Should You Go Contactless: Identifying Microbiome using Next Generation Sequencing

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Abstract

This project aimed to investigate the abundance of microbe species on given surfaces via NGS, as well as to investigate the effectiveness of various known bacterial disinfectants on the same surfaces. Multiple predetermined surfaces were prepared, and DNA of microbes were extracted from the surfaces for both culturing and NGS. Data collected from NGS was analysed using bioinformatic programmes, allowing for easy morphological identification of microbe species on samples. $2 Singaporean bank notes were found to be cleaner than previously expected, where more fungi than bacteria were found on them, which is likely due to the material of the note being polypropylene plastic. Coins had an average of 42 colonies, a mix of both bacterial and fungal. Those coins and notes obtained from wet market stalls handling dry, packaged foods had a lower colony count after incubation, while those handling fresh, wet foods had a greater number of microbe colonies. Bacteria was the main microbe on mobile phones, which had an average count of 106 bacterial colonies. The material of the phone casing was found to affect the number of bacterial colonies present.

1. Introduction

The COVID-19 pandemic has forced humanity to take steps to reduce viral exposure, and in the process, has brought attention to the cleanliness of surfaces around us. The principal modes of transmission of bacterial infection are contact, airborne, droplet, vectors, and vehicular (Doron et al., 2008). As such, along with the rapid commercialisation of science and technology that has made contactless payment a global trend, more people are turning to contactless paying options over cash payment, in a bid to reduce bacterial transmission via contact. In 2019, the number of mobile payments users stood at 0.95 billion worldwide. In Singapore alone, there has been a 12% increase in mobile contactless payment users last year (Finews, 2020). Furthermore, the Singapore government has taken major steps in recent years to support digital payments (Tan, 2021).

The microbiome, defined as a characteristic microbial community occupying a reasonable well-defined habitat which has distinct physicochemical properties (Berg et al., 2020), is present in all our living environments. Over 78% of all living organisms around us are bacteria (University of Oxford, 2021). To raise an example, there can be up to 3.2 million bacteria (a constituent of the environmental

microbiome) per square inch (BPSI) in an average toilet bowl, 200000 BPSI on a toothbrush, and 764 BPSI on an average floor (The Sun, 2017). However, contrary to popular belief, only a small percentage of the microbiome is harmful to the human body. The microbiome consists of microbes that are both helpful and potentially harmful. Most are symbiotic and some, in smaller numbers, are pathogenic (Harvard School of Public Health, 2021).

Our two surfaces of choice are cash notes and mobile phones. Across 80 $1 notes collected from a New York Bank, a total of 397 bacterial species representing more than 20 bacterial phyla were identified across aggregate samples (Maritz et al., 2017). Thus, cash notes have a notable flora of microbe species. For mobile phones, in a meta-analysis that reviewed 56 publications, the average contamination rate of mobile phones, as calculated, is 68%, and only 'clinically important' or 'pathogenic' bacteria were presented in the results (Olsen et al., 2020). This shows that mobile phone surfaces also have a significant microbiome population.

Our primary method of identifying the number of microbe species on a surface is Next-Generation Sequencing (NGS). It provides high speed and throughput that can produce an enormous volume of sequences with many possible applications in research and diagnostic settings (Barzon et al., 2011). NGS is massively parallel, sequencing millions of fragments simultaneously per run. This high-throughput process translates into sequencing hundreds to thousands of genes at one time. NGS also offers greater discovery power to detect novel or rare variants with deep sequencing (Illumina, Inc., 2021). Furthermore, due to time and resource constraints, not all bacteria detected will be culturable. NGS is able to analyze the bacterial species as long as there is genetic content present. Importantly, the analysis of data from NGS readings does not require morphological expertise, and can simply be compared to a genetic database to determine the species of bacteria present on a surface. Thus, NGS is the most suitable method of analysis for our project.

The antibacterial disinfectants of choice are 60% alcohol-based hand sanitizer, UVC light of wavelength 253.7nm and antibacterial wipes. For 60%-80% alcohol-based disinfectants, a total of 282 effectiveness tests were conducted, out of which 104 (36.9%) microorganism growth was found. Within the 92 efficacy tests, 23 of them (25.0%) detected microorganisms after alcohol disinfection (Ribeiro et al., 2015). For UVC light, upon exposure to UVC, a 99.9% inactivation rate was obtained at 3–5 s for the bacteria (*P. aeruginosa* and *Mycobacterium abscessus*) tested (Dai et al., 2012). Research for specific antibacterial wipes have yet to be conducted.

As such, this study aims to identify the abundance of various bacterial species present on mobile phones and cash notes via Next Generation Sequencing, which will henceforth be abbreviated as NGS. The latter part of this study aims to investigate the effectiveness of various disinfectants, as listed below, in eliminating bacterial populations on the given surfaces.

Our first objective was to use NGS to investigate the abundance of bacterial species present on the surfaces chosen, and our hypothesis was that there would be fewer bacterial species on mobile phones screens than on cash notes. Our second objective was to use NGS and bacterial culturing to evaluate the effectiveness of the chosen disinfectant techniques on the surfaces chosen, and our hypothesis was that the UVC disinfectant for mobile phones would be more effective than using antibacterial wipes.

2. Materials and Methods

2.1 Selection of surfaces

3 mobile phone models were used. As the sampling is randomized, mobile phones were obtained from individuals who had various and unchecked background and phone usage patterns. All mobile phone screens had a tempered glass protector, and phone cases for mobile phones 1 and 2 were made from polycarbonate plastic, while mobile phone 3 had a polyester flip case.

The coins of choice were the official $1 Singapore coins. All coins were obtained from a wet market. Coins from location 1 were from a dry, packaged food import and export store, those from location 2 were from a store handling fresh assorted foods for cooking (Yong Tau Foo), while those from location 3 were from a store handling fresh poultry.

The notes of choice were the official $2 Singapore notes. All notes were obtained from the same wet market as the coins. Notes from location 1 were obtained from the same dry, packaged food import and export store as coins, those from location 2 were from the same store handling fresh assorted foods for cooking (Yong Tau Foo), while those from location 3 were from the same store handling fresh poultry.

2.2 Swabbing of handphone and cash note surfaces

The swab of choice was a sterile cotton swab. For mobile phones, the regions of swabbing were the upper half of the screen, the lower half of the screen and the phone case. For coins, both front and rear surfaces were swabbed. For cash notes, they were split into 5 segments, as illustrated in the results section. For coins, both front and rear surfaces were entirely swabbed. The swabbing procedure was the same across all surfaces. Each region was swabbed in a vertical fashion 10 times in a downwards direction. Each swabbing motion lasted approximately 1 second.

2.3 Applying disinfectants

Hand sanitizer was chosen for cash notes. Antibacterial wipes and UVC disinfection were chosen for mobile phones. For alcohol based sanitizer, 0.5ml is to be applied to the center of the half-surface, and spread evenly across the entire half-surface. For antibacterial wipes, they are to be wiped in a downwards

vertical fashion 6 times down the length of the half-surface. For UVC disinfection, the object is to be placed in a UVC sterilizer and incubated inside for 3 minutes.

2.4 DNA extraction

24 ml ethanol (96-100%) was added to 18 ml buffer APW1 concentrate, and 42 ml of buffer APW1 was obtained. Solution was mixed well after ethanol was added. 35 ml ethanol (96–100%) was added to 15 ml buffer APW2 concentrate, and 50 ml of buffer APW2 was obtained. Solution was mixed well after adding ethanol. Samples were equilibrated to room temperature (15–25°C). If a precipitate was formed in Buffer ATL or Buffer APL2, it would be dissolved by incubation at 56°C. Buffer AVE was equilibrated to room temperature for elution. The tip of a swab was cut off and placed in a 2 ml microcentrifuge tube. 500 µl Buffer ATL was then added. The tube was placed onto a thermoshaker and incubated at 56°C for 10 min with continuous shaking at 600 rpm. The 2 ml tube was opened and all the liquid was carefully transferred into a fresh 2 ml tube. 40 µl Proteinase K was added and mixed by vortexing for 10 s. The sample was incubated at 56°C for 10 min using a thermoshaker. 200 µl of Buffer APL2 was added to the sample. The cap was closed and mixed using pulse-vortexing for 30 s. In order to ensure efficient pathogen lysis, the sample and Buffer APL2 were mixed thoroughly to yield a homogenous solution. The solution was then incubated at 70°C for 10 min in a water bath. The tube was spun briefly to remove droplets from the inside of the lid. 300 µl ethanol was added to the lysate. The cap was closed, and the solution was thoroughly mixed for 15–30 s using pulse-vortexing. 600 µl of the mixture from step 16 was applied to the QIAamp UCP Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed again, and centrifuged for 1 min at 6000 x g (8000 rpm). The QIAamp Mini spin column was placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded. In order to avoid aerosol formation during centrifugation, each spin column was closed. Step 17 was repeated, and the remaining mixture from step 16 was applied to the QIAamp UCP Mini spin column. The QIAamp UCP Mini spin column was carefully opened and 600 µl Buffer APW1 was added without wetting the rim. The cap was closed and centrifuged for 1 min at 6000 x g (8000 rpm). The QIAamp UCP Mini spin column was placed in a clean 2 ml collection tube (not provided), and the collection tube containing the filtrate was discarded. The QIAamp UCP Mini spin column was carefully opened and 750 µl Buffer APW2 was added without wetting the rim. The cap was then closed and centrifuged for 3 min at full speed (20,000 x g; 14,000 rpm). The QIAamp UCP Mini spin column was placed in a new 2 ml collection tube (not provided) and the old collection tube with the filtrate was discarded. The solution was centrifuged for 1 min at full speed. The QIAamp UCP Mini column was placed into a new 2 ml collection tube. To dry the membrane completely, the lid was closed and incubated for 3 min at 56°C. The QIAamp UCP Mini column was placed in a clean 1.5 ml elution tube and the

collection tube was discarded. 20–100 µl of Buffer AVE was applied to the center of the QIAamp UCP Mini membrane. The lid was closed and incubated for 1 min at room temperature. The elution buffer was equilibrated to room temperature. To elute the DNA, the solution was centrifuged for 1 min at full speed (20,000 x g; 14,000 rpm). Steps 23 and 24 were repeated.

2.5 PCR

The equipment used will be an amplicon PCR. All steps followed are provided in the handbook (pg 6-8). The DNA will first be mixed with Amplicon PCR Forward Primer, Amplicon PCR Reverse Primer, and 2x KAPA HiFi HotStart ReadyMix. The sample will then be sealed and PCR will be conducted in a thermal cycler

2.6 Next Generation Sequencing

Samples collected and prepared will be sent to Illumina's labs, where the samples will be sequenced according to the *16S Metagenomic Sequencing Library Preparation* document made by Illumina for their ISeq system (Illumina, 16s-metagenomic-library-prep-guide).

2.7 Data analysis

The data from the samples collected from NGS will be analysed by using programmes such as Mothur, which are bioinformatic websites and programmes that can process the data from NGS. This will allow for easy identification of the species of bacteria present in the samples.

2.8 Culturing of swabbed bacteria

Multiple sterile agar plates that were used for culturing of bacteria was prepared and labelled accordingly. A sterilized inoculating loop was then used to pick up bacteria from one of the samples, the bacteria was spread on one quarter of the surface of the agar plates from edge to edge. The inoculating loop was then heated over a flame to sterilize it. The quarter with bacteria was spread down one half of the agar plate. Then, after the loop was sterilized, the half of the agar plate with bacteria was spread out over the entire agar plate. This step was repeated until the entire surface was evenly covered. The agar plate was sealed and turned upside down in order to prevent dehydration of the samples. Agar plates were incubated at 37°C for a period of 3 days. Discrete colonies were counted manually using an analog clicker.

3. Results and discussions

3.1 Number of distinct colonies present on agar plates for coins

Three coins per location, from three different locations, as described in the methods, were swabbed and the number of colonies after 3 days of incubation are shown below.

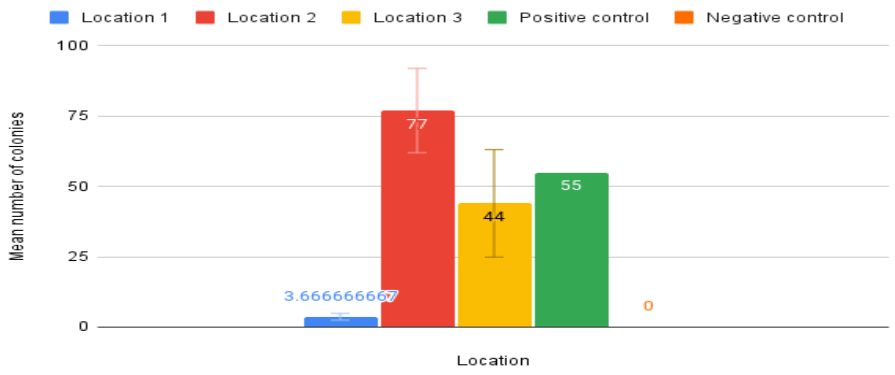


Fig 3.1.1 Graph showing mean number of colonies on agar plates for various locations of coins. Mean number of colonies from location 1 were 3.7, location 2 at 77 and location 3 at 44. 3 samples from 3 different coins were taken per location. For location 2, the second sample was not used in the computation of the mean and standard error as it was an outlier.

As shown in Fig 3.1.1, coins collected from different locations in the wet market all displayed different numbers of colonies ($p < 0.05$) after three days of incubation. Fig. 3.1.2 shows the colony growth from coins collected at different locations. The dry, packaged food import and export store had the lowest colony count. This is likely due to the lack of moisture retention of smooth plastic packaging surfaces. Bacterial attachment to a surface involves 2 phases, the first of which is reversible, occurs rapidly (on the order of ~1 min), and involves hydrodynamic and electrostatic interactions (Tuson et al,. 2013). Location 2, which was a store handling fresh assorted foods for cooking (Yong Tau Foo), had the highest bacterial colony count. This is likely due to these assorted foods being left out in the open for an extended period of time. Bacteria grow most rapidly in the range of temperatures between 40 and 140°F (4 to 60°C), doubling in number in as little as 20 minutes (USDA, Food Safety and Inspection Service, 2020). Furthermore, the shopkeepers from location 2 handled more items than location 1 and 3, thus the rate of bacterial and microbe transmission between surfaces is higher.

A B C

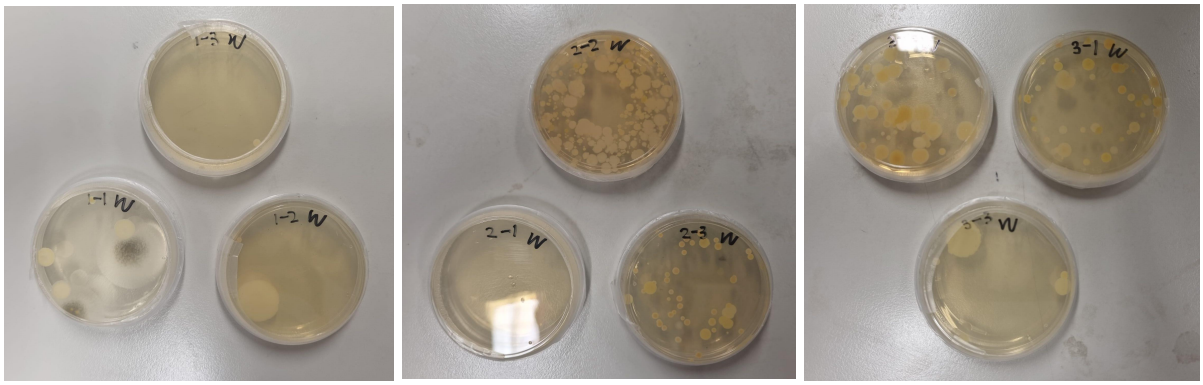


Fig. 3.1.2 Plates showing colony growth from coins collected from A. Location 1 B. Location 2 C. Location 3
Three coins from each location were swabbed with wetted cotton buds. Plates were incubated at 37 degrees C

3.2 Number of distinct colonies present on agar plates for cash notes

One note per location, from three different locations, as described in the methods, were swabbed and the number of colonies after 3 days of incubation are shown below. Notes were split into segments according to Fig 3.2.1B

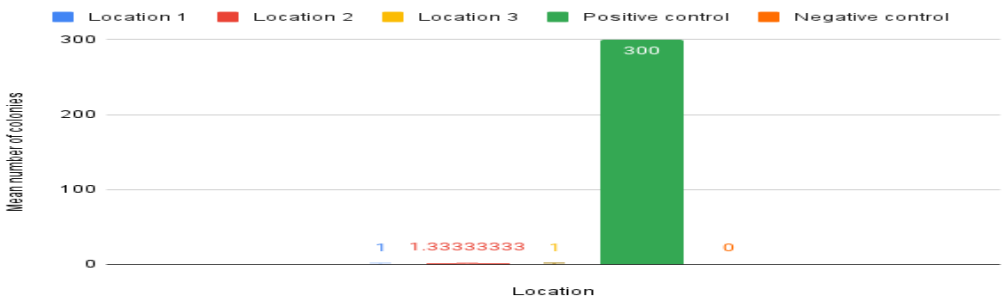
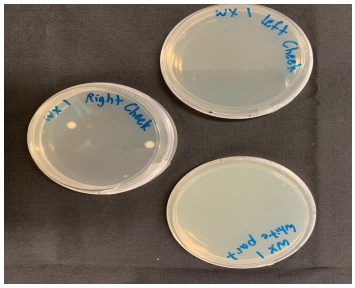


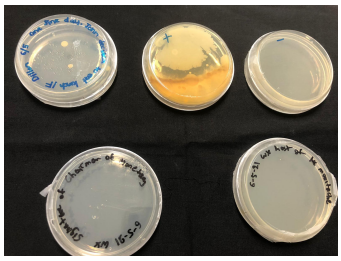
Fig. 3.2.1 Graph showing mean number of colonies on agar plates for various locations of notes. Mean number of colonies for all 3 locations were considerably lower than that of coins and mobile phones.

The mean number of colonies across all three locations were approximately constant, significantly ($p < 0.05$) smaller than the positive control (Fig. 3.2.1). Singapore notes are made of polymer plastics, more specifically, polypropylene, a type of plastic. Plastics are polymers, meaning they have a long repeating chain of molecules. These long chains are exceptionally durable, making them tough to break down and decompose naturally (Immago., 2020). Due to this, microbes were unable to adhere to the cash notes for a period of time long enough, such that culturing results reflected a very low number of microbe colonies. Fig. 3.2.2 shows the colony growth on different areas of the dollar notes.

A B



C



D

Fig. 3.2.2 Plates showing colonies growing from swabs of different areas of $2 notes A. Location 1 B. Location 2. C. Location C D. Different areas of swabbing

3.3 Number of distinct colonies present on agar plates for mobile phones

Samples from three different regions (top half of screen, lower half of screen and phone casing) of the mobile phone were swabbed. three mobile phones with various usage patterns and owner backgrounds were used for swabbing.

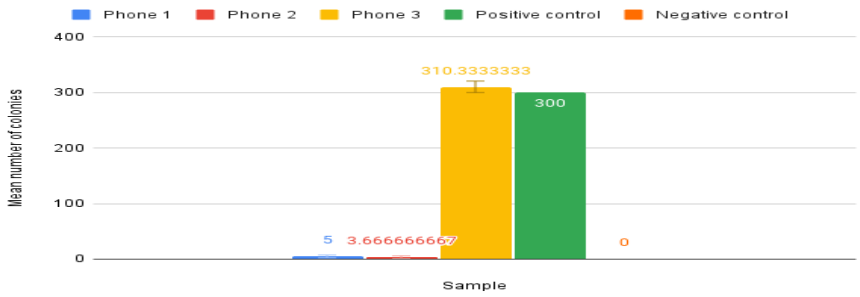


Fig 3.3.1 Graph showing mean number of colonies on agar plates across 3 phone samples. Mean number of colonies from sample 1 were 5, sample 2 at 3.67 and sample 3 at 310. The second and third results for phone number 3 were confluent, and thus set to an arbitrary value of 300 colonies.

As shown in Fig. 3.3.1, phones 1 and 2 had a significantly lower number of colonies than phone 3 ($p < 0.05$), which had a colony count higher than that of the positive control. A possible explanation for this is that phone 3 had a polyester flip phone case while the other 2 phones had a polycarbonate plastic, non-flip phone case. Plastics consist of individual chemical unit monomers of propylene that link together

by forming extremely strong carbon-carbon bonds with each other (livescience.com, 2011). Thus, they are extremely resistant to degradation, and thus few bacteria can multiply on it. On the other hand, contaminated textiles and fabrics are an excellent substrate for bacterial and fungal growth under the appropriate moisture and temperature conditions (Koca et al., 2012), and thus directly supporting results that agar plates swabbed with the microbiome from various surfaces of phone 3 had the greatest number of colonies. Fig. 3.3.2 shows the colony growth on these surfaces.

There is no observable correlation between the region of the mobile phones and the number of colonies that eventually populate agar plates after swabbing. The number of colonies observed across the 3 mobile phones for the same regions ranged from 0 to 331. The standard deviation value for the upper half of the screen is 190.8, that of the lower half of the screen is 168.9, while that for the phone case is 170.3. Considering that the sample size was minute, no meaningful comparisons can be observed between the three regions.

A B C

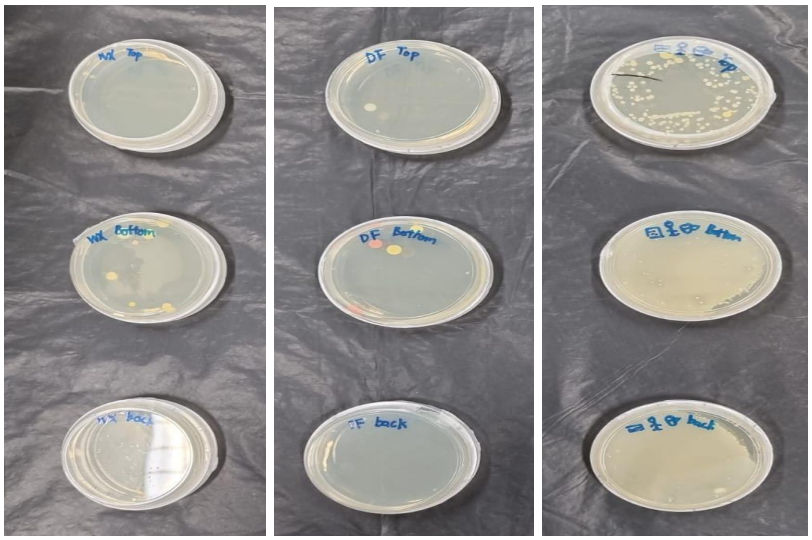


Fig. 3.3.2 Colony growth on three phones (A, B and C representing three different phones swabbed)

3.4 Morphology analysis

The morphology of the above colonies (Fig. 3.1.2, Fig. 3.2.2, Fig. 3.3.2) indicates that there are various microbes growing on each surface. The microbes observed on coins are mostly likely bacteria, although further investigations are needed to confirm their identity. Visual examination also showed that there were different types of bacteria growing based on the colony shape and size. This showed that the bacteria picked up from the coins were still viable and able to multiply on normal agar plates (Fig. 3.1.2). Microbes observed on cash notes are most likely fungi (Fig. 3.2.2), due to the colonies being more whitish

than yellowish, and they developed spores after prolonged incubation time. Microbes on mobile phones, especially phone 2 (Fig. 3.3.2B), is especially intriguing, as a combination of both fungi and bacteria colonies can be seen growing on the agar plates. Importantly, there are 3 observable different species of microbes on phone 2, that is, those white, yellow and red.

4. Limitations, Conclusion and Future Work

4.1 Limitations

Our first limitation was that many of the necessary chemicals for DNA extraction as well as PCR were missing, and we had to change much of the protocol, as well as push back some of the steps to wait for the chemicals to arrive. Due to this, only steps 2.1, 2.2, 2.4, 2.5 and 2.8 were performed, while only results from steps 2.1, 2.2 and 2.8 were included. Our second limitation was the lack of time. Due to the resurgence of COVID-19 cases in Singapore, there was a shortage of time, and we were unable to carry out NGS and testing with various antimicrobial disinfectants. Therefore, only the results from bacterial colony culturing were viable, and were substituted as our evidence to deduce how dirty each surface was. Our third limitation was our lack of knowledge on the PCR protocol. When we were extracting DNA, we were not aware of how much DNA was required for PCR, so we prepared an insufficient amount and conducted PCR with it, as a result, the PCR results were very faint and some were either not visible or just not present, leading to inconclusive results (Appendix 1).

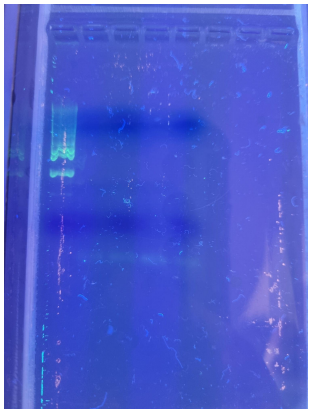
4.2 Conclusions and Future Work

Official Singaporean notes were cleaner than expected, with an average of approximately 1 observable colony across samples from notes. The samples from the notes consisted mostly of fungal colonies, rather than the expected bacterial colonies. Samples from coins and phones were dirtier than notes, with coins having an average of approximately 42 observable colonies across samples, and phones having the highest colony count with an average of approximately 106 observable colonies across samples. However, phones 1 and 2 had an average of approximately 4 observable colonies across samples, while phone 3 had an average of approximately 310 observable colonies across samples. This is likely due to the usage of a plastic polycarbonate phone casing in phones 1 and 2 rather than a fabric case such as in phone 3. Regardless, this procedure only shows the number of living or viable bacteria and fungi on each surface, therefore, there might be even more unknown bacteria and fungi that are dead and unable to be cultured. We plan to return to the lab to resume our experiments, and conduct NGS in order to discover the entire range of bacteria and fungi on the surfaces. We will introduce the previously mentioned disinfectants into the experiment, and use NGS and bacterial culturing to determine the effectiveness of the disinfectants.

Appendix

PCR results

Due to a lack of optimization of PCR, results were not definitive as most bands were too faint to be detected, and some had not appeared as expected. PCR protocol may need to be optimised or more DNA needs to be loaded for future work.



Appendix 1. Gel electrophoresis photo of PCR product

Samples were obtained from swabs taken from notes; 35 cycles of PCR were run. Lane 1: 1kb DNA ladder Lane 2-5: different samples from notes

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