Microbial Contamination according to the Numbers of Mask Worn in the Community

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Due to COVID-19 pandemic, wearing face masks is obligatory to prevent respiratory virus transmissions in the community. However, there are few studies of the desirable number of wearing a face mask, and how to store them for reuse. Therefore, in this study, a survey was conducted among 208 healthy adults, and 27 kf-94 masks worn for 1, 2, and 3 days were collected. To estimate the risk of bacterial contamination, we analyzed the extent of bacterial contamination of the BHI medium and 16S rRNA gene sequencing. With an increase in the number of days of using the mask, the degree of bacterial contamination of the used mask gradually increased. As a result of 16S rRNA PCR performed for strain identification, *Staphylococcus*, known as a pathogenic bacterium, was identified the most. In conclusion, we found that wearing a cotton KF mask provides an optimal environment for microbes, which are related to the skin and respiratory system, to thrive. Therefore, it is also important to reduce the risk of bacterial infection of the face mask with appropriate sterilization methods.

Key Words: Face masks, COVID-19, Microbiome contamination, Bacterial 16S rRNA sequencing

Coronavirus disease-2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has become a global health and social issue (Hu et al., 2021). Owing to this threatening respiratory disease, the World Health Organization (WHO, 2020) recommended that healthy people wear masks to control the spread of COVID-19 (Betsch et al., 2020). A meta-analysis concluded that the use of facial masks consistently prevented respiratory droplet transmission and reduced the risk of virus spread (Liang et al., 2020). Microorganisms from human saliva and exhaled breath can contaminate the masks. To date, no study has reported the effects of masks stored inappropriately or worn for long durations (Delanghe et al., 2021). Human saliva contains 100 million bacteria per milliliter,
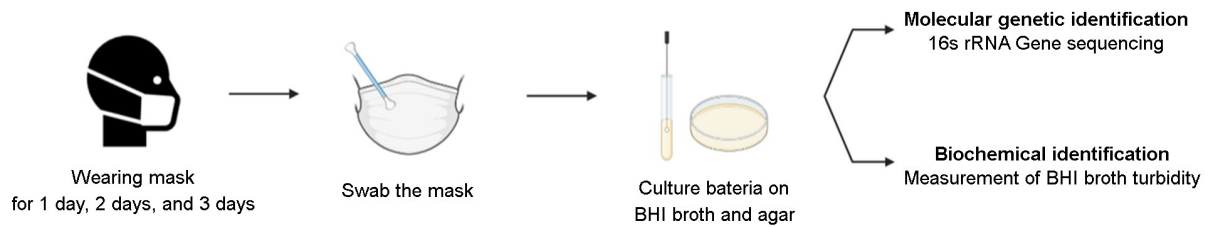
Fig. 1. Schematic representation of the experimental methodology of this study. This image was created with BioRender (https://biorender.com)

including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Klebsiella pneumoniae*, and *Veillonella* spp. (Curtis et al., 2011; Ogawa et al., 2012). In addition, cotton, the main material of the general face mask, can be an optimal environment for microorganisms to grow due to its moisture retention characteristics. Therefore, the reuse of a cotton-based mask or moisture retention from saliva can lead to a risk of the spread of respiratory viruses from the mask (MacIntyre et al., 2015).

In this study, we conducted a survey on how people use face masks, which has become an obligation due to the COVID-19 pandemic. We analyzed the biohazard caused by the inappropriate use of the same mask over several days by isolating microorganisms. Microbial species were identified by molecular genetic identification.

We surveyed 208 people, and the questions were as follows: how often they used a mask; how long they wore the mask, and how was the mask stored? Subsequently, 27 sheets of KF-94 masks worn for 1, 2, or 3 days were collected from healthy volunteers aged 20~24. Specimens were collected by swabbing inside the masks (Fig. 1). The samples were respectively cultured in Brain Heart Infusion (BHI) (KisanBio, Seoul, Korea) broth and agar, a nutrient-rich growth media. The samples were incubated overnight at 37℃ in a bacterial incubator. After primary culture, the shape and color of the colonies were observed. We measured the absorbance of the BHI broth at 600 nm to determine the extent of bacterial contamination of the medium.

We then cultured a single colony on nutrient agar (KisanBio, Seoul, Korea) to separate the aggregates of bacterial colonies and performed 16S rRNA sequencing to identify the bacterial species grown on the agar. Bacterial genomic DNA (gDNA) was extracted using 5% Chelex

Resin (Bio-Rad, Hercules, CA, USA). After dissolving the bacteria in 500 μL of 5% Chelex Resin and TBE mixture, the mixture was boiled at 90~95℃ for 10 min and cenrifuged at 3,000 ×g for 10 min. The supernatant was stored at -20℃ until use in subsequent assays.

The Ministry of Health and Welfare and the Ministry of Food and Drug Safety of the Republic of Korea approved face masks divided into four grades: KF80, KF94, KF99, and KF-ad, which were newly released during the COVID-19 pandemic (Lee et al., 2021). Of the respondents who participated in this study, 57.2% and 38.9% frequently wore KF-94 and KF-AD masks, respectively (Fig. 2A). Regarding the duration of wearing one mask, 44.2% wore it for one day; however, more than half of the survey respondents wore the same mask for more than two days (Fig. 2B). Regarding the storage of the mask, 32.7% answered that they used it for only one day and threw it away. However, 31.7% and 21.6% of participants responded that they hung it on the wall or placed it on a table, respectively (Fig. 2C).

We collected masks that were used for one day, a couple of days, and three days. First, to confirm the bacterial load which was considered the mask contaminant, mask swab culture medium turbidity was measured at 600 nm (Hazan et al., 2012). We observed that the OD gradually increased with the number of days of using the same mask (Fig. 2D). 16S rRNA sequencing was performed using 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R primer (5'-TACGYTACTTGTTACGACTT-3') to identify the bacteria that increase mask contamination turbidity. Then, by comparing the sequencing data with known 16S rRNA sequences using GenBank BLAST searches, we observed that *Staphylococcus*, a known pathogenic bacterial species, was most frequently identified (*Staphylococcus epidermidis*
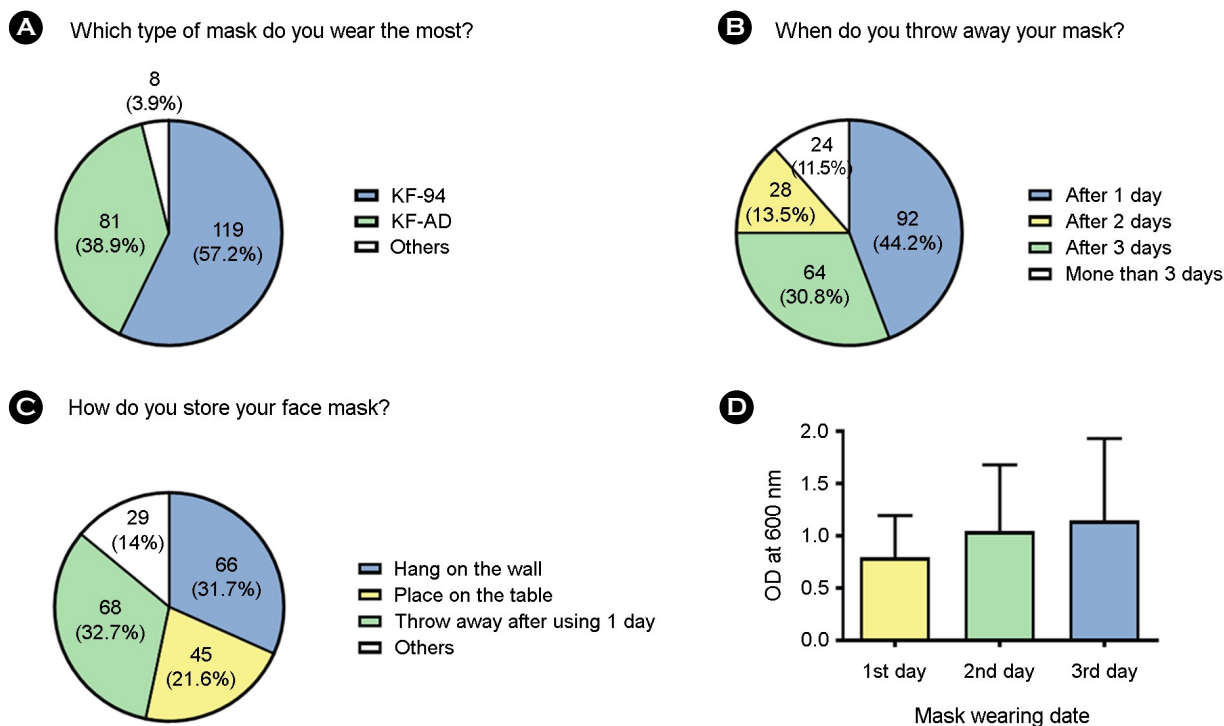
Fig. 2. Results of the survey filled in by 208 people and Bacterial turbidity according to the duration of masks worn. Distribution of answers to the questions (A) "Which type of mask do you wear the most?", (B) "When do you throw your mask away?", and (C) "How do you store your mask?". (D) Bacterial turbidity according to the duration of masks worn.

Table 1. Distribution of strains according to the duration of masks worn

Microorganism	1st day, n	2nd day, n	3rd day, n	Total, n (%)
Staphylococcus epidermidis	10	3	–	13 (21)
Staphylococcus hominis	1	–	1	2 (3.2)
Staphylococcus aureus	7	3	4	14 (22.6)
Staphylococcus capitis	–	1	–	1 (1.6)
Staphylococcus warneri	–	1	–	1 (1.6)
Staphylococcus spp.	1	–	–	1 (1.6)
Cutibacterium acnes	1	–	–	1 (1.6)
Micrococcus luteus	1	–	1	2 (3.2)
Lysinibacillus sp	–	2	–	2 (3.2)
Bacillus velezensis	–	4	3	7 (11.3)
Bacillus subtillis	–	1	–	1 (1.6)
Bacillus amyloliquefaciens	–	5	–	5 (8.1)
Bacillus atrophaeus	–	–	1	1 (1.6)
Bacillus licheniformis	–	–	1	1 (1.6)
Bacillus cereus	–	–	1	1 (1.6)
Bacillus tequilensis	–	–	1	1 (1.6)
Bacillus spp.	6	2	–	8 (13)
Total, n (%)	27 (43.5)	22 (35.5)	13 (21)	62 (100)

(21%) and *Staphylococcus aureus* (14%); Table 1) (Xie et al., 2016).

Many studies have focused on the importance of using masks to prevent the transmission of various respiratory viruses (Liang et al., 2020). However, the accumulation of human saliva-based pathogens in the mask may lead to some biosafety problems (Delanghe et al., 2021). An environment with a warm temperature, adequate moisture, and nutrients is required for the optimal growth of microorganisms. When wearing a mask, water vapor is generated from exhaled air (Lee et al., 2020), which can lead to the growth of micro-organisms; this can cause an increased risk of inflammation and infection by inhalation or transfer to the skin, as well as a disturbance in the resident flora of the skin and nasal passage (Wanke et al., 2013).

In this study, we found that wearing KF masks made of cotton provided an optimal environment for the growth of microorganisms. We confirmed that the number of bacteria increased according to the period of wearing the same mask by measuring the turbidity of mask swab culture media. In particular, *Staphylococcus* spp. and *Bacillus* spp., which are related to the skin and respiratory system, were predominantly detected (Zhiqing et al., 2018). Some *Staphylococcus* spp. are commensal human skin flora maintaining healthy skin. However, *S. epidermidis* and *S. aureus*, the most detected bacteria in worn mask swabs, are pathogenic bacteria causing inflammatory skin diseases (Findley and Grice, 2014). *S. aureus* causes serious infections via various routes, including the respiratory tract. In addition, *S. aureus* becomes resistant to various antibiotics, including Methicillin-resistant *Staphylococcus aureus* (MRSA), by acquiring the *mec*A gene (Parker and Prince, 2012). Some studies have shown that more people die from antibiotic-resistant bacterial infections than from cancer (WHO, 2014).

Overall, not reusing the mask is the best way to protect ourselves from microorganisms. However, the increasing use of cotton masks may lead to environmental pollution, especially if masks are not disposed of appropriately. Disinfection of used masks may be an appropriate strategy to mitigate the risk of bacterial infections and enable the reuse of the masks.

The present study has several limitations. More detailed measurements of media turbidity and analysis of isolated bacteria species, based on the mainly isolated cocci and the number of bacteria grown per mask, would be needed to more accurately show the trend of bacterial growth over days of use. Furthermore, flow-up research on antibiotic resistance tests would provide more detail on the risks of reusing masks.

CONFLICT OF INTEREST

The researcher claims no conflicts of interest.

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