to pooling of the blood in the lower area of the body by gravity [17]. Odor and swelling are also not found after death immediately. At this stage there was a sharp temperature decrease in the carcass after 24 hours PMI. The fresh stage ends after 24 hours after death. Early of 48 hours, the initial features of decomposition were present, bloating in body especially abdomen due to the accumulation of gas produced by bacteria in the intestine. Distention is also seen in the carcass bladder. In addition, the tongue began to soften. The carcass began to smell pungent and some green flies were spotted in the air and around but not observed to landed on the carcass. The body temperature of the carcass was lower than before but not founded larval activity in the body. A marbling and blue-green discoloration was also present on the abdomen**.** Discoloration and skin marbling after death are mainly related to the production of sulphmethhaemoglobin [18]. The early green discoloration, then to gray, while other looks relatively fresh. Discoloration was concentrated around the head, initially visible on the chin, chest and abdomen of specimen. Furthermore, blue-green discoloration was present on the head and the surface abdomen. Ants were observed swarming the surface**.** Bloating, in the abdomen and bladder, occurs until 72 hours after death with a maximum size (Figure 1). The larvae were first spotted especially in the protected area, such as the pulled abdomen with body weigh

and box. Ants are observed swarming around the carcass, especially the abdomen and bladder while flies are not found landing on the carcass because the specimen box is wrapped in gauze.

Figure 1 The carcass after 72 hours death

After 120 hours of PMI, deflation post bloating was present. A small hole is detected in the abdomen, caused gas accumulates out. The skin has changed to darkened, and this coincided with the maggots hatching on these specimens. Discoloration only observed in the head, abdomen and parts of the bladder, while other specimens have no discoloration. The inside of the mouth is damaged and large larvae are found out in the mouth and rectum, exfoliation of the skin and loose hair are seen (Figure 2).

Figure 2 The carcass after 120 hours death

A large amount of liquid decomposition was present around the carcass. Flies larvae were spotted in the abdomen and around**.** A strong stench from the carcass is getting stinging. Carcass temperature increased and associated by extensive larval activity.

Quantification of bacterial phyla was carried out by real time PCR from rectum sampled. The results of running Real Time PCR analyze the number of phyla contained in the sample to determine the dominant bacterial phyla at various stages of decomposition that occurs every serial time. Descriptively, Firmicutes can be concluded was the highest average concentration on the rectum which is equal to 4.63E7 units.

Our study presented (Figure 3**)** Firmicutes as the dominant phyla at the early (0-24 th hour) and end of PMI (120th hour) in the rectal sample while Actinobacteria dominant at 48-72 hours PMI.

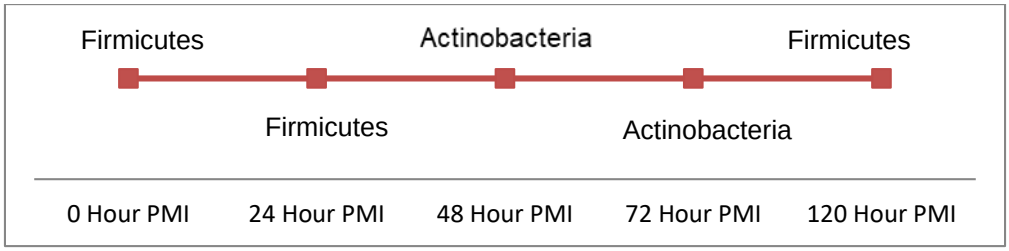

Figure 3 Bacterial phyla domination pattern in rectum

The concentration of the three phyla since the early observation not difference significantly. Firmicutes tend to be dominant in rectal samples from early to the end of PMI. Firmicutes were found to decline to 48 hours and increased again until the end of PMI. The highest Firmicutes concentration was found on the last day of PMI. Gammaproteobacteria and Actinobacteria were found in the rectum with concentrations and patterns that are not much different (Figure 4).

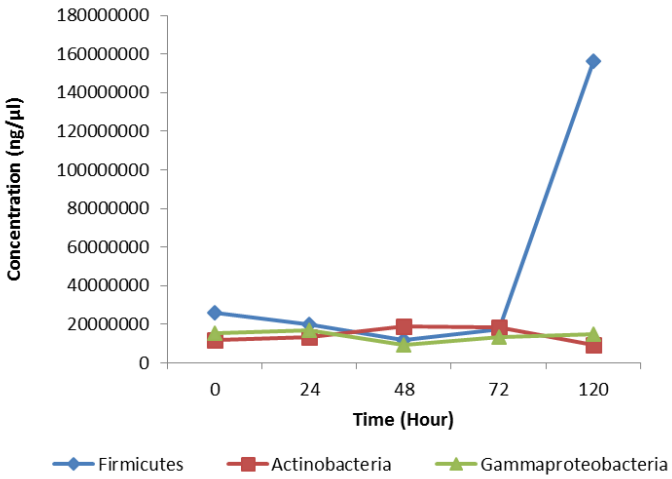

Figure 4 Average rectal phyla abundance

The dominant phyla in living rats is Bacteroidetes and Firmicutes [5]. These results were similar, Firmicutes dominated the rectal sample from rats sacrificed after 2 minutes (Figure 5). The results of this study present that the early to end of PMI is dominated by Firmicutes. This result is different as reported by Guo et al., [5] which show that dominance Firmicutes in the 1st post-mortem day rectum sample were replaced by Proteobacteria from the second day post-mortem onwards. These difference pattern is thought due to a different microbiome in the digestive tract [5]. The dominant phyla in living rats is Bacteroidetes and Firmicutes [5]. These results were similar, Firmicutes dominated the rectal sample from rats sacrificed after 2 minutes (Figure 5).

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The colon environment (large intestine) is generally anaerobic, and the most abundant strain in the human microbiota is obligate anaerobes. Cremer *et al*. [19] found that bacterial growth from the consumption of primary carbohydrates, which is one of the nutrients that comes from the small intestine, occurs in proximal colon. A large number of obligate anaerobes involved in fermentation in the large intestine, produce end products which are excreted mainly in the form of short chain fatty acids (SCFAs). The high density of bacteria involved in fermentation causes increased concentrations of fermented acids, causing a decrease in local pH and affecting bacterial growth [19]. The rapid decrease in pH is beneficial for Firmicutes growth, so the number of phylum bacteria tends to be large. Large bacterial growth is not possible in the distal colon (i.e., transverse and descending colon and rectum), because all major sources of nutrition have been used before. So the composition of the microbiota has not changed much. The composition of the microbiota in the rectum is a consequence of bacterial growth in the proximal colon. As a result, Firmicutes as an abundant bacterial phylum in the proximal colon also becomes an abundant bacterial phylum in the rectum.

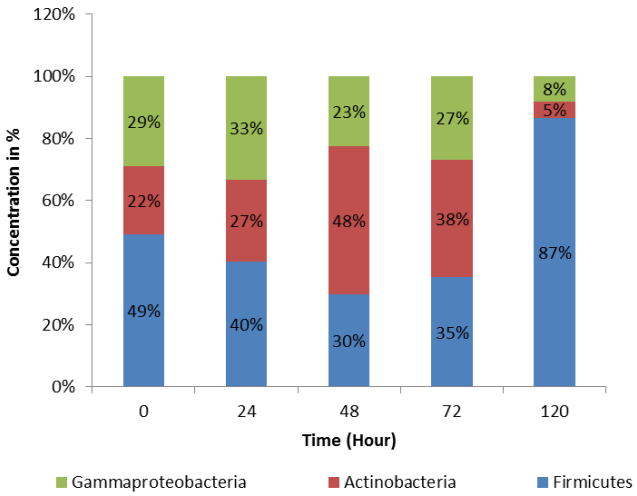

Figure 5 Rectal phyla domination at time point

There are some factors that can affect the diversity of intrinsic and extrinsic bacterial communities that are thought to potentially increase or retard decomposition in a person and corpse, such as normal flora, bodily factors, including trauma, disease, or weight and environmental factors, like scavenger activity and temperature. The dominant pattern of Actinobacteria shows that this phyla is dominant at early decomposition. Gammaproteobacteria form a widely distributed dominant pattern at the end of fresh stage and early decomposition stage, when bloating occur. This is probably due to the fact that members of this phylum are facultative anaerobic bacteria that can live in anaerobic or aerobic conditions. But the optimal growth is in anaerobic conditions, which is present by the high amount of this bacterium and increased in abundance on 24 hours.

Day 1 post mortem is early time process of carcass decomposition. The abundance of Gammaproteobacteria at the end of PMI was supported by previous studies which found that Gammaproteobacteria members, Proteus and Ignatzschineria, were dominant both in the buccal cavity and the rectum in the late stages of decomposition [5]. Our results supported the previous study that showed Gammaproteobacteria may be an important contributor to the decomposition process.

Known at healthy humans, the most abundant phyla in feces are Firmicutes and Bacteroidetes [5], thus, the dominant phylum of the rectal sample immediately after death, does not differ significantly from that found in living people. However, there is relatively no change in the dominance of bacterial phyla, because Firmicutes is dominant in rectal samples from the early to 72 hours PMI and only the numbers fluctuate but tend to increase. These results are supported by Tuomisto *et al*., [20] who observed that the number of bacteria increases while diversity decreases during the decomposition process. At the end of PMI, Firmicutes still appear to dominate (Figure 5), indicating there is migration of these bacteria during decomposition, because it is known that bacteria belonging to Firmicutes are normal intestinal flora.

The rectum area is the main site with bacteria that colonize the intestine meeting increasing aerobic conditions. This condition is certainly different from the anaerobic colon environment, bacteria recognize and modify themselves in different situations by monitoring the environment and also by creating signals and modifying appropriate gene expression for survival [21]. For example, type 1 bacterial fimbria antigens production is potentially more continuous under these conditions than in conditions where oxygen is almost completely eliminated [22]. Thus facultative anaerobic bacteria, including Firmicutes, can overcome different environmental challenges in the rectum rather than in anaerobic conditions of the inner intestine.

4. Conclusions

Our study is important for forensics investigation to show and estimate postmortem interval that could help researchers in identifying individuals. The results of this study provide an estimate that gastrointestinal tract bacteria such as Firmicutes, Actinobacteria and Gammaproteobacteria phyla were potential to estimate post mortem death intervals in terrestrial environment by forming a certain dominance pattern at each stage of decomposition at the rectal site.

Compliance with ethical standards

Acknowledgments

The author thanks to support staff from Central Laboratory of Biomedicine, University of Brawijaya and Genetic and Molecular Laboratory of Maulana Malik Ibrahim State Islamic University, Malang, East Java, Indonesia for helpful discussions.

Disclosure of conflict of interest

All authors declare no conflicts of interest.

Statement of ethical approval

The study was approved by the Medical Ethics Committee of Medical Faculty Brawijaya University (approval code: 21/EC/KEPK-S2/02/2018), and the methods were carried out in accordance with the approved guidelines.

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How to cite this article

Sholihah TP, Noorhamdani, Khotimah H and Prawestiningtyas E. (2019). Rectal Firmicutes, Actinobacteria and Gammaproteobacteria phyla fitness belong to post mortem intervals in the terrestrial environment death victims evaluation in Malang, East Java, Indonesia. GSC Biological and Pharmaceutical Sciences, 6(3), 61-68.