Preliminary Study on Bacterial Diversity Causing Human Foot Odor

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Abstract. This preliminary study was aimed at studying on microbial diversity causing odor of the human foot. The objective was to know the main bacteria species causing foot odor. The method used in this study was foot swab by using cotton buds for bacterial isolation, tests for H2S gas formation by using Kligler Iron Agar solid medium, odor test and optical density value. There were 204 bacterial isolates collected from 30 people ranged from 7-40 years old. They were identified based on polyphasic characterization. Based on 50 cultures of 25 isolates, it was indicated that sweat filtrate was better medium for bacterial growth than shocks filtrate. This was indicated that the odor problem was from sweat which was consisted of bacterial growth producing unpleasant odor. These were shown by the H2S producing-bacteria such as Bacillus cereus and Proteus mirabilis were isolated from the foot. Those bacteria were identified based on their partial sequences of 16sDNA. Other bacteria isolated were Acinetobacter pittii, Enterobacter hormaecheii subsp. xiangfangensis, Enterobacter morii, Glutamicibacter creatinolyticus, Klebsiella pneumonia, Leclercia adecarboxylata, and Staphylococcus warneri. Further study will be carried out to use bacteriophages for eliminating bacterial biofilm in vitro. Bacteriophage uses for prevention application for human foot odor by bacterial growth is much prospective than other application such as sanitizer or disinfectant.

1. Introduction
Bromodosis or body odor is a very common problem naturally in human. This unpleasant odor is naturally occurred when sweat is excreted causing body provide high levels of humidity and a rich supply of nutrients that favor for microbial growth. Foot odor also have a similar condition as foot are also good habitat for microbes to grow and make colonization [1].

Bacteria which are ubiquitous organisms can grow by using nutrition from sweat produced by foot. Hence, the bacteria produce various unpleasant odor. Methanethiol and dimethylsulphide, volatile one carbon compounds were produced by bacteria in the human mouth or foot habitat [1][2][3]. The existence of methylotrophic bacteria in that habitat have capability of utilizing volatile one carbon compounds as their energy source. The enzymes of methylotrophic metabolism play an important role in methylotrophic bacteria’s ability to use one carbon compounds as energy source) [4].

Several studies on culture and genomic have revealed that Staphylococcus was the principal organism existent on the plantar surface of the foot, included Corynebacterium made an important role. Other bacteria, Betaproteobacterium, Brevibacterium, Micrococcus, and Propionibacterium also were reported that they played an important role to the whole foot microflora in some subjects [5][6][7][8][9][10]. The spatial distribution of these bacterial populations across the foot has not been studied in any great detail. Eccrine sweat glands are the primary source of foot secretions and are present on both the plantar and dorsal surface, secreting a primarily aqueous solution containing inorganic electrolytes, free amino acids,
lactic acid and urea [11]. Unlike most areas of the body, the plantar surface of the feet has no sebaceous glands and also lack apocrine glands which are associated with odor production in the axilla[3].

The purpose of the study was a preliminary study on bacterial diversity and to know the foot odor causing-bacteria from Indonesian peoples. There is a few report on similar study conducted in Indonesia in particular. This preliminary study is important so as to do further study which will be focus on the foot odor causing-bacteria so as to eliminate the problem.

2. Materials and Methods

2.1. Isolation and collection of human foot odor causing-bacteria

A total of 30 volunteers had been chosen for bacterial isolation purposes from their toes. swabbed their foot using a cotton bud with two treatments. Before swabbing for microbial isolation purposes, each volunteer was treated or not treated by rinsing with a citrus lemon peel, continued rinsing by a soapand subsequently cleaning by running tap water for several minutes.

2.1.1. The media used for bacterial isolation. The media used for bacterial isolation were Kligler Iron Agar (KIA). KIA media is known as a solid medium recommended for use in qualitative procedures for differentiation of enteric gram-negative bacilli on the basis of dextrose and lactose fermentation and hydrogen sulfide (H2S) production. KIA media was composed of reagent such as casein peptone, 10.0 g; sodium thiosulfate, 0.3 g; lactose, 10.0 g; ferric ammonium citrate, 0.2 g; meat peptone, 10.0 g; phenol red, 25.0 mg; sodium chloride, 5.0 g; agar; 12.5 g; dextrose, 1.0 g; demineralized water, 1000.0 ml. The steps for preparation of the dehydrated culture medium were suspending 49 grams of medium in 1000 ml of demineralized water, heating to boiling with agitation to completely dissolve, sterilizing by autoclaving at 121°C for 15 minutes, dispensing into appropriate containers and cooling tubes in a slanted position so that deep butts are formed.[11][12][13][14][15][16][17].

2.1.2. Bacterial isolation, collection and preservation. An area of between toe was swabbed by sterile cotton bud and streaked on media. Each cultures grown on KIA medias were incubated for 24 hours. The isolates that were successfully grown were then purified by transferred on to new KIA media and then incubated for 12-24 hours. For preservation purposes, bacterial collection was transferred in HTR (heterotroph) glycerol media in cryotube and stored at -80°C.

2.2. Comparison of sweat and socks as source of bacterial growth

Isolates that were successfully grown were then purified by growing on KIA media which Congo red had added, then incubated for 12-24 hours.

2.2.1. Bacterial density

A number of 25 bacteria isolates were selected based on that cause foot odor, sweatand soaking aquadest of socks were collected from volunteers. Sweat or soaking water of socks were filtered (0.2 µm) as medium for bacterial growth. Each filtrates were transferred in to a sterile test tube and each bacterial isolates inoculated in to it. The density of each bacterial cultures were measured by using iMark microplate reader (Biorad) at λ295 nm.

2.2.2. Organoleptic test

For organoleptic test purpose, a number of 46 bacteria isolates were selected and tested paneliststo compare odor types betweensweat filtrateand socks filtrate as bacterial medium. Organoleptic test was performed by. They were given a sheet that had to be filled with several possible odors. The method was each panelists smells the odor of each test tube randomly.

2.3. Characterization of the bacterial isolates

The polyphasic characterization was based on morphology, physiological/biochemical test and molecular identification.

2.3.1. Gram staining test. A small masses of bacterial isolate was placed on a sterile slide. Subsequently, sterile distilled water was dripped on top and then fixed using bunsen, by
passing the above slides on Bunsen 2-3 times. Crystal violet was dripped on the isolate until evenly distributed and allowed to stand for three minutes. Rinse with sterile distilled water and dried. Lugol was dripped evenly and left for one minute. Rinsed again with sterile distilled water and dried. Ethanol is dropped on the isolate for 10 seconds. Rinse and dried. Safranin was dripped and left for 1 minute. Rinse with sterile distilled water and dried. Cell structure that appears to be was observed under a light microscope at 1000 times magnification.

As the KIA media was used to characterization of bacterial isolates, following (Table 1) was the interpretation of the KIA test.

2.3.2. Physiological/biochemical test.
KIA media was used as a physiological/biochemical test was applied. To perform H2S test, a mass colony of each isolates was inserted using needle N in to KIA media agar slant on the ¾ part of the media. The changes were observed for 24 hours. If the media was raised it shows an indication that isolate produces H2S.

| Carbohydrate fermentation | Hydrogen sulfide production: |
|---------------------------|------------------------------|
| Positive test for slant reaction - yellow (acid) | positive test - black color throughout medium, a black ring at the juncture of the slant and butt, or a black precipitate in the butt. |
| negative test for slant reaction - red (alkaline) | negative test - no black color development |
| positive test for butt reaction - yellow (acid) | Gas production: |
| negative test for butt reaction - red (alkaline) | Positive test - bubbles in the medium, cracking and displacement of the medium, or separation of the medium from the side and bottom of the tube. |
| Kia color reactions: | negative test - no bubbles and no separation or displacement of the medium. |
| red slant/ yellow butt | - dextrose (+), lactose (-) |
| yellow slant/ yellow butt | - dextrose (+), lactose (+) |
| red slant/ red butt | - dextrose (-), lactose (-) |

2.3.3. Molecular identification
Molecular identification was carried out on several bacterial isolates mainly based on H2S production. Initially, DNA extraction of each isolates was performed followed the instruction from the manufacture (MyTaq Red Mix, Bioline). The PCR reaction contained template (2µl), each primers (27 F 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R 5' TACGGYTACCTTGTTACGACTT 3'; 20 µM each) 1 µL, MyTaq Red Mix, 12.5 µL; Water (ddH2O) up to 25 µL. The temperature profile consisted of initial denaturation at 95°C for 1’, followed by denaturation at 95°C for 15’, 30 cycles of annealing at 55°C for 15” and extension 72°C for 10”. Each PCR DNA products was sequenced. Each sequences were analysis its quality and trimmed to have a good sequence by Bioedit before making BLAST searching using database of National Center of Biology Informatics (NCBI) web.
3. Result and Discussion

3.1. Isolation and collection of foot causing odor-bacteria

There were 204 bacterial isolates collected from 30 people ranged from 7-40 years old. *Bacillus cereus* and *Proteus mirabilis* were recognized as a main cause of foot odor. Other bacteria isolated were *Acinetobacter pittii*, *Enterobacter hormaechei* subsp. *xiangfangensis*, *Enterobacter mori*, *Glutamicibacter creatinolyticus*, *Klebsiella pneumonia*, *Leclercia adecarboxylata*, and *Staphylococcus warnerii*.

Isolation from foot without treated resulted 137 isolates and with treatment resulted 107 isolates and the total isolates were 244 isolates (Table 1). All isolates were collected for further study. All isolates were observed microscopically after gram staining test.

Direct swabbing method showed that 137 bacterial isolates was collected. It was 50% more than pre-treatment (72). This mean, the pre-treatment successfully removed bacterial inhabitants on an area of between toes.

**Table 2.a.** Bacterial isolation from volunteer’s foot.

| Panelists number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | Total Isolates (A) |
|------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|-------------------|
| Direct swabbing  | 4 | 3 | 3 | 2 | 5 | 4 | 2 | 4 | 3 | 4 | 3 | 3 | 2 | 8 | 8 | 58                |
| Pre-treatment    | 5 | 3 | 2 | 3 | 2 | 4 | 3 | 5 | 3 | 3 | 3 | 3 | 5 | 10 | 57              |

| Panelists number | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | Total Isolates (B) |
|------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-------------------|
| Direct swabbing  | 7  | 5  | 7  | 2  | 10 | 5  | 6  | 2  | 3  | 7  | 3  | 6  | 6  | 2  | 79               |
| Pre-treatment    | 4  | 0  | 9  | 2  | 9  | 6  | 3  | 0  | 2  | 1  | 2  | 3  | 1  | 8  | 15               |

**Table 2.b.** Comparison between direct swabbing and pre-treatment method.

| Methods          | Total Isolates A | Total Isolates B | total | Effect of Methods                  |
|------------------|------------------|------------------|-------|-----------------------------------|
| Direct swabbing  | 58               | 79               | 137   | Many bacteria isolated            |
| Pre-treatment    | 57               | 15               | 72    | Reduction to 53% bacteria isolated compared to direct swabbing |

The inoculation result of 25 isolates originated from foot showed that sweat filtrate was better medium for bacterial growth than shocks filtrate (Table 2). This was indicated that the odor problem was from sweat which was consisted of bacterial growth producing unpleasant odor.

3.2. Detection on foot odor causing-bacteria

There were different densities between bacterial cultures cultivated in foot sweat filtrate and shock filtrate medium showed (Figure 1).
Figure 1. Density of bacterial growth on foot sweat filtrate and shock filtrate medium.

At figure 1 and Table 2), *Bacillus cereus*, *Proteus mirabilis* and two other isolates produced H2S which indicated by black color on the slant agar. Other isolates were
Table 3 shows bacterial characterization based on morphology, Gram staining and physiological/biochemical test on KIA media. Production of H₂S gas indicated that bacteria reacted with iron. If there is a color change from yellow to red, it means that the bacteria use lactose, but if it is above it means aerobic and if the media is below it means microaerophilic.

Based on the result of molecular identification, *Acinetobacter pittii*, *Bacillus cereus*, *Enterobacter*, *Glutamicibacter*, *Klebsiella*, *Leclercia*, *Proteus mirabili*, *Staphylococcus warnerii*, were suspected as the causing foot odor bacteria. Interestingly, they were unique bacteria found from human foot. Other bacteria were recognized after morphological and biochemical tests were *Pseudomonas*.

The result of 46 isolates inoculation into sweat filtrate and shocks filtrate medium showed that sweat filtrate medium was better medium for bacterial growth. It indicated that sweat filtrate was more nutritious and more favorable for bacterial growth than shock filtrate (Figure 3).
Table 3. Bacterial Characters based on Morphology, Gram staining and physiological/biochemical test on KIA Media.

| Isolates | Name of bacteria          | Shape | Gram Stain | Carbohydrate fermentation | Dex -trose | La -tose | H2S (Gas production) | Others (Gas, Media) |
|----------|---------------------------|-------|------------|---------------------------|------------|----------|---------------------|---------------------|
| MK.7/B2  | Enterobacter hormaechei subsp. xiangfangensis* | Bacil | +          | -                         | -          | -        | -                   | -                   |
| MK.6/B8  | unknown                   | cocci | +          | +                         | -          | +        | -                   | ++                  |
| MK.14/B  | unknown                   | Bacil | -          | +                         | +          | -        | -                   | +                   |
| MK.6/B1  | Staphylococcus            | cocci | -          | -                         | +          | -        | -                   | ++                  |
| MK.12/B  | unknown                   | cocci | -          | +                         | +          | -        | -                   | -                   |
| MK.26/B  | unknown                   | Bacil | +          | +                         | +          | -        | -                   | -                   |
| MK.12/B  | Acinetobacter pittii *    | Coccus| -          | -                         | -          | -        | -                   | full, orange        |
| MK.15/B  | Acinetobacter pittii *    | Coccus| -          | -                         | +          | -        | -                   | yellow, red at top part |
| MK.11/B  | Leclercia adecarboxylata *| Coccus| +          | +                         | +          | -        | -                   | -                   |
| MK.17/B  | Glutamicibacter creatinolyticus* | Bacil | +          | +                         | +          | +        | -                   | ++++, media divided in two parts |
| MK.2/B1  | unknown                   | Coccus| +          | +                         | +          | -        | ?                   | ++++, media lifted   |
| MK.26/B  | unknown                   | Coccus| -          | -                         | -          | -        | -                   | +, media cracked    |
| MK.20/B  | Staphylococcus warnerii*  | Coccus| +          | +                         | +          | +        | -                   | -                   |
| MK.20/B  | Proteus mirabilis*        | Coccus| +          | +                         | +          | +        | +                   | -                   |
| MK.20/B  | unknown                   | Coccus| +          | +                         | +          | +        | +                   | ++, media full, black |
| MK.20/B  | unknown                   | Bacil | +          | +                         | +          | +        | +                   | -                   |
| MK.22/B  | Enterobacter mori*        | Coccus| +          | -                         | -          | -        | -                   | -                   |
| MK.25/B  | unknown                   | Coccus| -          | -                         | -          | -        | -                   | -                   |
| MK.13/B  | Klebsiella pneumoniae*    | +     | +          | +                         | -          | -        | -                   | +++                 |
| MK.16/B  | Bacillus cereus*          | Bacil | +          | -                         | +          | -        | -                   | -                   |
| MK.16/B  | Bacillus cereus*          | Bacil | +          | +                         | +          | +        | +                   | black               |
| MK.16/B  | Bacillus                  | Bacil | -          | +                         | +          | -        | -                   | -                   |
| MK.26/B  | Bacillus                  | Bacil | +          | +                         | +          | -        | -                   | -                   |
| MK.26/B  | Bacillus cereus*          | Bacil | +          | +                         | +          | +        | -                   | -                   |

Note:  - : Indicates negative reaction results; +: indicates positive reaction results
*): Based on 16sDNA identification.

High microbial capacity as one of the principal concerns of this is the production of an unpleasant odor which has a distinct cheesy/acidic note [17]. Previous work has demonstrated that this odor emanates from the bacterial
Biotransformation of branched-chain amino acids, such as leucine and valine into volatile fatty acids (VFAs), in particular isovaleric acid [3][18]. The branched-chain amino acids are released by the deprivation of the exfoliated layers of the stratum corneum under the influence of extra-cellular bacterial proteases [6]. Otherwise, they originate as a soluble element of the secreted eccrine sweat [4][19]. The key causative bacteria responsible for this biotransformation on the foot are Staphylococcus spp., but some species of Brevibacterium, Micrococcus and Kytooccus are biochemically competent at converting branched-chain amino acids to VFAs [3][17][20][21].

Comparison of odor produced between two bacterial cultures, sweat filtrate medium was more odorous than shocks filtrate medium (Figure 3). This result indicated that sweat was more nutritious and more favorable for bacterial growth.

![Figure 3](image1.png)

**Figure 3.** Comparison of odor produced by foot inhabiting-bacteria cultivated in foot and shocks filtrate medium.

Further study is needed to clarify the identity of odor causing bacteria of foot and the necessity of removal or eradication of those bacteria by using several methods including application of bacteriophages. Bacteriophages is known have a powerful to break bacterial biofilm and kill the bacteria which are resistant to antibiotics or disinfectant. This application will be promising in the very near future mainly to clear the odor foot problem.

![Figure 4](image2.png)

**Figure 4.** Unknown bacteriophage was found to lysis Bacillus sp. on Lurie Bertani agar. Inset is plaques caused lytic activities by phages on Bacillus cells.
4. Conclusion
There was diverse of bacterial inhabitant on foot. Bacterial growth in sweat was suspected as foot odor causing bacteria. *Bacillus cereus*, *Proteus mirabilis* and two other isolates were detected as the foot odor causing bacteria. Sweat and shocks were the sources of foot odor problem because of the bacterial growth. Sanitation such as by rinsing or cleaning was good preventive or effectively reduction of bacterial growth causing foot odor.

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