

## Contaminant bacteria in traditional-packed honey

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### ABSTRACT

Honey may be contaminated by microorganisms during its harvesting, processing, and packaging. Honey selected for clinical purposes must be safe, sterile, and contain antimicrobial activity, so it must be evaluated using laboratory testing. The aim of this descriptive laboratory study was to isolate and identify the bacterial contaminant in the traditional-packed honey dealing with the use of honey for medical purposes. The colony forming units of honey sample cultured on blood agar were counted using Stuart bacterial colony counter. The suspected bacterial colonies were isolated and identified based on cultural morphology characteristics. The isolates of suspected bacterial colonies were stained according to Gram and Klein method and then were examined by biochemical reaction. The results showed that there were two contaminant bacteria. Gram-positive cocci which were presumptively identified as coagulase-negative *Staphylococci* and gram-positive rods which were presumptively identified as *Bacillus subtilis*. In conclusion, the contaminant bacteria were regarded as low pathogen bacteria. Subtilin enzyme of *B subtilis* may cause allergic reaction and coagulase-negative *Staphylococci*, *Staphylococcus epidermidis* is also an opportunist pathogen. Inevitably, for medical purposes traditional-packed honey must be well filtered, water content above 18%, and standardized sterilization without loss of an antibacterial activity or change in properties.

**Key words:** Bacterial contaminant, traditional-packed honey

### INTRODUCTION

Honey is a floral nectar or secretions of plants that is gathered and processed by honeybees<sup>1,2</sup> or one of bee honey products besides bee wax, royal jelly, and propolis.<sup>3</sup> It has been used for thousands of years in traditional medicine for several purposes. The ancient Greeks, Roman, Chinese and Egyptians used honey to heal wounds and cure disease of the gut.<sup>4</sup>

Another author reported that 2000 years before bacteria were discovered to be the cause of infection, honey was used to treat infected wounds.<sup>5</sup> Then, the ancient African of West Africa

ate honey daily as supplementing diet that might occur in their daily food intake. They also prescribed it when rearranging a patient's daily food regimen.<sup>6</sup>

Recently, there were scientific evidences supporting therapeutic uses of honey. Honey has been used as modern therapeutic agent because of its antibacterial activity. It has been proven that 91% of infected wounds treated with honey were free of infection within 7 days. Patients treated with honey experienced less irritation, more relief of pain, and no adverse effects.<sup>4,5</sup> The anti-inflammatory activity of honey also reduces edema and exudates and prevents or minimizes

hypertrophic scarring.<sup>7</sup>

Furthermore, honey has been reported to have an inhibitory effect to around 60 species of bacteria including aerobes and anaerobes, gram-positives and gram-negatives bacteria.<sup>5</sup>

Some antibiotic-resistant bacteria, such as MRSA (Methicillin Resistant *Staphylococcus aureus*) and VRE (Vancomycin Resistant *Enterococci*) have been reported sensitive to honey.<sup>5,8</sup> Specifically, Manuka and Pasture honeys at concentration of 2.7-5% (v/v) inhibited coagulase-negative Staphylococci.<sup>9</sup> In Netherlands, a sterile wound ointment with a honey basis 50% (v/v) was used in a multi-centre case report study of 139 nursing homes and General Practitioners practice patients suffering from ulcer, decubital ulcers, abrasion and burns.<sup>8</sup>

In dentistry, honey has been used to treat oral ulcer and periodontal disease.<sup>5</sup> It has anticariogenic as well. Even though it contains more than 70% sugar, honey at high concentration had inhibitory effect on oral streptococci in vitro and salivary counts of total bacteria and *Streptococcus mutants* decreased after 1 hour application of honey.<sup>10</sup> The antibacterial activity of honey can be caused by osmotic effect of its high sugar content, low 3.9 pH, and a thermo unstable substance named *inhibine*, hydrogen peroxide and enzyme such as catalysts.<sup>5,8,11</sup>

Honey is presumed to be sterile. However, it is a misconception. Honey may contain *Bacillus sp*, *Clostridium*, viable spore.<sup>5,8</sup>

It may also contain pesticides as well as antibiotic residues such as tetracycline used on bee.<sup>8</sup> Commercially honey is sold in traditional-packed and factory-packed honey. It is widely believed that factory-packed honey are more hygiene than traditional-packed honey due to have a better process or standardized in harvesting, processing, and packaging.<sup>3</sup> Meanwhile, traditional-packed honey are usually processed by hand-pressing of a honeycomb. Then, honey are filtered using cloth.<sup>12</sup> On the contrary, using traditional packed-honey as therapeutic agent is still an attractive option, may be because it is cheaper than factory-packed honey.

The use of honey for medical purposes, especially for the treatment of human, needs a sterile honey. It must be emphasized before problem arise for man. To achieve best results that

traditional-packed honey can safely be applied as therapeutic agent, therefore the aim of this study was to isolate and identify contaminant bacteria in traditional-packed honey sold in Bandung, Indonesia.

## MATERIALS AND METHODS

### Choosing of honey sample

In the preliminary study we used and selected randomly three samples (Asy-syifa, Sumbawa, and Danau Sentarum) of Indonesian traditional-packed honey that were sold in Bandung city. Traditional-packed honey is natural honeybee that is harvested, processed, and packed by beekeepers using hands in traditional way. To proof that contaminant bacteria are from honey sample, all honey samples were prepared for bacterial smear preparation on an object glass, and stained using gram-staining.<sup>13,14</sup>

Bacterial smear stained was examined using light microscope. It showed that all samples of honey were similar containing gram-positive rods (purple color). Then, one inoculating loop of each sample honey was cultured on blood agar plate, incubated for 24 hours at 37°C in incubator.

The result showed that the density of contaminant bacteria colonies of Danau Sentarum honey was lighter than the other honey samples. So, it is easily to isolate the suspected bacteria. Next, according to the density of bacterial colony, this descriptive laboratory study used Danau Sentarum honey as sample of study. This study was conducted in the Laboratory of Microbiology at Faculty of Dentistry, Universitas Padjadjaran in Jatinangor-Bandung.

### Characteristics of contaminant bacteria

To determine the characteristic of contaminant bacteria in honey sample, one inoculating loop of honey was cultured on blood agar plate in Petri dish, incubated aerobically at 37° C for 18-24 hours in incubator.

Culture of honey was made triple of different bottles of honey. Bacteria colonies or the colony forming units that grew on each of blood agar plate were counted using Stuart bacteria colony counter. After that, the characteristic of bacteria colonies were identified based on morphologies such as form, margin, elevation, size, color or

pigment and the ability of microorganism to lyses blood in culture.<sup>13,14,15</sup>

#### Microscopic and biochemical identification

One inoculating loop of isolate of each suspected bacterial colony was smeared on an object glass for bacterial smear preparation and stained using Gram staining. If Gram-staining of bacterial colony shows gram-positive (purple color) coccus, next, the isolate of bacterial colony are prepared for biochemical identification, these are catalase and coagulase test. Catalase test was used to differ genus of *Staphylococcus* (catalase-positive) and *Streptococcus* (catalase-negative). Hydrogen peroxide 3% was dropped on bacterial suspension in 0.85% NaCl on an object glass.

The reaction is catalase-positive if air or oxygen bubbles are produced. Coagulase test was used to differ *Staphylococcus aureus* (coagulase-positive) and *Staphylococcus epidermidis* or *Staphylococcus saprophyticus* (coagulase-negative *Staphylococci*). On object glass containing a heavy bacterial suspension was dropped 0.1 ml blood plasma. *Staphylococcus aureus* can coagulate blood plasma or coagulase test is positive. If the result of coagulase test is negative, then it is suspected that isolate bacteria is *Staphylococcus epidermidis* or *Staphylococcus saprophyticus*. In this study, we did not differentiate both of last species. Next, these last species are called coagulase-negative *staphylococci*.

If the gram-staining result shows gram-positive rods, the isolate of bacterial colony are prepared for further identification, spore staining and mannitol test. Spore staining according to Klein's method was used to differentiate spore-forming bacilli and non spore-forming bacilli. Suspected bacterial smeared on object glass was stained with Klein-spore staining. Spores will appear red and vegetative cells will appear blue. One inoculating loop of isolate of bacterial colony added to mannitol solution in glass tube, incubated for 24 hours at 37°C.

Thereafter, if phenol red indicator in mannitol solution turn to yellow color, it is identified that bacteria are mannitol fermenter (mannitol-positive) or bacteria is suspected as *Bacillus subtilis* or *Bacillus megaterium*. Next, Voges-Proskauer test is used to differentiate these bacteria. Mannitol test is negative if isolate

bacteria is *Bacillus cereus*.<sup>13,14,15</sup>

The schema for the identification of contaminant bacteria in traditional-packed honey in this study is presented in figure 1.

## RESULTS

### Characteristics of contaminant bacteria

The blood agar culture of three traditional-packed honey can be seen in Figure 2. Then, there were two different colonies of contaminant bacterial of honey samples (Table 1 and Figure 3). First type colonies or A type colonies are round, about 1-5 mm in diameter, and have an irregular margin, a white grey pigment, a flat elevation. The A type colonies were  $\beta$ -hemolytic on blood agar plates.

The second type colonies or B type colonies are round, about 1-4 mm in diameter, and have an entire margin, a white pigment, a convex elevation. In contrast, B type colonies were unhemolytic on blood agar plates. The first replication of honey sample contains both A type colonies and B type colonies. While the second and third replication of honey sample contain only B type colonies (Table 2 and Figure 3).

The number of colonies on each honey sample on blood agar plate were presented in Table 2. In first replication of honey sample showed that the number of A type colony were greater than the number of B type colony. Then, the number of A type colony in the second and third replication are almost twice of the number of A colony in the first replication.

### Microscopic examination and biochemical test

The Gram-staining result showed that the isolate of A type bacterial colony is gram-positive rods and arranged in chains. Klein staining result showed that they are spore-former. Then, the mannitol test showed that A bacterial colony is positive or they change phenol red indicator to yellow color (Figure 4).

The isolate of bacteria of B type colony showed gram-positive coccus and arranged in grape fruit-like clusters. Then, the bacteria produced air bubbles in catalase test or positive-catalase. Coagulase test performed that bacteria suspension did not coagulate blood plasma or coagulase-negative (Figure 5).

Figure 1. The scheme for the identification of contaminant bacterial in traditional-packed honey

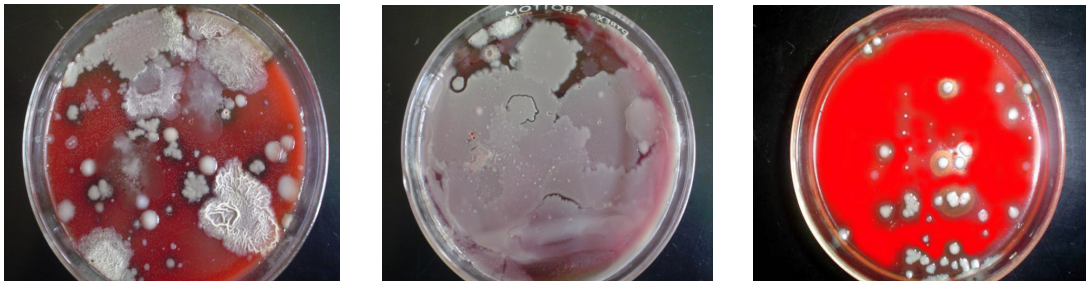


Figure 2. Characteristics of three kinds of traditional-packed honey that are sold in Bandung city  
a. Asy-syifa; b. Sumbawa; c. Danau Sentarum



Figure 3. A and B types colonies of three replications of honey samples cultured on blood agar plate

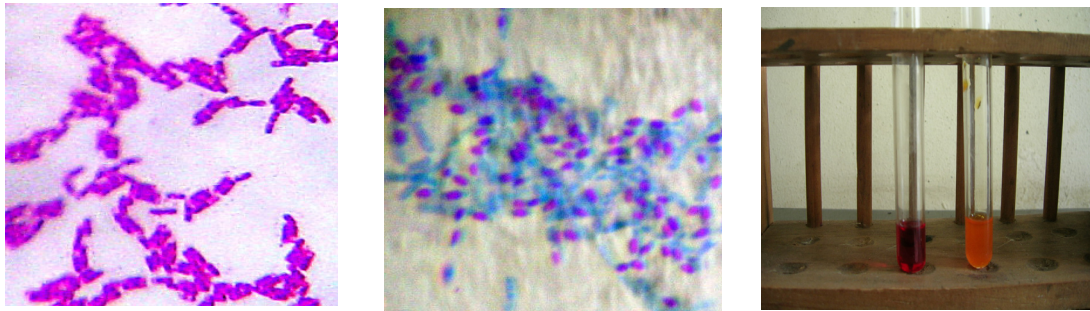


Figure 4. The results of microscopic examination and biochemical test of A type colony. a. Gram-staining positive rods (purple); b. Klein-staining (white arrow-spores-red/pink); c. Mannitol test (white arrow-sample)



Figure 5. The results of microscopic examination and biochemical test of B type colony. a. Gram-staining positive coccus (purple); b. Catalase test-positive (air bubbles); c. Coagulate test-negative or no cult blood plasma

Table 1. Characteristics of contaminant bacterial colonies of honey samples on blood agar plates

Colony	Characteristics of culture	Replication of honey sample		
		1	2	3
A	Form	Round	Round	Round
	Diameter	1-5 mm	1-5 mm	1-5 mm
	Margin	Irregular	Irregular	Irregular
	Pigment	Grey-White	Grey-White	Grey-White
	Elevation	Flat	Flat	Flat
	Ability to lyses blood	Hemolytic	Hemolytic	Hemolytic
B	Form	Round		
	Diameter	1-4 mm		
	Margin	Entire		
	Elevation	White		
	Ability to lyses blood	Convex		
		Unhemolytic		

Table 2. The number of colonies of on blood agar plates

Colony	The number of colonies		
	Sample 1	Sample 2	Sample 3
A	52	101	104
B	10	-	-
Total	62	101	104

Based on characteristic on blood agar plate, gram-staining and Klein-staining examination, and mannitol test as well, the isolate of A type colony was presumptively identified as *Bacillus subtilis*. While, the result of characteristic of B type colony on blood agar plate, catalase and coagulase test, it was presumptively identified as coagulase-

negative Staphylococci.<sup>13,14,15</sup>

## DISCUSSION

It has been proved that samples of Indonesian traditional-packed honey contain gram-positive rods bacteria. In further identification, sample

honey cultured on blood agar plate showed that the growth of bacteria were vary. It is presented by the number of colony in each blood agar plate. The number of *Bacillus subtilis* colonies are greater than the number of coagulase-negative Staphylococci colonies.

It can be assumed that *B. subtilis* is the highest contaminant bacteria in Indonesian traditional-packed honey. *B. subtilis* are saprophytic organism prevalent in soil, water, and air.<sup>13,16,17</sup> So, it is possible that honey can also be contaminated by *B. subtilis* from air or non-sterile bottle. Honey may occasionally contain *B. subtilis* result of non-sterile condition during harvesting, processing, and packaging.<sup>3</sup> Another explanation it may be caused by *B. subtilis* endospore contaminating honey and it germinated during incubation. Honey do not allow vegetative bacteria to survive, but it does contain viable spores.<sup>5</sup> Spores display a spectacular ability to survive in hostile environment for long periods. They also resistant to many of chemical agents used as disinfectant, physical environments including high temperature, ionizing radiation, and desiccation.<sup>5,18,19,20</sup>

Coagulase-negative Staphylococci found in traditional-packed honey may due to Coagulase-negative Staphylococci of beekeeper skin contaminated honey while harvesting, processing or packaging. One of human coagulase-negative staphylococcal species, *S. epidermidis* is a human normal flora on the skin surface, respiratory and gastrointestinal tract and it is spread by contact.<sup>13,16,17,20,21</sup>

Contaminant bacteria are found in Indonesian traditional-packed honey even honey scientifically proof has an antibacterial activity. It could be explained that honey is produced from many different floral sources and its antibacterial activity varies with origin and processing.<sup>5,9</sup> Another explanation is that there is a tendency some traditional-packed honey have been added water to increase volume. Diluting honey with water will decrease the antibacterial activity and it will promote the microbial growth. Honey containing water above 18% is easily to get a fermentation.<sup>3</sup>

*B. subtilis* is not considered as human pathogen, it may contaminated food but rarely cause food poisoning. However, it produces the

proteolytic enzyme, subtilin which has been shown to be a potent occupational allergen.<sup>17</sup> On the other hand, coagulase-negative Staphylococci species, especially, *S. epidermidis* is opportunist pathogen.<sup>16,17,20,21</sup>

It means that the bacteria cause infection if the opportunity arises. *S. epidermidis* may infect man with low immunity or specific conditions. For example *S. epidermidis* can cause infection of man with intravenous catheter or prosthetic implants.<sup>16,21</sup> Inevitably, honey especially for medical uses must sterile and has antimicrobial activity. Gamma radiation has been used to sterilize some brands of honey. It kills clostridial spores without loss of any of the antibacterial activity or changes in honey properties.<sup>5,8</sup>

## CONCLUSION

Contaminant bacteria found in Indonesian traditional-packed honey and were presumptively identified belong to *Bacillus subtilis* and coagulase-negative Staphylococci species. To enhance the quality of Indonesian traditional-packed honey for medical purposes, honey must be well filtered, contain no water above 18% v/v, and treated by standardized sterilization.

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