RESEARCH ARTICLE

Multi-drug resistant microbes are resident on nose masks used as preventive protocols for COVID-19 in selected Ghanaian cohort

[v1; peer review: awaiting peer review]

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\textbf{Abstract}

\textbf{Background}: Use of nose masks was an effective and significant part of the World Health Organization (WHO) coronavirus disease (COVID-19) preventive protocols; however, unhygienic practices by the user could lead to microbial contamination of masks. This study profiled bacteria and fungi resident on nose masks and established unhygienic practices; which was not prioritized during the COVID-19 pandemic.

\textbf{Methods}: This was a cross-sectional exploratory study, and questionnaires on unhygienic practices toward mask use were completed by 100 consenting participants from which their nose masks were collected for microbial assessment. The isolated microorganisms were characterized with phenotypic and molecular assays. Data were analyzed with descriptive statistics and presented in graphs.

\textbf{Results}: Overall, 65\% out of the 100 participants reported using a single nose mask for more than a day and 31\% washed the mask to reuse. The bacterial load on the exterior (9.9 \times 10^4 CFU/ml) mask interior (9.1 \times 10^4 CFU/ml) was higher than the threshold outlined by WHO. \textit{Streptococcus}, \textit{Staphylococcus}, \textit{Bacillus}, \textit{Proteus}, \textit{Citrobacter}, \textit{Salmonella}, \textit{Penicillium}, and \textit{Aspergillus} species were isolated from the mask, and about 80-100\% of these isolates were resistant to 18 antimicrobials tested. Uncommon bacterial and fungal isolates, including \textit{Providencia}, \textit{Morganella}, \textit{Edwardsiella}, \textit{Rhodotorula} and \textit{Fusarium} species, were also resident on the masks.

\textbf{Conclusions}: Diverse multidrug-resistant pathobionts resided on the used mask. These microbes can be opportunistic and cause infections.
Nose masks have become a common social accessory; thus strategies are required to ease user discomfort and encourage hygiene practices.

**Keywords**
Multi-drug Resistant, Antibiotic Resistant, Nose Masks, COVID-19, Antimicrobials

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**Introduction**

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a global issue that originated in Wuhan, China, in December 2019. The wide spread of the virus within a short time caused the World Health Organization (WHO) to declare it a pandemic by March 2020 (Cacchiotti & Vanelli, 2020); as a result, different public health control measures were implemented to flatten the epidemic curve and reduce the disease burden. Countries, including Ghana, initiated mandatory nose mask-wearing (Rab et al., 2020). Prior to coronavirus disease (COVID-19), nose masks were commonly used by healthcare personnel, researchers and patients; and have been described as effective in reducing disease transmission (Brooks & Butler, 2021). However, WHO asserts that nose masks are only effective when combined with hand hygiene, proper mask use, and disposal (World Health, 2020). This suggests that nose mask-associated side effects (Gyapong et al., 2022; Rosner, 2020), coupled with unhygienic practices towards mask use, pose a risk of contamination, especially for regular users.

Human saliva and exhaled breath contain bacterial cells and harbor various pathobionts, such as Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans, and Staphylococcus aureus (Delanghe et al., 2021). These opportunistic pathogens have the tendency to accumulate on the exterior and interior surfaces of the nose mask (Monalisa et al., 2017). For example, E. coli, S. aureus, C. albicans, Klebsiella spp., Enterobacter spp., Streptococcus spp., and Staphylococcus have been isolated from nose masks (Luksamijarulukul et al., 2014; Monalisa et al., 2017). The warm and humid environment underneath the mask may favor the accumulation and growth of these pathogens. Although, most of the isolated microbes are normal respiratory tract and skin commensals, some have been associated with inflammatory skin conditions (acne and eczema), in particular S. epidermidis and S. aureus (Zheng et al., 2022) (Findley & Grice, 2014; Khorvash et al., 2012). Unhygienic or user practices such as the prolonged wearing of the mask, improper storage and reusing the mask without proper disinfection have been associated with microbial contamination.

Since azithromycin and doxycycline are used as part of COVID-19 treatment therapy in Ghana, there is a tendency for these opportunistic pathogens to develop resistance. Metagenomic analysis of nose masks (healthy and chronic pulmonary diseased individuals) showed antimicrobial resistance (AMR) genes, including mecA, tetM and ermB (Kennedy et al., 2018); as such, respiratory droplets associated with nose mask usage could facilitate the spread of AMR genes. Subsequently, overuse, and continuous and improper use of these antibiotics could increase AMR. For instance, Streptococcus, Bacillus, and Staphylococcus species isolated from used nose masks of healthy volunteers were resistant to erythromycin and ampicillin (Delanghe et al., 2021). This is a public health concern, especially when there is limited information on the contamination of nose masks in community settings. Also, there is a paucity of data on the resistance of fungi and bacteria resident on nose masks. As a result, the study aims to profile microbes resident on nose masks in the COVID-19 era. The specific objectives include:

1. Isolation and characterization of bacteria and fungi resident on the nose mask samples.
2. Determination of the antimicrobial profiles of identified bacterial and fungal isolates.

**Methods**

**Study design**

This was a cross-sectional exploratory study aimed at identifying unhygienic/user practices and microbial contamination related to nose mask usage.

**Settings**

The study enrolled 100 participants (students) at the University of Ghana between September and December 2021. University of Ghana has over 40,000 registered students and it is situated to the north-east of the city center (Legon) in the Ayawaso West Municipal District in the Greater Accra Region of Ghana (UG, 2023). Participants completed a structured questionnaire on unhygienic/user practices relating to mask use, including the source of the mask, frequency of use, packaging style, reuse by washing and storage practices. The nose masks were collected aseptically in a sterile Falcon tube for microbial analysis. The questionnaire can be found in ‘Supplementary 1’ as Underlying data (Isawumi, 2023).

**Ethics statement**

Ethical clearance was sought and approved on September 12, 2021 by the Ethics Committee for Basic and Applied Sciences (ECBAS 078/20-21). University of Ghana, Legon Accra Ghana, West Africa before sampling and data collection.

**Participants**

Participants for the study included both sexes at the University of Ghana. Participants in good health who regularly used masks were recruited for the study and informed written consent was obtained. Participants were randomly selected and their participation was entirely voluntary. The sample size was determined as previously described (Daniel & Cross, 2013; Pourhoseingholi et al., 2013) using the formula below with known population size.

\[
 n = \frac{N \times X}{(X + N - 1)}
\]

Where

\[
 X = \frac{Z^2 (P)(1-P)}{\text{Error}^2}
\]

[Sample size, n; Population size, N; Sample proportion, P; Z (95% confidence level), Error (E); the margin of error],

\[
 [N=340; Z= 1.96; P= 0.5; N= 340; E= 0.0825].
\]

**Variables**

Potential confounders, particularly individuals suffering from medical conditions such as respiratory infections, were declared ineligible. This is a cross-sectional study and not a case-control...
study as such there were no defined risks, exposures or outcomes.

Bias
While the study did not make conclusions on gender differences, an equal number of male and female participants were recruited to avoid gender bias.

Quantitative variables
All the quantitative variables were distinguished and measured with the appropriate measurement scale. Descriptive statistics were used to summarize the basic features of a dataset through measures of central tendency (mean, mode, and median). Depending on the nature of the variables and their scale of measurement, the appropriate statistical test was applied for further analysis.

Culturing and isolation of microbes
The nose mask was aseptically separated into the interior and exterior parts with a sterile scissor and enriched with 25 ml of LB broth and incubated at 37°C for 24 h with shaking at 225 rpm to dislodge the resident microbes. Then, 10 µl of serially diluted broth culture was inoculated by spreading on Luria‑Bertani, Mannitol Salt, Blood and MacConkey agar for bacterial isolation and incubated (37°C for 18–24 h). The fungi were isolated on Sabouraud Dextrose Agar (SDA, Oxoid – CM0041T) at 29°C for 5–7 days. The bacterial load was determined using a colony counter and expressed in CFU/ml. Morphological characterization (Gram staining and lactophenol cotton blue) and biochemical assays were performed for presumptive microbial identification.

Molecular analysis
The DNA of presumptive bacterial and fungal isolates from the nose masks were extracted using Quick‑DNA Fungal/ Bacterial Kit (cat. no. D6005) as outlined by the manufacturer (Zymo Research). PCR was performed in a final volume of 25 µl; 12.5 µl of One‑Taq® Quick‑Load® 2X Master Mix with Standard Buffer (cat. no. M0486S; New England Biolabs, Inc.), reverse and forward primers (0.5 µl), nuclease‑free water (9.5 µl) and template DNA (2 µl). The 2720 Thermal Cycler (Applied Biosystems, USA, California) was used for the PCR reaction alongside controls. All primer sequences and amplification conditions for the 16S rRNA gene and ITS region are listed in ‘Supplementary 1’ as Underlying data (Isawumi, 2023). Amplified products were resolved on 1.5% agarose gel stained with ethidium bromide and run at 100 V for 45 min. The gel was visualized with Gel Doc™ imager (Amersham Imager 600, Tokyo, Japan). The bacterial PCR amplicons were purified using a QIAquick PCR purification kit before Sanger sequencing (Eurofins Genomics, India, https://eurofinsgenomics.eu/en/custom‑dna‑sequencing/additional‑services/sequencing‑projects/, details of sequencing protocol will be made available under request by Eurofins). The isolates were identified using the NCBI BLAST (RRID:SCR_004870) algorithm.

Antimicrobial susceptibility testing
A total of 16 antibiotics (ceftriaxone (30 µg), doxycycline (30 µg), azithromycin (15 µg), ciprofloxacin (5 µg), cefuroxime (30 µg), levofloxacin (5 µg), amoxiclav (10 µg), gentamicin (10 µg), trimethoprim (23.75 µg), tetracycline (30 µg), penicillin (10 µg), ampicillin (10 µg), cloxacillin (1 µg), amoxicillin (10 µg), erythromycin (15 µg), meropenem (10 µg)) were tested with Kirby–Bauer disk diffusion assay on Sterile Mueller Hinton agar and incubated (18–24 h at 37°C). Fluconazole (10 µg) and itraconazole (10 µg) antifungals, Dettol® (Reckitt Benckiser, Nigeria), So‑Klin® (PT. Sayap Mas Utama, Indonesia) and bleach (Power Zone‑Thick Perfumed Bleach, Tema‑Accra), disinfectants were tested against the fungi isolates using microbroth dilution assay. Briefly, 1:10 (0.1), 1:100 (0.01), and 1:1,000 (0.001) (v/v) of disinfectants were prepared using sterile double distilled water. Then, 100 µl of each concentration and the isolates were transferred into a sterile 96-well microtiter plate alongside controls. The microtiter plate was incubated at 37°C for 16–20 h, and the absorbance was measured using the microplate reader (Varioskan LUX) at 600 nm. Further details on the identification and characterization of the isolates are included in ‘Supplementary 3’ as Underlying information (Isawumi, 2023).

Statistical analysis
The epidemiological data were expressed as percentages. Paired t‑test was used to compare the bacterial load on the interior and exterior sides of the nose mask. The bacterial load was expressed as log CFU/ml and AMR profiles expressed as percentages. Data analysis was performed using SPSS (RRID: SCR_002865) 16.0 and GraphPad (RRID:SCR_000306) 6.0 software. Values were considered significant at P<0.05 (5%).

Results

Unhygienic / user practices associated with mask‑wearing
Assessment of the responses expressed in the questionnaire, indicates 60% of 100 participants obtained their masks from the market (Figure 1A) (Isawumi, 2023). About 35% of the participants reported having bought a mask packaged in rubber (Figure 1B). Also, 65% of the respondents used a single nose mask for more than a day (Figure 1C). Moreover, 31% indicated that they washed to reuse the mask (Figure 1D), citing cost as a major reason. About 44% of the respondents stored the mask in their pockets after use, and 30% in their wardrobes (Figure 1E). These practices could expose the mask to microbial contamination.

High bacterial load on the nose masks
The exterior and interior of the nose mask had 4.0–5.0 log CFU/ml bacterial load relative to the unused mask controls with little or no bacterial growth (Figure 2A and Figure 2B). All the five COVID‑19 patient masks had a high bacterial load, with 5.0 log CFU/ml as highest for the exterior as the least 4.34 log CFU/ml for interior (Figure 2B). Overall, the bacterial loads were higher on the exterior as compared to the interior of the masks (‘Supplementary 1’ as Underlying data (Isawumi, 2023)).

Diverse bacteria and fungi are resident on nose masks
Based on the colony morphology, 454 isolates were obtained from the 200 samples analyzed, with an average of two isolates
per sample; 56% were Gram-negative, and 44% were Gram-positive. Phenotypic and 16S rRNA gene-based Sanger sequencing identified Streptococcus, Staphylococcus, Bacillus, Citrobacter, Proteus, Pseudomonas, Klebsiella, E. coli, Shigella, Salmonella and Campylobacter species on the exterior and interior of the nose masks. However, Providencia, Morganella, and Edwardsiella species were particular to the exterior. Streptococcus spp. was the most common genus on the mask (Figure 3A). E. coli was identified on the exterior of an unused new mask, which was insignificant compared to those on the used masks. The interior of the COVID-19 patient mask had species of Streptococcus, Staphylococcus and Bacillus, while the exterior Bacillus, Staphylococcus and E. coli. The fungi were identified based on the colony morphology, spore and hypha formation microscopically (X100) using lactophenol cotton blue staining (‘Supplementary 2’ as Underlying data (Isawumi, 2023)). Most of the fungal isolates on the interior and exterior were Penicillium spp. (52% and 46%) and Aspergillus spp. (33% and 54%), respectively (Figure 3B). Also, Fusarium (5%) and Rhodotorula (10%) species, which are rare fungal isolates, were particular to the interior. However, there was no fungal growth on the controls or the COVID-19 nose mask.
Nose masks have been reported to be effective in reducing COVID-19 infection (Brooks & Butler, 2021); however, unhygienic practices associated with mask usage represent a major public health concern. In this study, about 60% of the participants claimed to use a single mask for more than a day. This might be attributed to the high cost of nose masks during the COVID-19 pandemic, and the repeated use of a single mask posed an increased risk of infection to the user (Kisielinski et al., 2021). Despite the fact that people wash their nose masks, this does not reduce the microbial load on the mask (Delanghe et al., 2021). In addition, nose mask storage practices could expose the mask to potential pathogens and might increase the microbial load (Monalisa et al., 2017).

Similar to earlier studies (Luksamijarulkul et al., 2014; Monalisa et al., 2017; Park et al., 2022), the bacterial count on the exterior surface of the mask was higher than the interior. This might support the evidence that nose masks filter potential pathogens from inhaled air (Luksamijarulkul et al., 2014). Also, it could be attributed to improper mask usage, such as the regular removal of the mask to place it on open and contaminated surfaces. The increased bacterial count is linked to the higher temperature beneath the mask, which might interfere with the nasal and skin microbiome (Delanghe et al., 2021; Gyapong et al., 2022). For instance, S. aureus, which is part of the skin microbiome, has been associated with acne development due to its high accumulation on the mask (Foo et al., 2006; Han et al., 2020; Nowicka & Grywalska, 2019).

In addition to the bacterial load, some of the identified bacteria resident on the interior and exterior surfaces of the mask, *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Klebsiella*, and *E. coli* species, have been previously reported (Delanghe et al., 2021; Monalisa et al., 2017). However, *Providencia*, *Morganella* and *Edwardsiella* species were particular to this study. All the fungal isolates (*Aspergillus*, *Penicillium* *Fusarium* and *Rhodotorula* spp.) identified were similar to studies conducted in Asia and Europe as established opportunistic pathogens (Kisielinski et al., 2021; Park et al., 2022). These isolates may cause life-threatening infections due to their virulence and ability to adapt to different environmental conditions (Han et al., 2020; Park et al., 2022).

*Staphylococcus* and *Bacillus* species isolated in this study are associated with nosocomial and community-associated skin infections (Otto, 2010). Studies have reported *Streptococcus*, *Staphylococcus*, and *Bacillus* species from masks in Belgium,
with a prevalence rate comparable to this present study (Delanghe et al., 2021). Members of the genus Proteus, Pseudomonas, Citrobacter, E. coli, and Klebsiella are high-risk pathogens linked with diverse human infections (urinary tract, dermatitis, bloodstream, cystic fibrosis, septic arthritis, meningitis and ophthalmic infections) (Liu et al., 2018; Monalisa et al., 2017; Schaffer & Pearson, 2015). Salmonella, Shigella and Campylobacter species were also isolated in this study; these strains are notable pathogens associated with diarrheal, shigellosis, stomach cramps and typhoid fever (Lake, 2017; Silva et al., 2014). Species of Providencia, Morganella and Edwardsiella, which are uncommon isolated bacterial strains, although relatively low in this study, have been implicated in septicemia, gastroenteritis, and cellulitis (Cho et al., 2010; Liu et al., 2016; Spencer et al., 2008).

Data from this study indicated that the nose mask harbors community and hospital-associated opportunistic fungi, and there

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**Figure 4.** Percentage resistance of isolated bacteria and fungi to tested antibiotics/antifungals. (A) Exterior and interior for bacteria. (B) Fluconazole and Itraconazole for fungi. The error bar represents the percentage resistance of the strain to antibiotics/antifungals.

**Figure 5.** Resistance profiles of fungal isolates to disinfectants. Error bars showed percentage resistance to the tested disinfectants.
is a probability of community-hospital transmission and vice-versa (Nicolle et al., 2011). Aspergillus and Penicillium spp., represent about 80% of the fungi isolated and have been attributed to invasive fungal infections, especially in immunocompromised hosts with approximately two million cases annually (Brown et al., 2012; Obar, 2020). For instance, A. fumigatus, the most common fungal pathogen in this study, causes life-threatening infections, such as invasive pulmonary aspergillosis (Mousavi et al., 2016; Patterson & Strek, 2010) and P. chrysogenum has been implicated in endophthalmitis (Walsh et al., 2004). Also, there is a gradual rise in the number of rare human fungal pathogens, such as Fusarium and Rhodotorula species, as reported in this study. For example, Fusarium solani, a plant pathogen, has been linked to sinusitis infections (Egbuta et al., 2017; Jain et al., 2011) and Rhodotorula mucilaginosa to endophthalmitis in immunocompromised and immunocompetent patients (Saha et al., 2014; Wirth & Goldani, 2012). Fungal pathogens cause secondary infections and could increase disease severity, as observed during the second wave of the COVID-19 pandemic in Pakistan and India (Ghazi et al., 2021).

This study showed a high prevalence of resistant bacteria from the interior and exterior parts of the mask. Similar to previous studies, the Gram-negative and Gram-positive bacteria displayed resistance within 80–100% to the tested 16 conventional antibiotics (Delanghe et al., 2021; Nightingale et al., 2022). The strains are multi-drug resistant to at least two of the eight classes of antibiotics tested. Levels of resistance reported in this study in resistant Enterobacteriaceae, were similar to previous studies (Iredell et al., 2016; Nightingale et al., 2022; Singh et al., 2020). More than 80% of the isolated E. coli, Proteus, Salmonella, Citrobacter, Klebsiella, and Shigella species were resistant to conventional antibiotics. Fungal resistance is not as prevalent as bacterial resistance; however, the economic impacts associated with fungal infections remain relatively high, probably due to the limited number of antifungal drugs (Pai et al., 2018; Srinivasan et al., 2014). All the isolated fungal species in this study showed between 60–100% levels of resistance to fluconazole and itraconazole. A similar resistance pattern has been reported elsewhere, accounting for therapeutic failure (Cowen et al., 2014; Denning, 2022). Resistance of Aspergillus sp. to azole antifungals such as itraconazole and fluconazole has been linked to changes in the drug target and efflux pump mechanism (Arastehfar et al., 2020). Also, the increased prevalence of fungal resistance suggests that individuals with immunosuppression may require long-term antifungal combination therapy (Pai et al., 2018).

Disinfectants are commonly used in homes, hospitals and research institutions to reduce microbial load; and, they are usually recommended during outbreaks like COVID-19 to prevent disease transmission (Isawumi et al., 2021). The excessive use of disinfectants during the COVID-19 pandemic has been linked to the development of drug resistance (Chen et al., 2021). In this study, So-Klin® and Dettol® were effective against fungi isolates at the user recommended concentrations. Additionally, the fungal isolates could not survive in the recommended concentration of Bleach. Chloroxyleneol and halo-phenol, which are present in Dettol®, denature proteins, modify the cell wall permeability and result in the leakage of cell components (Isawumi et al., 2021). It is known to be effective against fungus and bacteria (Poger & Mark, 2019); hence it is not surprising that the fungal isolates were 100% susceptible to Dettol® at the appropriate user concentration. The continuous use of these disinfectants, especially for washing nose masks, has been linked to reduced filtration and potentially degraded masks (Dewey et al., 2022). This may defile the purpose of mask usage and expose the user to COVID-19 infection.

Conclusions
Unhygienic or user practices such as using a single mask frequently and touching the mask at regular intervals could lead to contamination of the hands and mask, further spreading infections. The interior and exterior of the mask harbor potential pathobionts that are highly resistant to antimicrobials. Since nose masks have become a common social accessory, shorter breaks for prolonged mask use and discouraging the repeated use of a single mask should be prioritized. Also, appropriate antimicrobial stewardship should be encouraged to minimize the overuse of antimicrobials to avoid therapeutic failure.

Data availability
Underlying data

This project contains the following underlying data:
- Raw Data on Microbes Resident on Nose Masks-1.rar [raw data generated on the microbial profiling of nose masks in selected Ghanaian cohort, including images of agar plates/gel and spreadsheet data]
- Supplementary 1.docx [bacterial loads, primer sequences, PCR conditions and structured questionnaire for participants]
- Supplementary 2.docx [fungal isolates characterization (culturing, staining and ITS) and 16S bacterial profiling]
- Supplementary 3.docx [detailed isolation, identification and characterization experimental approaches and methods]

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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