Long-term Storage of Bacterial Isolates by Using Tryptic Soy Broth with 15% Glycerol in The Deep Freezer (-70 to -80 °C)

Sunarno1*, S Nursofiah1, Y Hartoyo1, N Amalia1, T Febrianti1, D Febriyana1, R D Saraswati1, N Puspandari1, K Sariadji1, Khariri1, Y Rukminiat1, F Muna1, I Susanti1, P Multihartina2

1Centre for Research and Development of Biomedical and Basic Health of Technology, National Institute of Health Research and Development
2Centre for Research and Development of Human Resources and Health Services, National Institute of Health Research and Development

*Corresponding author’s e-mail: no_nar@yahoo.com

Abstract. For different bacterial preservation techniques, there is no single method applicable for all bacteria. This study aimed to assess the viability of seven species/species groups of clinical bacteria isolates on the long-term storage (more than 5 years) by using Tryptic Soy Broth with 15% glycerol in the deep freezer (-70 to -80°C). A total 10,654 clinical bacteria isolates used as samples in this study. The isolates consisted of seven species/species groups (i.e. *Escherichia coli*, *Campylobacter* spp, *Shigella* spp, *Vibrio* spp, *Salmonella* spp, *Aeromonas hydrophila*, and *Neisseria gonorhoeae*). The isolates were collected from some previous studies and preserved in the Tryptic Soy Broth (TSB) with 15% glycerol and stored in the deep freezer (-70 to -80°C) for more than five years. The samples were revived on the suitable medium to evaluate the viability of bacteria. Identification conducted by microscopic examination, biochemical test, and latex agglutination. The study showed that the viability of *Salmonella* spp, *Shigella* spp, *A. hydrophila*, and *E. coli* was 100%, while *Campylobacter* spp, *Vibrio* spp, and *N. gonorhoeae* were 66.7%, 66.4%, and 52.5% respectively. We concluded that viability of *Salmonella* spp, *Shigella* spp, *A. hydrophila*, and *E. coli* was optimum thus better than *Campylobacter* spp, *Vibrio* spp, and *N. gonorhoeae* for more than 5 years storage by using TSB with 15% glycerol in the deep freezer (-70 to -80 °C).

1. Introduction
Indonesia is a tropical country with mega biodiversity, ranging from plants, animals, and also microbes. The diversity of microbes can further increase through their mutation into a different strain [1], adding to the sum of total biodiversity. This high level of biodiversity is a precious asset, we find it as useful resources for research and the development of science and technology [2,3]. The development in science and technology allows humankind to obtain many benefits from microbes that makes life easier and more convenient, e.g., vaccine production in medicine [4], enzyme production in biochemistry [5], and probiotic microorganism addition in biotechnology of food production [6]. Polymerase Chain Reaction (PCR) assessment method is becoming easier and faster using the Taq polymerase enzyme, which was isolated from the bacterium *Thermus aquaticus* [7]. The development of DNA vaccine is more effective using cloning method with *Escherichia coli* as vector [8]. Various fermented foods (e.g., yogurt, cheese) were manufactured using microorganisms such as bacteria [9].
Nevertheless, several microbes could be misused as a threat, such as bioterrorism that can threaten the safety and health of humankind. Since the first world war, there were several microorganisms utilized as biological weapons [10]. The Center for Diseases Control and Prevention (CDC) has assigned three different categories of microorganism-based biological weapons. The most harmful one is the category A which consisted of bacteria and virus, such as *Bacillus anthracis*, *Francisella tularensis*, Smallpox virus, *Clostridium botulinum*, *Yersinia pestis*, and Viral hemorrhagic virus (Ebola, Marburg, Lassa, and Machupo) [10,11].

In order to gain benefits and avoid the harmful possibilities of various microorganisms, special attention and specific efforts are needed for storage and maintaining them. The microorganism preservation and maintenance techniques are customized based on their intended utilization, i.e., daily, short-term, or long-term. Short-term utilization is usually related to research purposes that were daily needed. Long-term utilization is related to collection and conservation purposes [2]. There are several microorganism preservation methods and each of it has its own advantages and/or disadvantages. Thus, there is no single method applicable for all kinds of microorganisms. Some microorganisms can be easily stored and be able to survive with various preservation methods. Meanwhile, any other microorganisms have preservation limitations, for instance, their sensitivity for changes in temperature, pH, or moisture [12,13]. This study aimed to assess the viability of seven species/species groups of clinical bacteria isolates on the long-term storage (more than 5 years) by using TSB with 15% glycerol in the deep freezer (-70 to -80°C).

2. Material and Methods

The study was descriptive in terms of the research design. Ethical approval was obtained from the Ethics Committee of the National Institutes of Health Research and Development, Ministry of Health, Indonesia (No: LB.02.01/2/KE014/2019). No: KE.01.05/EC/359/2011.

2.1. Sample

A total 10,654 archive samples belong to the National Institute of Health Research and Development, Ministry of Health were used as samples in this study. The samples consisted of seven species/species groups of clinical bacteria isolates, including *Salmonella* spp, *Shigella* spp, *Campylobacter* spp, *Vibrio* spp, *Escherichia coli*, *Aeromonas hydrophila*, and *Neisseria gonorhoeae* (Table 1). The isolates collected from some previous studies. The isolates were preserved in 1.5 ml tubes containing Tryptic Soy Broth (TSB) with 15% glycerol and stored in the deep freezer (-70 to -80 °C) for more than five years. Sample selections were conducted based on inclusion criteria, including their packagings and label were still intact and not disintegrate.

Table 1. Sample proportion.

| No. | BACTERIA         | NUMBER OF SAMPLE | %  |
|-----|-----------------|-----------------|----|
| 1   | *Salmonella* spp| 1,388           | 13.0|
| 2   | *Shigella* spp  | 1,129           | 10.6|
| 3   | *Campylobacter* spp | 439          | 4.1 |
| 4   | *Vibrio* spp    | 903             | 8.5 |
| 5   | *E. coli*       | 6,060           | 56.9|
| 6   | *A. hydrophila* | 135             | 1.3 |
| 7   | *N. gonorhoeae* | 600             | 5.6 |

A half of the samples were *E. coli* isolates. Indeed, most isolates were collected from studies on diarrhea disease.
2.2. Laboratory Examinations
All samples were revived on the suitable growth or selective medium. Salmonella spp, Shigella spp, Campylobacter spp, Vibrio spp, E. coli, and A. hydrophila, were revived on the MacConkey agar and blood agar. Meanwhile, N. gonorrhoeae were revived on the chocolate agar. The inoculated medium was incubated at 37°C for 24 hours. The isolates of three bacteria species/species groups (Campylobacter spp, A. hydrophila, and N. gonorrhoeae) were incubated in the CO₂ incubator. The bacterial colonies on culture medium were identified by microscopic examination dan biochemical test. Latex agglutination was used to determine serotype of some bacteria. The conclusion of bacterial viability was determined in the presence or the absence of bacterial colonies on the culture medium by ignoring number of colonies. When we found at least one bacterial colony and identified correctly by microscopic and biochemical test, it mean the isolate viable. Hence, if there was no bacterial colony found on the culture medium, the samples were re-cultured. And when there was still no bacterial colony for second reviving, it concluded that isolates were non-viable.

3. Results
There were 9,920 (93%) out of a total 10,654 samples containing viable bacteria. Description of the viable bacteria by species/species groups were shown in Table 2.

| Table 2. Viability of bacterial isolates |
|----------------------------------------|
| No | Bacteria       | Number of samples | Viable bacteria | % Viable |
|----|----------------|-------------------|-----------------|----------|
| 1  | Salmonella spp | 1.388             | 1.388           | 100      |
| 2  | Shigella spp   | 1.129             | 1.129           | 100      |
| 3  | Campylobacter spp | 439             | 293             | 66.7     |
| 4  | Vibrio spp     | 903               | 600             | 66.4     |
| 5  | E. coli        | 6.060             | 6.060           | 100      |
| 6  | A. hydrophila  | 135               | 135             | 100      |
| 7  | N. gonorrhoeae | 600               | 315             | 52.5     |

Compared to the other six bacteria in this study, N. gonorrhoeae has the most proportion of non-viable isolates (Table 2). The other bacteria showing a relatively sensitive were Campylobacter spp and Vibrio spp (Table 2). On the other hand, viability of aerobic bacteria better than anaerobic bacteria relatively as well as diarrhea-causing bacteria better than sexually transmitted infection-causing bacteria (Table 3).

| Table 3. Viability of the isolates based on bacterial group |
|------------------------------------------------------------|
| Bacterial group    | Number of species | Viable bacteria | % Viable |
|-------------------|-------------------|-----------------|----------|
| Gram staining     |                   |                 |          |
| Gram negative bacteria | 10.654     | 9.920           | 93       |
| Gram positive bacteria | 0         | 0               | UC       |
| Aerobic/anaerobic |                   |                 |          |
| Aerobic bacteria  | 9.480             | 9.177           | 96.8     |
| Anaerobic bacteria| 1,174             | 743             | 63.3     |

Diseases
4. Discussion

Based on the theory, all methods of preserving bacteria are carried out by limiting the metabolism of bacterial isolate. Bacterial isolate preservation using medium TSB + 15% glycerol in the ultra-low temperature freezer has several advantages, e.g., relatively easy, space-saving, and applicable for aerobic and anaerobic bacteria. However, some bacteria cannot be stored using this medium. For instance, *Neisseria meningitidis* has shown better compatibility with Greaves medium and *Mycobacterium tuberculosis* that was better stored in medium containing skim milk [14,15]. Therefore, the preservation technique needs to be customized based on the bacterial isolates since the bacterial viability depends on the preservation method and bacterial species.

Table 2 showed that the viability of bacterial isolates may vary. One factor that influences the viability of bacteria during ultra-low temperature preservation is the temperature instability [16]. A broken or less-functional freezer and the high frequency of opening and closing the freezer door can cause freeze-thawing and temperature instability [17]. Furthermore, an electrical problem can also cause temperature loss. Several bacteria, e.g., *Vibrio* spp, *Campylobacter* spp, and *Neisseria* spp were showing a sign of sensitivity towards the temperature changes. A study by Mils & Gherna showed the optimum viability of *Campylobacter* spp was gained with the liquid-nitrogen preservation method [18]. One limitation of this study was the absence of information regarding the freezer history condition (whether it was broken before or not), the frequency of opening the freezer door, freeze-thawing, and electrical instability during the five years of preservation. Even though there was an electricity generator available in the laboratory where samples were kept, this generator needs some times to start generates electricity. On the other hand, qualitative data of the bacterial viability (presence or absence of bacterial colony) only observed in this study is another limitation.

All bacterial isolates were Gram-negative bacteria in this study and all samples (94.4%), but *N. gonorrhoeae* were bacteria causing gastroenteritis diseases (Table 3). The Gram-negative bacteria has a relatively thin and fragile cell wall compared to the Gram-positive bacteria. This characteristic can influence bacterial viability during preservation. A study by Mai-Prochnow, et al showed that the thickness of the cell wall influenced the sensitivity and resistance towards cold atmospheric-pressure plasma (CAP). Another study by Miyamoto-Shinohara, et al showed a higher survival rate of Gram-negative bacteria compared to Gram-positive bacteria during preservation with freeze-drying [19,20].

Based on the results of this study, we concluded that viability of *Salmonella* spp, *Shigella* spp, *A. hydrophila*, and *E. coli* was optimum thus better than *Campylobacter* spp, *Vibrio* spp, and *N. gonorrhoeae* for more than 5 years storage by using TSB with 15% glycerol in the deep freezer (-70 to -80 °C). Laboratories storing bacterial isolates, especially *Vibrio* spp, *Campylobacter* spp, *N. gonorrhoeae*, and other bacteria that were sensitive to temperature changes need to pay attention to the factors affecting the bacterial viability, especially related to the freezer and indoor temperature stability [17]. The scheduled monitoring of freezer and indoor temperature is required to be done, so it can be used as early warning system to detect the risk of damage or death of bacterial isolates.

References

[1] Denamur E, Matic I. Evolution of mutation rates in bacteria. *Mol Microbiol.* 2006;60(4):820-827. doi:10.1111/j.1365-2958.2006.05150.x.

[2] Machmud M. Teknik Penyimpanan dan Pemeliharaan Mikroba. *Buletin AgroBio*. 2001;4(1):24-32.
[3] Vitorino LC, Bessa LA. Technological Microbiology: Development and Applications. *Front Microbiol*. 2017;8:827. Published 2017 May 10. doi:10.3389/fmicb.2017.00827

[4] Weinstock GM, Smajs D, Hardham J, Norris SJ. From microbial genome sequence to applications. *Res Microbiol*. 2000;151(2):151-158. doi:10.1016/s0923-2508(00)00115-7.

[5] Spasic J, Mandic M, Djokic L, Nikodinovic-Runic J. Streptomyces spp. in the biocatalysis toolbox. *Appl Microbiol Biotechnol.* 2018;102(8):3513-3536. doi:10.1007/s00253-018-8884-x.

[6] Seminario-Amez M, López-López J, Estrugo-Devesa A, Ayuso-Montero R, Jané-Salas E. Probiotics and oral health: A systematic review. *Med Oral Patol Oral Cir Bucal*. 2017;22(3):e822-e288. Published 2017 May 1. doi:10.4317/medoral.21494.

[7] Olszewski M, Rebala K, Szczerkowska Z, Kur J. Application of SSB-like protein from Thermus aquaticus in multiplex PCR of human Y-STR markers identification. *Mol Cell Probes*. 2005;19(3):203-205. doi:10.1016/j.mcp.2004.11.006.

[8] Howe C. Gene Cloning and Manipulation. 2nd ed. 2007. New York: Cambridge University Press.

[9] Azam M, Mohsin M, Ijaz H, et al. Review - Lactic acid bacteria in traditional fermented Asian foods. *Pak J Pharm Sci*. 2017;30(5):1803-1814.

[10] Jansen HJ, Breeveld FJ, Stijnis C, Grobusch MP. Biological warfare, bioterrorism, and biocrime. *Clin Microbiol Infect*. 2014;20(6):488-496. doi:10.1111/1469-0691.12699.

[11] CDC. Laboratory Methods for the Diagnosis of Meningitis Caused by Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae 2nd ed. 2011.

[12] Chen H, Jin RC. Summary of the preservation techniques and the evolution of the anammox bacteria characteristics during preservation. *Appl Microbiol Biotechnol*. 2017;101(11):4349-4362. doi:10.1007/s00253-017-8289-2.

[13] WAGMAN J, WENECK EJ. Preservation of bacteria by circulating-gas freeze drying. *Appl Microbiol*. 1963;11(3):244-248. doi:10.1128/am.11.3.244-248.1963.

[14] CDC. Bioterrorism Agent/Disease by Category. Available from: http://www.bt.cdc.gov/agent/agentlist-category.asp

[15] Sleight SC, Wigginton NS, Lenski RE. Increased susceptibility to repeated freeze-thaw cycles in Escherichia coli following long-term evolution in a benign environment. *BMC Evol Biol*. 2006;6:104. Published 2006 Dec 5. doi:10.1186/1471-2148-6-104.

[16] Mills CK, Gherna RL. Cryopreservation studies of Campylobacter. *Cryobiology*. 1988;25(2):148-152. doi:10.1016/0011-2240(88)90008-9.

[17] Miyamoto-Shinozara Y, Imaizumi T, Sukenobe J, Murakami Y, Kawamura S, Komatsu Y. Survival rate of microbes after freeze-drying and long-term storage. *Cryobiology*. 2000;41(3):251-255. doi:10.1006/cryo.2000.2282.

[18] Mai-Prochnow A, Clausen M, Hong J, Murphy AB. Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. *Sci Rep*. 2016;6:38610. Published 2016 Dec 9. doi:10.1038/srep38610.

Acknowledgments
We thanked the head of Centre for Research and Development of Biomedical and Basic Health of Technology for allowing us to conduct this study. This study was financially supported by DIPA Centre for Research and Development of Biomedical and Basic Health of Technology 2011. We also thanked our colleagues at Bacteriology Laboratory and all parties involved.