AETIOLOGIC AGENT OF ACUTE UPPER RESPIRATORY INFECTION IN HUMAN INFLUENZA AND SARS-CoV-2 NEGATIVE CASES IN TANZANIA

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By

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A Dissertation Submitted in (Partial) Fulfilment of the Requirements for the Degree of Master of Science (Epidemiology and Laboratory Management) of

Muhimbili University of Health and Allied Sciences
October, 2024

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled; **‘‘Aetiologic agent of acute upper respiratory infection in Human Influenza and SARS-CoV-2 negative cases in Tanzania’’**, in (partial) fulfilment of the requirements for the degree of Master of Science (Epidemiology and Laboratory Management) of Muhimbili University of Health and Allied Sciences.

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Prof. Agricola Joachim

(Supervisor)

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Date

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Ms. Loveness Urio

(Co- Supervisor)

_____

Date

DECLARATION AND COPYRIGHT

I, **Peter Torokaa**, declare that this **dissertation** is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

Signature……..... **Date**…….……

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DEDICATION

This work is dedicated to all my family members; mother, my late father, wife and my sons, brother and sisters for their continuous love, prayer, support, and encouragement.

ABSTRACT

Background: Viral aetiologies are among the most frequent causes of respiratory disease in both children and adults worldwide. The annual epidemics causes estimated 290,000 to 650,000 deaths and 3 to 5 million cases of serious respiratory illness globally. There are several respiratory pathogens that cause acute respiratory infections (ARIs), however adequate data on aetiologic agents are lacking.

Objective: To determine aetiological agent of ARI among patient tested negative for human influenza and SARS-CoV-2 in Tanzania.

Method: This was a retrospective cross-sectional study carried out using archived nasopharyngeal swab samples of patients confirmed of SARS-CoV-2 and Human Influenza virus tested negative. The study was conducted at National Public Health Laboratory (NPHL), samples were collected from 27 sentinel site from October 2023 to March 2024. Total nucleic acid (RNA and DNA) were extracted using Qiagen-QIA/80204 All-Prep DNA/RNA Mini Kit. The profiling of genomes of pathogen was quantified using Illumina Miseq reagents kit and DNA libraries were sequenced using the Illumina sequencer.

Results: A total of 172 patient samples were include in this study of which 89% (153/172) were positive with at least one pathogen detected. A high proportion 84.9% (146/172) of samples had bacteria pathogen, 18.0% (31/172) viral pathogen and 18.6% (32/172) had fungi. A total of 406 of respiratory pathogen were detected. *S. pneumoniae* was the predominant bacterial pathogen 78.2% (115/147) detected while Human rhinovirus 38.7% (12/31) was the prevalent virus. Individuals aged between 18 and 64 had 3.1 times the odds of having ARI caused by single pathogen (aOR 3.1, 95%CI: 1.1–8.8) compared to patients aged ≥65 years while the odds of having ARI due multiple pathogens for the same age group (aOR 6.1, 95%CI: 2.3–16.3) were 6.1 times higher than those aged ≥ 65 years..

Conclusion: The study found a high proportion of other pathogens causing ARI. Particularly *S. pneumoniae* were the predominant cause with Human rhinovirus emerged as the prevalent viral cause of ARI. Age played a significant role in susceptibility to respiratory infections.

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LIST OF ABBREVIATIONS

AdV	Adenovirus
aOR	Adjusted odds ratio
APHL	Association of Public Health Laboratory
ARI	Acute Respiratory Infection
CDC	Centre for Disease Control and Prevention
cDNA	Complementary DNA
CI	Confidence interval
COPD	Chronic Obstructive Pulmonary Disease
cOR	Crude odds ratio
CoV	Coronavirus
COVID-19	Corona Virus Disease
DNA	Deoxyribonucleic Acid
dsDNA	Double stranded DNA
HCoV-NL63	Human coronavirus NL63
HCoV-OC43	Human coronavirus OC43
HIV	Human immunodeficiency virus
HKU1	Human coronavirus HKU1
hMPV	Human metapneumovirus
HRV	Human rhinovirus
HS	High Sensitive

IFV	Influenza viruses
ILI	Influenza Like Illness
IRB	Institutional Review Board
MNH	Muhimbili National Hospital
nCoV	Novel coronavirus
NGS	Next Generation Sequencing
NPHL	National Public Health Laboratory
PCR	Polymerase Chain Reaction
PIV	Parainfluenza virus
QC	Quality control
qPCR	quantitative PCR
RNA	Ribonucleic Acid
RPIP	Respiratory Pathogen ID/AMR Enrichment Panel
RSV	Respiratory syncytial virus
RTI	Respiratory Tract Infection
RT-PCR	Real Time Polymerase Chain Reaction
RV	Rhinoviruses
SARI	Severe Acute Respiratory Illness
SRA	Sequence reads archive
VAP	Ventilator-associated pneumonia

DEFINITION OF TERMS

Quality control	These are materials prepared to check if the technique produces the intended results.
Sequencing	The procedure of identifying the nucleotide bases in a fragment of DNA's sequence.
Acute respiratory infection	In this study means the detection of other aetiological agents that cause acute respiratory infection apart from Influenza A&B virus and SARS-CoV-2

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Viral etiologies are among the most common causes of respiratory disease in both children and adults (1). There have been cases of respiratory illnesses that can be severely caused by coronaviruses and influenza viruses (1). Acute respiratory infections (ARIs) are the most leading cause of morbidity and mortality both in children and adults globally (2). The annual epidemics causes estimated 290,000 to 650,000 deaths and 3 to 5 million cases of serious respiratory illness globally (3,4). Viral and bacterial agents can both cause ARIs, but viruses like human respiratory syncytial virus (RSV), influenza, coronavirus (CoV), human metapneumovirus (hMPV), parainfluenza virus (PIV), adenovirus (AdV), human rhinovirus (HRV) are the most frequent reported culprits (2). Respiratory diseases usually involve the organisms that are spread by droplets formed during coughing and sneezing. Uninfected persons usually contract these pathogens during inhalation. Some people might get infected by touching their mouth or nose after contact with fomites (5–7). The respiratory symptoms of these diseases, such as coughing, coryza, fever, and shortness of breath. (8).

It has been reported that younger age groups are more susceptible than middle-aged adults to ARIs. Studies have shown that individuals at high risk of developing serious illness includes older age 65 years and more, young children and pregnant women (4,9,10).

Although up to 80% of ARI cases have been reported to be a viral infection, the majority of causative agents are still unknown (11). There have been reports of co-infections with other viral and bacterial agents that show clinical manifestations similar to other severe acute respiratory illnesses (SARI) during the COVID-19 pandemic dominance era and its genetic variation (8,12–16). There are several other respiratory pathogens that can cause ARIs, however adequate epidemiological data is lacking (11). Additionally, among the prevalent viruses linked to respiratory tract infections, include HCoV-HKU1, which was initially discovered in 2005 in a Hong Kong adult with chronic pulmonary disease, and HCoV-NL63, which was discovered in

a Dutch infant with bronchiolitis in 2004 (17,18). The prevalence and epidemiology of influenza have been mostly studied in developed countries, however untreated co-morbidities, malnutrition, and other factors may lead to a distinct aetiology in less-resourced situations, such as Africa.

In line with the implementation of International Health Regulation (IHR) core capacities, Tanzania had initiated the influenza prevention and preparedness and control activities in late 2000s. In doing so, the country has been implementing influenza sentinel surveillance since the year 2008 through the existing twenty-seven (27) sentinel sites (Figure 1). Since the onset of COVID 19 pandemic, the ongoing influenza surveillance in the country was expanded to include the non-influenza viruses which are COVID in early 2021(Figure 1).The sentinel sites are being supported to identify patients using standard case definition for ARI, collect samples and transported them to the National Influenza Centre in Dar es Salaam for testing. The expansion involved review of case definition to include the category of "ARI, laboratory testing of COVID 19 using combo kits and also testing of other non-influenza viruses.

Since December 2022, there has been an increasing number of cases of ARI, with most of patients sample tested negative for Human Influenza and SARS CoV-2. Notably, in a study done at Muhimbili National Hospital (MNH) revealed an increase of respiratory illness during the COVID-19 pandemic (19). The available routine diagnostic methods/assays in the surveillance system in Tanzania allow the detection of Influenza virus A&B and SARS CoV-2 virus by using Real Time Polymerase Chain Reaction (RT-PCR), however the challenge of this technique is that, it miss the other causes of ARI. Since the routine available laboratory test is limited to the number of pathogens that can be detected, there is a need to expand testing in order to detect other aetiological cause of ARI especially when patient with symptom and sign of ARI, samples test negative Next Generation Sequencing (NGS) and multiplex PCR are important diagnostic platforms in detecting pathogens causing ARI. This study was therefore, deploy NGS to determine the various cause of ARI pathogens. The importance of looking for other aetiological causes of ARI cannot be emphasized.

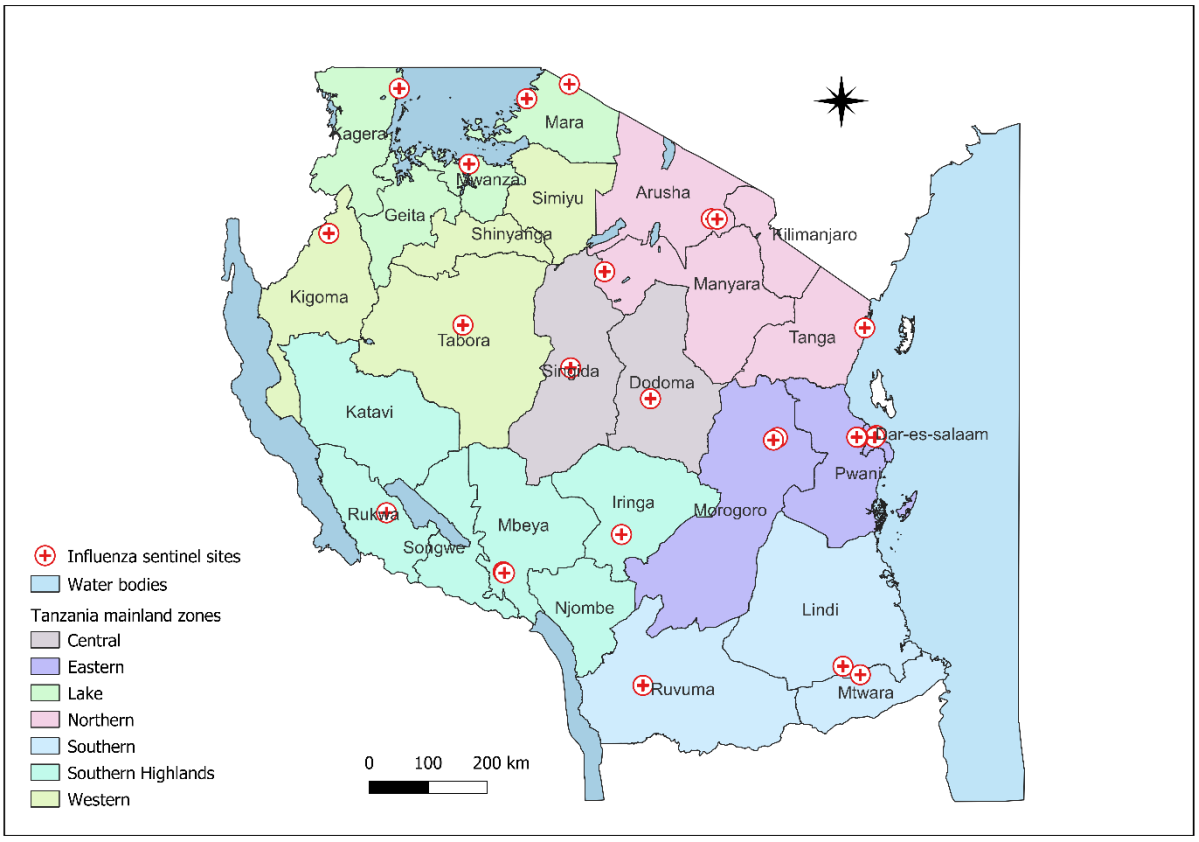


Figure 1: Map showing distribution of influenza sentinel sites in each zone

1.2 Problem Statement

Viruses are the most common cause of acute respiratory infections in many parts of the world (1). However, making a diagnosis of a respiratory viral infection based on the symptoms is difficult since most of these infections have overlapping symptoms. A recent study done at MNH, showed that the COVID-19 pandemic has caused a rise of respiratory illnesses that lead to an increase of morbidity and mortality since 2020 nevertheless, the aetiological agents were not studied (19).

Recently, there have been an increase in a number of patients in our setting presenting with symptoms and sign of SARS COV-2 and /or ARI. However, on testing patient's samples turn out negative for the two-illness making difficult to manage the patients as the causes are not known. The available testing algorithm allow for testing only two viruses; SARS-CoV-2 and

Influenza virus A&B only making it difficult to know the causative agent of the illness when sample test negative for the two viruses. This has caused more fear and unnecessary use of antibiotics increasing the risk of antimicrobial resistance. There is need to know the causative agents of these illnesses in order to guide patient management and to strategize prevention and control measures in Tanzania.

1.3 Conceptual Framework

Demographic characteristics including age, gender, and geographical location, clinical characteristics such as underlying medical conditions and clinical presentation or characteristic of ARI, climatic conditions such as changes of seasons are among the indirect variables linked to other ARI etiological causes. The climatic changes characteristics will determine the different strains, variants, and variations of the viruses in geographical locations. The associated factors of ARI other than human influenza, SARS-CoV-2 will be determined (Figure 2).

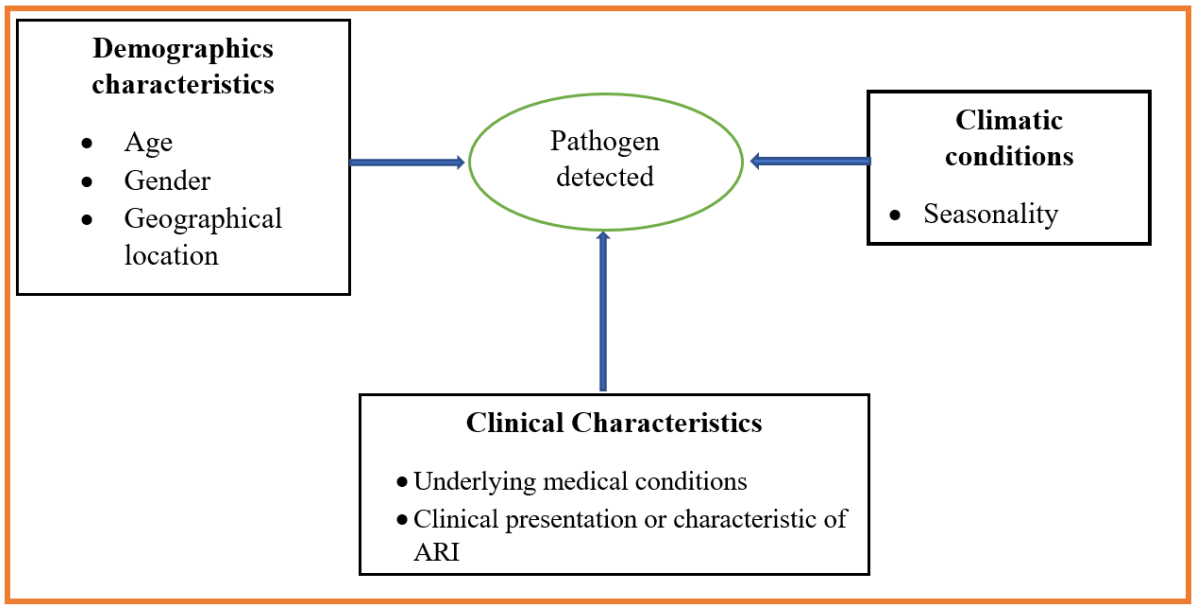


Figure 2: Conceptual framework on factors associated ARI other than human influenza and SARS-CoV-2.

1.4 Rationale

The findings from this study identified the other aetiological agents of ARI. Information about these other aetiological agents of ARI will be useful in improving diagnostic strategies and development of testing algorithm i.e. including next generation sequencing or multiplex PCR to include testing all possible causes of ARI in Tanzania.

1.5 Research Questions

1.5.1 Broad Research Question

What are aetiological agents of upper ARI and associated factors among patient tested negative for human influenza and SARS-CoV-2 in Tanzania year 2023/2024?

1.6 Objectives

1.6.1 Broad objective

To determine aetiological agents of upper ARI and associated factors among patient who tested negative for human influenza and SARS-CoV-2 in Tanzania year 2023/2024

1.6.2 Specific objectives

1. To determine aetiological agents of upper ARI among patient who tested negative for human influenza and SARS-CoV-2 in Tanzania 2023/2024.
2. To determine the associated factors of upper ARI among patient who tested negative for human influenza and SARS-CoV-2 in Tanzania 2023/2024.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aetiological cause of ARI among patient who tested negative for human influenza and SARS-CoV-2

Particularly in young children under five, ARIs are a leading cause of morbidity and death globally (22). The World Health Organization (WHO) estimates that ARIs account for about 6% of all disease burden globally (23). Pneumonia is the most common and severe type of ARI, which account for roughly 2.5 million deaths in 2019 and account for the majority of the disease burden in older adults (>65 years) (24,25). Around 20% of all fatalities in children under the age of five years worldwide occurred in 2000 alone as a result of ARIs and the majority of these deaths nearly 70% happened in Sub-Saharan Africa and the southern parts of Asia (26). Parainfluenza virus is an important cause of acute respiratory illness (ARI) in children, especially in the Middle East (27). Respiratory syncytial virus (RSV) is one of the main pathogens responsible for ARIs, causing over 41,000 deaths in children aged 0 to 60 months in 2016 (28). Several studies shows that in Negative Human Influenza cases, the other causes like RVs, RSVs, PIVs, AdVs, and IFVs are the primary viral agents associated with RTIs (14,29). According to previous studies, ARI appears to be the leading cause of death for children in developed countries, where it is estimated that more than 15 million children under the age of five pass died annually (30). PIV was found in children hospitalized with ARI at 7.0% in the study conducted in Jordan, although PIV-3 was the most common serotype (57% in ARIs) and the primary cause of mortality and disability globally, particularly in young children under the age of five (27).

Study done in Indonesia reported that 57.6% of patients had additional SARI aetiologies, including influenza virus cases in 12.1% of patients, systemic infections in 28.8% of cases, respiratory infections in 16.7% of cases, and systemic infections in 16.7% of cases (11). According to a study conducted in north India, influenza A (H3N2) and rhinovirus were the most often reported non-SARS CoV-2 respiratory viruses during the COVID-19 pandemic,

while hMPV and influenza A (H1N1) remained the most frequently reported respiratory viruses during the pre-pandemic period in North India. This finding suggests that non-SARS CoV-2 circulated differently throughout the epidemic (31).

2.2 The associated factors of ARI among patient who tested negative for human influenza and SARS-CoV-2.

2.2.1 Age

Around the world, ARIs are a frequent cause of illness and death in children (33). Several studies have shown that younger age groups are more susceptible than middle-aged adults to SARIs. Adults of 65 years of age and older, young children, pregnant women are all at risk for developing serious illnesses (4,20,34). A study in Indonesia reported that individuals hospitalized with fever between the ages of ≤5 years and above the ages of ≥60 years had an overall higher rate of SARI cases (52.7% and 48.6%, respectively) than patients between the ages group of 5 to ≤18, and 18 to <60 (25.5% and 22.3%, respectively) (21).

Additionally, a study done in Mexico reveals similar results: the age of 0 to 9 years had the upmost percentage of positive cases (62.5%), while the group of those 60 years and older was the second-highest percentage (20.5%) (12). Furthermore, study done in Niger among children without influenza viruses reveals an overall of 86% children, tested positive for one or more pathogens (bacterial or viral). In 78% of samples, at least one respiratory virus was found, and in 64% of samples, at least one bacteria (35). Both studies found other etiological factors for ARI/SARI beyond human influenza and noted the most typical viral combination.

2.2.2 Gender

According to a Guatemalan study, men are nearly twice as likely as women under the age of 18 to develop ARI (36). According to the Mexican study, 47.1% of patients who tested positive for at minimum one virus were female and 52.9% were male, making up 35.8% of the total number of patients. Similar findings resulted from a study conducted in Tanzania in 2022, where males and young kids of less than five years made up the majority of SARI cases in the country (12,17). Furthermore, males had a higher median age at death (35 years) than females (28 years) of

respiratory diseases (37). For various causes of ARI, it is important to determine whether the findings from several studies remain relevant in our community.

2.2.3 Geographical location

Based on data from the WHO, developing nations bear a disproportionately heavy burden of ARIs, accounting for more than 90% of all cases worldwide (34). According to a Tanzanian research, the likelihood of hospitalization (SARI vs. ILI) differed depending on the patient's gender, age, geographical region, period of diagnosis, and organism detected (18). Based to a study conducted in Tanzania, the Eastern, Central, and Western regions of the country had more cases of SARI among male children under the age of five in 2017 compared to what they experienced in 2018–2019. In general, compared to other zones in the country, the Northern zone (27.4% and 36.7%) and the Eastern zone (31.4% and 25.8%) had higher proportions of SARI and ILI cases, respectively (18). However, the study done in Mexico revealed different distribution of ARI in their study (12). Determine the geographic distribution of other ARI causes across the country related with the changing of seasons in the context of the disparity in distribution observed in various studies.

2.2.4 Underlying medical conditions

According to reports, the severity of a disease infection is associated with the existence of co-morbidities. Diabetes, cardiovascular disease, hypertension, cancer, oxidative stress, vitamin deficiency, and renal disease are among the most prevalent co-morbidities, as is the case with SARS-CoV-2 illness (35). The systematic review study done in India and the study done in Indonesia show that among ARI and SARI individuals, one or more co-morbidities have increased the severity of the disease (11,36). In participants with respiratory illness diagnoses (54.1%) versus those with non-respiratory disease diagnoses (22.1%), these were more prevalent (11). The study done in Mexico shows that, compared to individuals with co-infections, clinical symptoms and comorbidities was higher in ARIs positive for a single virus (12). Co-morbid illnesses were a factor in 9.1% of all respiratory disease-related deaths in Tanzania and the common is the immunodeficiency. HIV accounted for 60.4% of co-morbid conditions, making it the most prevalent (33).

2.2.5 Seasonality

The seasonal cycle of respiratory viral infections has long been understood since common cold and influenza epidemics annually attack individuals in the winter in temperate countries (37). The study in Mexico found that the period of high and low positives varied considerably in terms of the general behaviour of the non-influenza respiratory viruses in cases of acute respiratory illness during the study period (12). According to a study conducted in Tanzania, the majority of samples were sent in for testing from December to April, which also happened to be the country's rainy season. Several viral diseases, including RSV, hMPV, influenza A and B, and PIV, all showed seasonal change (18).

Despite this use small sample size, the study conducted in Niger reveals that RV and HBV are other commonly detected throughout the raining period compared to RV and PIV through the warm period (October to December) in cases of SARI in children who do not have influenza (13). Studying the seasonality of respiratory viruses can help allocate scarce resources more effectively toward disease control and prevention at the most advantageous times of the year (38).

2.2.6 Clinical presentation or characteristic of ARI

A huge number of ARIs are triggered by viral infections, but specific viruses that cause them have largely remained unknown due to challenges like the wide range of potential etiological agents, an overlap of symptoms and emergence of new strain of viruses (12,39). ARI is most common observed to children under five years (40). The study conducted in Northwest Ethiopia found that 37.5% had respiratory problems and the three most prevalent respiratory symptoms were wheezing (9.72%), phlegm (10.48%), and runny nose (25.63%). Chest tightness (3.03%) was the least common respiratory symptom reported (41).

Without a clear diagnosis, there is a tendency to prescribe antibiotics as a precautionary measure. This practice is not only often ineffective against viral infections but also contributes to the growing problem of antimicrobial resistance (AMR).

This challenge have caused overuse of antibiotic due to unknown causative agent increasing the risk of emerging. Addressing these issues by identifying the causative agents of ARI can help improve patient care, reduce unnecessary antibiotic use, and combat the threat of AMR. This is essential for ensuring better health outcomes and maintaining the effectiveness of antibiotics for future generations.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Design

This was a retrospective cross-sectional study using archived nasopharyngeal swab samples of patients confirmed of COVID or flu according to case definition (42,43) but both tested negative by PCR. Samples are regularly collected for routine national surveillance of human influenza virus type A and B and SARS-CoV-2 across the country and submitted to National Public Health Laboratory (NPHL) at Dar es Salaam for testing. The study used the remnant of human nasopharyngeal swab samples which were stored for further use. In this study we used a combination of retrospective samples and prospective samples to determine the other aetiologies. The retrospective samples from October to December 2023 were obtained from archived specimens at NPHL. Prospective samples consisted of routine remnant samples from January to March 2024.

3.2 Study Population and Study Area

The study involved archived and routine samples collected from patients confirmed SARS-CoV-2 and the Human Influenza virus tested negative for both tests. The study was conducted at NPHL which receives samples from all the twenty-seven sentinel sites including; seven zones which are Western zone, Eastern zone, Central zone, Northern zone, Southern zone, Lake Zone and Southern Highlands zone in Tanzania.

3.3 Sample size determination

The sample size was calculated using single proportion formula (Kish and Leslie formula) for cross-sectional studies. With the analyses we considered reporting 95% confidence intervals (corresponding z-score=1.96), and we conservatively assumed that samples yield at least 30% of outcomes of interest i.e., the prevalence of either virus, bacteria or fungi. The maximum error accepted (Margin of error) was set at 5 and assuming 5% of the sequenced samples will be indeterminate results) the final sample size calculated was 484. The sample size was calculated from the formula below:

$$S = \frac{Z^2 x\ P(1-P)}{e^2}$$

Where:

S=Sample size

P=Prevalence

e=Standard error

Due to the available funding and resources a total of 172 samples were tested.

3.3.1 Sampling Technique

A stratified sampling was used to select the samples. Three levels: retrospective/prospective, zones, and time of sample collection i.e., month of collection, was considered in selecting the samples. Of the samples selected, 50% were archived samples, and 50% were prospectively sampled. From each zone, samples were allocated proportional to size for data collected monthly.

3.4 Variables

The dependent variable in the study were detection of the other aetiological causes of respiratory infection. The independent variables were the demographic characteristics such as; age, and gender. For clinical characteristics such as; underlying medical conditions and the clinical presentation or characteristic of ARI, and climatic conditions such as seasonality.

3.5 Inclusion and Exclusion Criteria

3.5.1 Inclusion criteria

1. Samples of subjects who met the WHO case definition for ILI/ARI collected from sentinel sites in Tanzania for surveillance purposes (44).
2. Archived nasopharyngeal samples tested negative for Influenza A&B and SARS-CoV-2 using Polymerase Chain Reaction (PCR).

3.5.1.1 Standard case definitions

Influenza like Illness

An acute respiratory illness with fever (temperature of ≥ 38 °C) and cough, with onset within the past 10 days (42).

SARS-CoV-2

Acute onset of fever AND cough (ILI) OR acute onset of ANY THREE OR MORE of the following signs or symptoms: fever, cough, general weakness/fatigue, headache, myalgia, sore throat, coryza, dyspnoea, nausea/diarrhoea/anorexia OR Epidemiological criteria contact of a probable or confirmed case, or linked to a COVID-19 cluster (43).

SARI

An acute respiratory infection with: history of fever or measured temperature of ≥ 38 C°; and cough; with onset within the last 10 days; and requires hospitalization(42).

3.5.2 Exclusion criteria

1. All samples with incomplete information about the variable of interest were excluded from this study.

3.6 Data collection technique

Data were extracted from case investigation and laboratory request forms. The laboratory request form contained facility information, patients' demographic data (patient ID, name, date of birth, sex, case classification, and test requested), details on specimen collection, and result. The case investigation form contained clinical data (i.e., patient particular, signs/symptoms and underlying medical conditions, vaccination status, risk factors, and case classification).

3.7 Laboratory Procedures

3.7.1 Sample Collection

The archived samples stored at -80˚C were identified for negative status of influenza types and B and SARSCoV-2 and available volume in the viral transport media (VTM). Prospective samples were shipped at 2-8^{o}C to NPHL through expedited mail service (EMS) within 96 hrs after collection and stored at -80°C. All samples were kept at -80˚C until testing was performed.

3.7.2 Nucleic acid extraction

Total nucleic acid, including RNA and bacterial DNA, were extracted from nasopharyngeal swab specimens using Qiagen-QIA/80204 All-Prep DNA/RNA Mini Kit (37,45) (Germany) as per the manufacturer's instructions. Briefly, sample of 200ul were lysed and homogenized in buffer, which immediately inactivates DNases and RNases as well as proteases to ensure isolation of intact DNA, RNA, and proteins. The lysate is then passed through an All-Prep DNA spin column. This column, in combination with the high-salt buffer, allows selective and efficient binding of genomic DNA. The column is washed and pure, ready-to-use DNA is then eluted. . Ethanol was added to the flow-through from the All-Prep DNA spin column to provide appropriate binding conditions for RNA, and the sample was then applied to an RNeasy spin column, where total RNA binds to the membrane and contaminants were efficiently washed away. High-quality RNA was then eluted in RNase-free water. Buffer APP, a novel aqueous protein precipitation solution, was added to the flow-through of the RNeasy spin column, and the precipitated proteins were pelleted by centrifugation. Intact total proteins were redissolved in an appropriate buffer and then ready to use in downstream applications.

3.7.3 Library preparation

The library preparation was prepared using the Illumina Respiratory Pathogen ID/AMR (RPIP) assay for library preparation, tagmentation with enrichment kit pairs with an index kit and enrichment panel to generate enriched libraries for dual-indexed, pared-end sequencing. Reverse transcription converts RNA into complementary DNA (cDNA), which is then tagmented and amplified to add indexes and other adapters. The resulting libraries were normalized for three-plex enrichment and further amplification. Sequence-specific biotinylated probes combined with magnetic beads to capture regions of interest. The captured sequences were washed, eluted, and amplified to generate copies of the enriched library. A limited-cycle PCR program exponentially amplifies the enriched fragments, copying each fragment to increase the amount of library.

3.7.4 Sequencing

The profiling of genomes and nucleic acids were quantified using a Qubit 3.0 fluorometer with dsDNA high-sensitivity (HS) reagents. Additionally, cDNA synthesis, tagmentation, and library preparation were conducted using the Respiratory Pathogen ID/AMR Enrichment (RPIP) assay. Lastly, the genomic DNA libraries were sequenced using the Illumina MiSeq system (Illumina Inc., San Diego, CA, United States) (46). The kit is capable of sequencing targeted 280 respiratory pathogens. For viruses includes: Adenovirus, human corona viruses (Coronavirus 229E, HKU1, NL63, OC43), Cytomegalovirus, Enterovirus D68, Influenza A virus (H1N1, H3N2, avian), Influenza B virus, Metapneumovirus, Parainfluenza virus 1-4, Respiratory syncytial virus A + B, Rhinovirus A, B, C and SARS-CoV-2). The list of respiratory bacteria includes; *Bordetella pertussis, Chlamydia pneumoniae, Coxiella burnetii, Enterobacter cloacae* complex, *Francisella tularensis, Klebsiella pneumoniae, Legionella pneumophila, Mycobacterium tuberculosis, Nocardia farcinica, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae.* The fungi that are detected by the kit includes*; Aspergillus fumigatus, Candida auris, Coccidioides immitis, Fusarium solani, Histoplasma capsulatum, Mucor racemosus, Paracoccidioides brasiliensis, Pneumocystis jirovecii, Rhizopus oryzae, Sporothrix schenckii* and *Talaromyces marneffei* (47). The kit is capable for comprehensive whole genome sequencing of SARS-CoV-2 and Influenza A/B viruses which enables detection of variants and lineages.

3.7.5 Quality control

Sample quality before extraction process was assessed by checking the proper sample storage and those meet the accepted and rejection criteria including sample transportation temperature of 2-8^{o}C. Sample quality for library preparation after DNA/RNA extraction were assessed by checking the quality and quantity of the starting biological samples (e.g., DNA, RNA) using fluorometry (Qubit) to ensure sufficient and intact genetic material. Library Preparation QC a verification of the efficiency and accuracy of the library preparation steps using quantitative PCR (qPCR) were used to determine the library concentration and validate the adapter ligation. Negative Control Library included a negative control library prepared without template DNA

(e.g., water or buffer only) to identify any potential contamination or artefacts during library preparation process.

3.8 Investigation tools, Validity and Reliability issues

FastQC tools was used to assess the quality of raw sequences, Phred Score >30 was used as minimum score for downstream analysis. Fastp was used to filter out low quality sequenced bases and adapters sequences.

3.9 Pre-testing/piloting of tools

Pre-testing of the testing protocol was done to validate the quality of the library preparation process and DNA/RNA extraction process before the starting testing of samples.

3.10 Bioinformatics analysis

Sequence reads were checked for quality using Fastqc then trimming of low-quality reads (Qscore less than 30) and sequencing adapters were trimmed using Fastp (48). The reads were then mapped to Human genome for removing host reads using Sequence reads archive- (SRA) human scrubber (https://github.com/ncbi/sra-human-scrubber) thereafter the dehosted reads were used for downstream analysis. De novo assembly was then performed using Megahit (49,50) software and the resulting fasta files were then processed using Kraken2 (51) for Taxonomic profiling, later kraken output was parsed using Stata software.

3.11 Data analysis and Data management

Epidemiological data were extracted from Ministry of Health database and laboratory information system at NPHL. Data were cleaned using Microsoft excel. The Stata version 15.1 was used for data analysis. Descriptive analysis was performed, and frequency distributions (%) for categorical variables and a measure of central tendency median for age was presented. P-values of < 0.05 was considered significant. Multinomial logistic regression was performed to assess the relationship between independent variables and the aetiological agent of ARI (dependent). Factors with a p-value of < 0.20 in the bivariate analysis was included in the

multivariable model using forward selection. The 95% confidence intervals (CI) was presented, and a significance level of $p < 0.05$ was used.

3.12 Ethical Issues

Ethical clearance was obtained from Muhimbili University of Health and Allied Sciences (MUHAS) with reference no. MUHAS-REC-01-2024-2003. The permission to use the remnant stored and routine sample was obtained from NPHL. The study only involved the use of specimens and data collected through the routine diagnostic procedure of SARS-CoV-2 (standard of care) and sentinel sites ILI/SARI surveillance program, and did not in either way pose risk to subjects or their regular care, therefore consent waiver were granted by the Ethical Review Board at MUHAS. Confidentiality were maintained by the research team as per ethical regulations governing research on human subjects, and no patient identifier were disclosed to an unauthorized person or the public.

CHAPTER FOUR

4.0 RESULTS

4.1 General characteristics of study participants

A total of 172 patient's samples were included in the study. Nearly half of the participants 83/172 (48.3%) were children under the age of five years with a median age of 6 years (interquartile range (IQR: 1-39) years, while 52.3% (90/172) were female. The Eastern Zone had the high number of cases 30.8% (53/172) whereas the month of November 2023 had more cases of 20.9% (36/172). Most common underlying medical conditions were lung disease 10.5% (18/172) and Hypertension 8.7% (15/172). Majority 95.9% (165/172) of patients presented combination of symptoms of fever, cough and running nose (Table 1). Most participants, 90.7% (156/172), experienced cough symptoms, followed by those who had runny noses, 68.0% (117/172) (Figure 3). The most common clinical signs were nasal discharge 45.9% (79/172) (Figure 4).

Table 1: Study participants characteristics among specimens tested from October 2023 to March 2024 (N=172)

Characteristic	Frequency (n)	Proportion (%)
Age groups (years)		
<5	83	48.3
5-17	19	11.1
18-64	60	34.8
≥65	10	5.8
Median 6 years (IQR:1-39)		
Gender		
Female	90	52.3
Male	82	47.7
Geographical location		
Eastern	53	30.8
Northern	35	20.4
Southern highlands	32	18.6
Central	18	10.5
Western	18	10.5
Lake	10	5.8
Southern	6	3.5
Months of sample collection		
October 2023	18	10.5
November 2023	36	20.9
December 2023	34	19.8
January 2024	28	16.3
February 2024	25	14.5
March 2024	31	18.0
Underlying medical conditions		
Yes	52	30.2
No	120	69.8
Types of underlying medical condition		
Lung disease	18	10.5
Heart disease	12	7.0
Hypertension	15	8.7
Infectious disease (HIV)	7	4.1
Combination of symptoms		**Percent**
Fever, Cough and Difficult breathing	163	94.8
Fever, Cough and running nose	165	95.9
Fever, Running nose and headache	158	91.9
Fever, cough and general body weakness	163	94.8

HIV –Human Immunodeficiency Virus

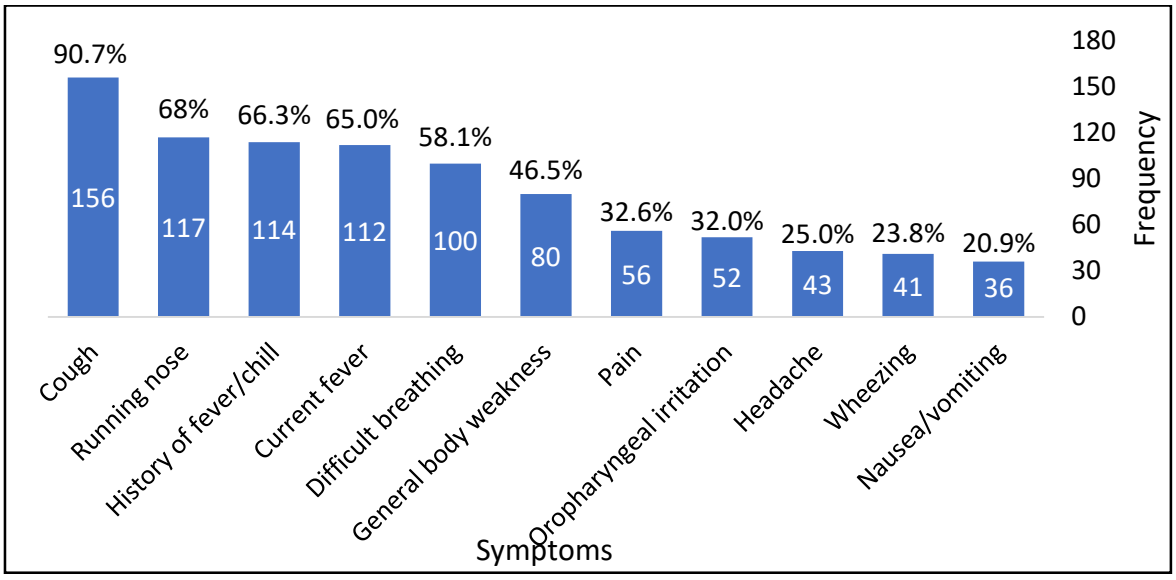


Figure 3: Distribution of Symptoms among the study participants

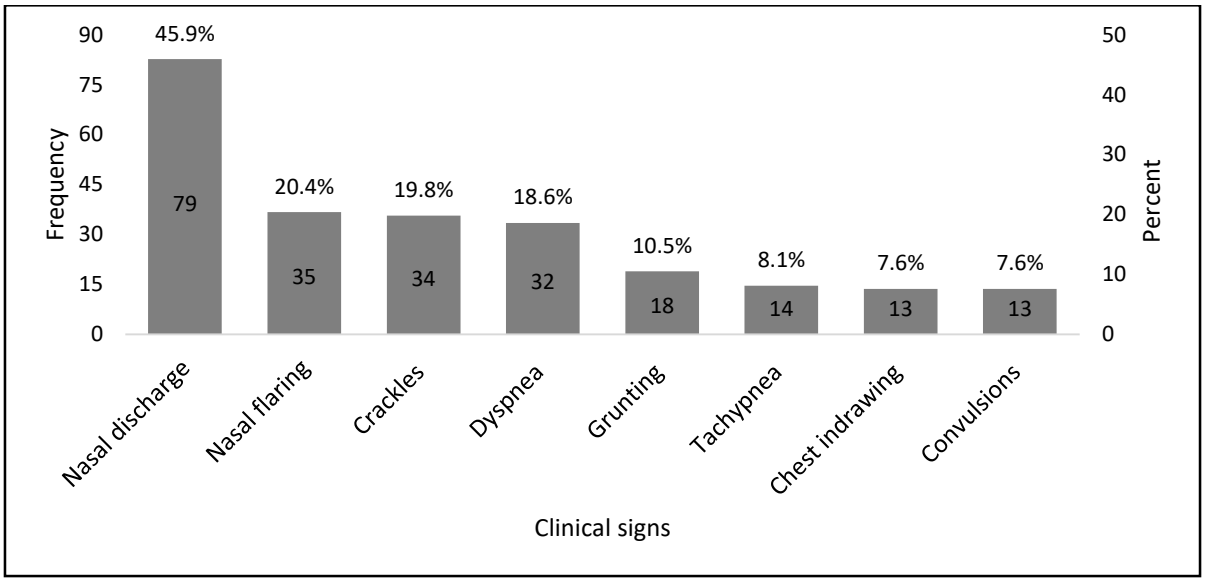


Figure 4: Distribution of clinical signs among study participants

4.2 Frequency of Respiratory pathogens detected among samples tested

Of the total 172 samples included 153 (89%) samples tested positive with at least one pathogen detected while 19 (11%) of samples had no pathogen detected. A total of 406 pathogen were detected in samples tested. Majority of the pathogens detected 83.7% (340/406) were bacteria while 8.4% (34/406) were viruses and 7.9% (32/406) were fungi which was *Aspergillus*

fumigatus (Figure 5). A high proportion of co-infection 39.0% (67/172) were due to bacterial pathogens followed by bacteria-viral co-infection 12.8% (22/172) (Appendix III).

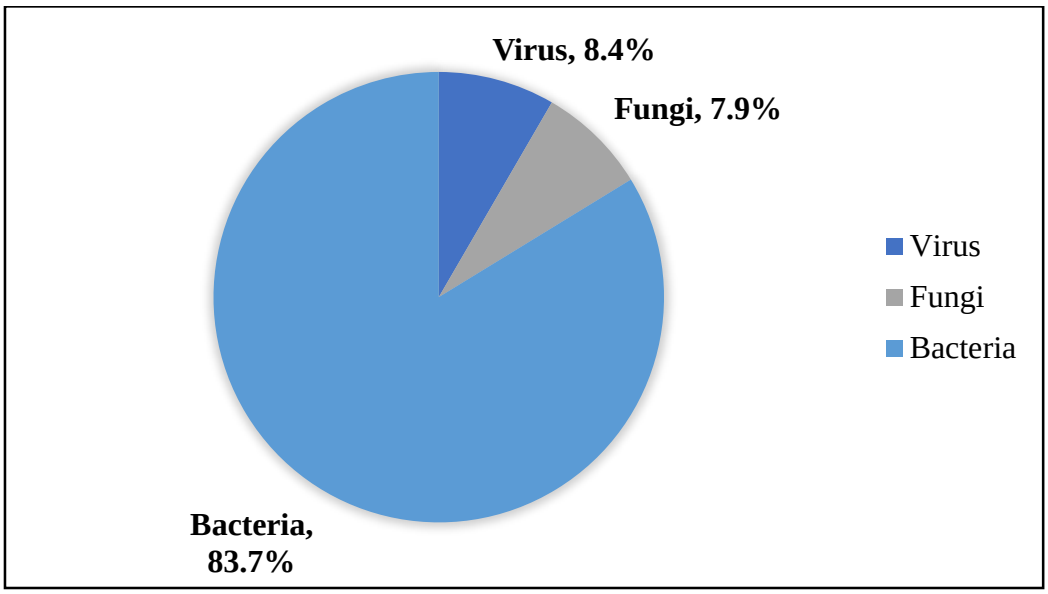


Figure 5: Distribution of respiratory pathogen detected

4.3 Type and frequency of Viral Respiratory pathogens detected by age group

Of 172 samples tested, 18.0% (31/172) had viral pathogens. We detected a total of 34 viruses in our study (where 4/34 (11.8%) were DNA viruses, the adenovirus and other were RNA viruses). Of the detected viruses, human rhinovirus had the highest frequency, 38.7% (12/31), followed by enterovirus, 29.0% (9/31). The last detected virus was parainfluenza virus 3.2% (1/31) and human coronavirus NL-63 3.2% (1/31). The distribution of the virus detected shows that rhinovirus and adenovirus were detected more in individuals aged between 5-17 years, 10.5% and 15.8%, respectively, while respiratory syncytial virus and enterovirus were detected in children under five years at 4.2% and 6.0% respectively. Co-infection was detected in two individuals on 2/31 (6.5%). the co-infection were between (Adenovirus and Rhinovirus) and (Adenovirus, Rhinovirus and Enterovirus). There was no viral pathogens detected in age group ≥65 (Table 2).

Table 2: Frequency of viral respiratory pathogens by age group among participants' samples tested (N=172)

Virus detected		Age group (years) (N=172) n (%)			
	Virus Positivity	**<5 (n=83)**	**5-17 (n=19)**	**18-64 (n=60)**	**≥65 (n=10)**
Negative	141(82.0)	66 (79.5)	14 (73.7)	51 (85.0)	10 (100.0)
Virus detected	31(18.0)	17 (20.5)	5 (26.3)	9 (15.0)	0
Rhinovirus	12 (38.7)	7 (8.4)	2 (10.5)	3 (5.0)	0
Enterovirus	9 (29.0)	5 (6.0)	1 (5.3)	3 (5.0)	0
Adenovirus	4 (12.9)	1 (1.2)	3 (15.8)	0	0
Respiratory syncytial virus	4 (12.9)	4 (4.2)	0	0	0
Human metapneumovirus	3 (9.6)	1 (1.2)	0	2 (3.3)	0
Parainfluenza virus-4	1 (3.2)	1 (1.2)	0	0	0
Human coronavirus NL63	1 (3.2)	0	0	1 (1.7)	0
Co-infection	2 (6.5)	1 (1.2)	1 (5.3)	0	0
Adenovirus + Rhinovirus	1 (3.2)	0	1 (5.3)	0	0
Adenovirus + Rhinovirus + Enterovirus	1 (3.2)	1 (1.2)	0	0	0

4.3.1 The frequencies of respiratory viruses' subspecies detected

The leading subclasses of the virus detected were human rhinovirus A 29.7% (11/37), followed by Respiratory syncytial virus 10.8% (4/37) and Enterovirus D68 10.8% (4/37) and human metapneumovirus 8.1% (3/37) (Figure 6).

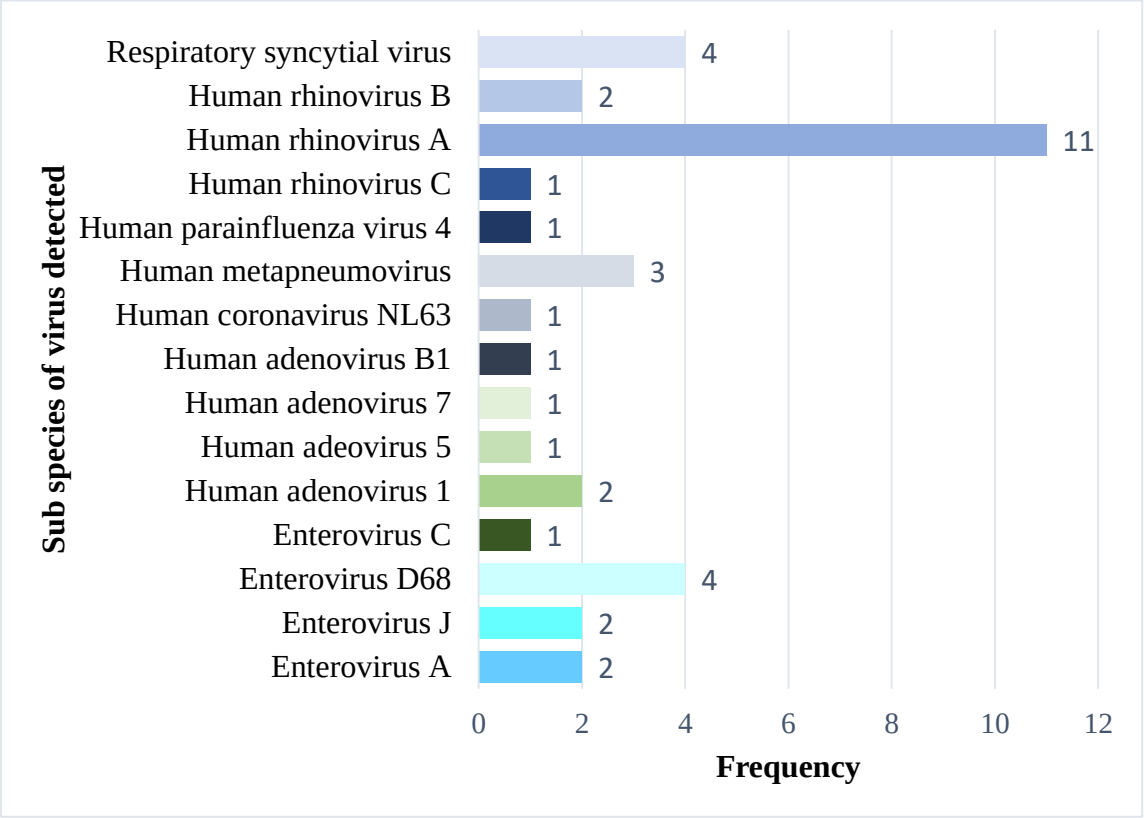


Figure 6: Clustered bar chart of the frequencies of the detected respiratory viruses sub species via next-generation sequencing

4.4 Frequency of Respiratory bacteria pathogen detected, by age group

A total of 85.5% (147/172) samples were detected with bacteria while a total of 340 bacteria were detected in all samples tested. Of the detected bacteria *S. pneumoniae* 78.2% (115/147) had high proportion followed by *Enterobacter cloacae* 51.0% (75/147) and *Staphylococcus aureus* 47.0% (70/147). We found that more children under the age of five years 74.7% (62/83) detected *S. pneumoniae* (Table 3).

Table 3: Frequency of bacteria detected causing ARI, by age group, among specimens tested (N=340)

Bacteria detected		Age group (years) (N=172) n (%)			
	Bacteria positivity	**<5 (n=83)**	**5-17 (n=19)**	**18-64 (n=60)**	**≥65 (n=10)**
Negative	25 (14.5)	11 (13.3)	3 (15.8)	9 (15.0)	2 (20.0)
Bacteria detected	147 (85.5)	72 (86.7)	16 (84.2)	51 (85.0)	8 (80.0)
Streptococcus pneumoniae	115 (78.2)	62 (74.7)	12 (63.2)	37 (61.7)	4 (40.0)
Enterobacter cloacae	75 (51.0)	38 (45.8)	8 (42.1)	23 (38.3)	6 (60.0)
Staphylococcus aureus	70 (47.6)	32 (38.6)	10 (52.6)	23 (38.3)	5 (50.0)
Moraxella catarrhalis	23 (15.6)	15 (18.1)	4 (21.1)	3 (5.0)	1 (10.0)
Klebsiella pneumoniae	29 (19.7)	12 (14.5)	2 (10.5)	11 (18.3)	4 (40.0)
Pseudomonas aeruginosa	25 (17.0)	12 (14.5)	5 (26.3)	7 (11.7)	1 (10.0)
Bordetella pertussis	1 (0.7)	0	1 (5.3)	0	0
Coxiella burnetii	1 (0.7)	0	1 (5.3)	0	0
Nocardia farcinica	1 (0.7)	1 (1.2)	0	0	0

4.4.1 Serotypes distribution of *Streptococcus pneumoniae* carriage

Among 115 detected *S. pneumoniae* strains, the most commonly observed serotypes were serotype 19A, 31.6% (80/253); serotype 14, 21.3% (54/253).We found that 8.7% (22/253) strains were nontypeable (Table 4).

Table 4: Serotype distribution of Streptococcus pneumoniae carriage (N=253)

Serotype	Frequency	%
19A	80	31.6
14	54	21.3
19F	49	19.4
6B	31	12.3
11	16	6.3
3	1	0.4
Non-typeable	22	8.7

4.4.2 Serotype distribution of Streptococcus pneumoniae carriage by age groups

Among of 253 of invasive serotypes detected, most of 19A serotype were detected from children under the age of 5 years followed by 14 serotype and serotype 19F. The least detected serotype was 3 serotype (Figure 7).

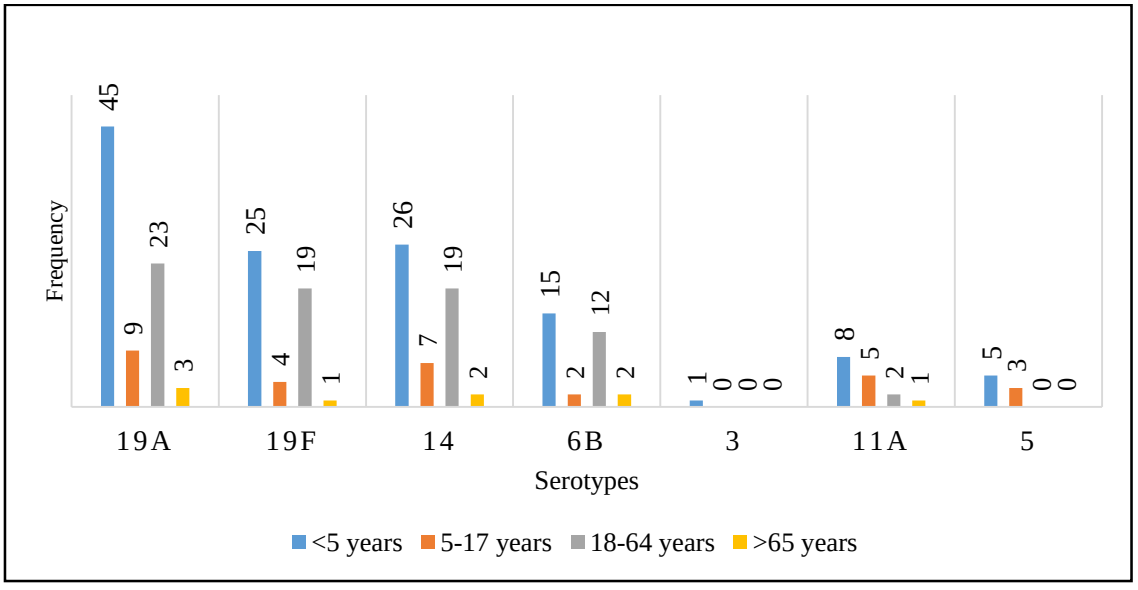


Figure 7: Serotype dis*tribution of Streptococcus pneumoniae carriage by age groups*

4.5 The relationship of detected pathogen and participants characteristics

There was no any statistical relationship of the detected pathogen among gender, age, geographical distribution and period of sample collection (Table 5).

Table 5: Demographics, geographic zone and months of sample collection characteristics and detected pathogens

	Detection categories n (%)			
Variables	**No pathogen n=19 (11.1)**	**Single pathogen n=35 (20.4)**	**Multiple pathogens N=118 (68.6)**	**P-value**
Age groups				
<5	10 (12.1)	15 (18.1)	58 (69.8)	
5-17	2 (10.5)	3 (15.8)	14 (73.7)	
18-64	5 (8.3)	16 (26.7)	39 (65.0)	
≥65	2 (20.0)	1 (10.0)	7 (70.0)	0.746
Gender				
Female	11 (12.2)	20 (22.2)	59 (65.6)	
Male	8 (9.8)	15 (18.3)	59 (72.0)	0.703
Geographical location				
Eastern	2 (3.8)	9 (16.9)	42 (79.3)	
Northern	7 (20.0)	7 (20.0)	21 (60.0)	
Southern highlands	5 (15.6)	6 (18.8)	21 (65.6)	
Central	0	5 (27.8)	13 (72.2)	
Western	2 (11.1)	5 (27.8)	11 61.1)	
Lake	3 (30.0)	2 (20.0)	5 (50.0)	
Southern	0	1 (16.7)	5 (83.3)	0.209
Sample collection period				

	Detection categories n (%)			
Variables	**No pathogen n=19 (11.1)**	**Single pathogen n=35 (20.4)**	**Multiple pathogens N=118 (68.6)**	**P-value**
October 2023	1 (5.6)	3 (16.7)	14 (77.8)	
November 2023	1 (2.8)	5 (13.9)	30 (83.3)	
December 2023	4 (11.8)	11 (32.4)	19 (55.9)	
January 2024	3 (10.7)	4 (14.3)	21 (75.0)	
February 2024	5 (20.0)	7 (28.0)	13 (52.0)	
March 2024	5 (16.1)	5 (16.1)	21 (67.7)	0.201
Medical conditions				
No medical condition	15 (11.5)	28 (21.5)	87 (66.9)	
Cardiovascular disease	1 (6.3)	4 (25.0)	11 (68.8)	
Chronic disease	1 (50.0)	0 (0)	1 (50.0)	
Infectious disease	1 (25.0)	2 (50.0)	1 (25.0)	
Special conditions	0 (0)	0 (0)	5 (100.0)	
Lung disease	1 (5.3)	1(2.9)	13 (11.0)	0.276

4.6 The associated factors of ARI among patient who tested negative for human influenza and SARS-CoV-2

Bivariate analysis were performed for single pathogen, multiple pathogens vs no pathogen detected. Individuals aged between 18 and 64 years had 3.2 times the odds of having ARI caused by single pathogen (cOR 3.2, 95%CI: 1.2-8.7) compared to patients aged ≥65 years while the odds of having ARI due multiple pathogens for the same age group (cOR 7.8 95%CI: 3.1-19.8) were 7.8 times higher than those aged ≥ 65 years. Males had 1.9 times the odds of having ARI caused by single pathogen (cOR 1.9, 95%CI: 0.8-4.4) compared to females though were not statistically significant while the odds of having ARI due to multiple pathogens (cOR 7.4, 95CI: 3.5-15.4) were 7.4 times higher than females.

After adjusting for potential confounding variables for single pathogen, multiple pathogens vs. no pathogen detected factors that were independently associated with ARI were as follows; Individuals aged between 18 and 64 had 3.1 times the odds of having ARI caused by single pathogen (aOR 3.1, 95%CI: 1.1–8.8) compared to patients aged ≥65 years while the odds of having ARI due multiple pathogens for the same age group (aOR 6.1, 95%CI: 2.3–16.3) were 6.1 times higher than those aged ≥ 65 years. Males had 1.1 times the odds of having ARI caused by single pathogen (aOR 1.1 95%CI: 0.4–3.2) compared to females while the odds of having ARI due to multiple pathogens (aOR 1.9, 95%CI: 0.8–4.6) were 1.9 times higher than female though were not statistically significant (Table 6).

Table 6: Bivariate and multivariate, multinomial logistic regression of associated factors for acute respiratory infection

	Detection categories n (%)			**Crude OR(95%CI)**		**Adjusted OR (95%CI)**	
Variables	**No pathogen n=19**	**Single pathogen n=35**	**Multiple pathogens n=118**	**Single pathogen**	**Multiple pathogens**	**Single pathogen**	**Multiple pathogens**
Age groups**							
<5	10 (12.1)	15 (18.1)	58 (69.8)	1.5 (0.7-3.3)	5.8 (2.9-11.3)	1.4 (0.6-3.5)	**4.3 (2.0-9.3)**
5-17	2 (10.5)	3 (15.8)	14 (73.7)	1.5 (0.3-9.0)	7.0 (1.6-30.8)	1.4 (0.2-8.8)	**5.7 (1.3-25.7)**
18-64	5 (8.3)	16 (26.7)	39 (65.0)	3.2 (1.2-8.7)	7.8 (3.1-19.8)	**3.1 (1.1-8.8)**	**6.1 (2.3-16.3)**
≥65	2 (20.0)	1 (10.0)	7 (70.0)	**Ref.**	**Ref**	**Ref**	**Ref**
Gender**							
Female	11 (12.2)	20 (22.2)	59 (65.6)	**Ref.**	**Ref.**	**Ref**	**Ref**
Male	8 (9.8)	15 (18.3)	59 (72.0)	1.9 (0.8-4.4)	7.4 (3.5-15.4)	1.1 (0.4-3.2)	1.9 (0.8-4.6)
Geographical location							
Eastern zone	2 (3.8)	9 (16.9)	42 (79.3)	4.5 (0.9-20.8)	20.9 (5.1-86.7)	0.9 (0.1-7.1)	2.4 (0.4-15.4)
Northern zone	7 (20.0)	7 (20.0)	21 (60.0)	1.0 (0.4-2.9)	2.9 (1.2-7.1)	0.2 (0.1-1.3)	0.3 (0.1-1.4)
Southern highlands	5 (15.6)	6 (18.8)	21 (65.6)	1.2 (0.4-3.9)	4.2 (1.6-11.1)	0.2 (0.1-1.5)	0.4 (0.1-2.1)
Central zone	0	5 (27.8)	13 (72.2)	**Ref.**	**Ref.**	**Ref**	**Ref**
Western zone	2 (11.1)	5 (27.8)	11 61.1)	2.5 (0.5-12.9)	5.5 (1.2-24.8)	0.5 (0.1-6.3)	0.4 (0.1-4.1)
Lake zone	3 (30.0)	2 (20.0)	5 (50.0)	0.7 (0.1-4.0)	1.7(0.4-7.0)	0.2 (0.1-1.6)	0.2 (0.1-1.4)
*Southern zone	0	1 (16.7)	5 (83.3)	-	-	-	-
Sample collection period							
October 2023	1 (5.6)	3 (16.7)	14 (77.8)	3.0 (0.3-28.8)	13.9 (1.8-106.5)	0.9 (0.1-13.6)	0.8 (0.1-9.5)
November 2023	1 (2.8)	5 (13.9)	30 (83.3)	**Ref**	**Ref.**	**Ref**	**Ref**
December 2023	4 (11.8)	11 (32.4)	19 (55.9)	2.8(0.9-8.6)	4.8 (1.6-14.0)	0.9 (0.2-4.4)	0.5 (0.1-2.1)

	Detection categories n (%)			Crude OR(95%CI)		Adjusted OR (95%CI)	
Variables	**No pathogen n=19**	**Single pathogen n=35**	**Multiple pathogens n=118**	**Single pathogen**	**Multiple pathogens**	**Single pathogen**	**Multiple pathogens**
January 2024	3 (10.7)	4 (14.3)	21 (75.0)	1.3 (0.3-5.9)	7.0 (2.1-23.5)	0.4 (0.1-3.0)	0.5 (0.1-3.0)
February 2024	5 (20.0)	7 (28.0)	13 (52.0)	1.4 (0.4-4.4)	2.6 (0.9-7.3)	0.5 (0.1-2.6)	0.2 (0.1-2.6)
March 2024	5 (16.1)	5 (16.1)	21 (67.7)	1.0 (0.3-3.5)	4.2 (1.6-11.1)	0.3 (0.1-1.9)	0.4 (0.1-1.7)
Medical conditions							
No medical condition	15 (11.5)	28 (21.5)	87 (66.9)	**Ref.**	**Ref.**	**Ref**	**Ref**
Cardiovascular disease	1 (6.3)	4 (25.0)	11 (68.8)	4.0 (0.4-35.8)	11.0 (1.4-85.3)	2.2 (0.2-21.5)	3.2 (0.4-27.0)
*Chronic disease	1 (50.0)	0 (0)	1 (50.0)	-	-	-	
Infectious disease	1 (25.0)	2 (50.0)	1 (25.0)	2.0 (0.2-22.1)	1.0 (0.1-16.1)	0.7 (0.1-9.5)	0.2 (0.01-4.3)
*Special conditions	0 (0)	0 (0)	5 (100.0)	-	-		
Lung disease	1 (5.3)	1(2.9)	13 (11.0)	1.0 (0.1-16.0)	13.0 (1.7-99.4)	0.7 (0.04-11.7)	4.4 (0.5-36.7)

*The study participants were few

** Included in multivariate analysis as potential confounder

CHAPTER FIVE

5.0 DISCUSSION

The present study deployed next-generation sequencing to identify the other etiological agents of acute upper respiratory infection among patients with signs and symptoms of influenza-like illness and SARS-CoV-2 tested negative for both tests. The study revealed that a high proportion (89%) of nasopharyngeal samples, negative for human influenza and SARS-CoV-2 tested positive for at least one pathogen as other causes of ARI. The common detected pathogen were bacteria (83.7%), viruses (8.4%) and fungi (7.9%). The most common detected bacteria were *Streptococcus pneumoniae*, while rhinoviruses were the frequent viruses detected. *Aspergillus fumigatus* was the prevalent fungus detected. This study found that the co-infection (39%) was commonly caused by *Streptococcus pneumoniae and Enterobacter cloacae*.

The study found that a high proportion (89%) of patient's samples had at least one pathogen detected as other cause of ARI. The findings were almost similar to the study done in Ifakara which reported 81% of at least one pathogen were detected in the study participants (52). However, low rates have been reported in the studies done in Tanzania (46.9% and 62%) and Kenya (66.6%) respectively (16,27,53). The variations in pathogen detection across different studies can be attributed to factor like sample size and testing methods used.

The current study found that bacterial were the predominant causes (84%) of ARI among all pathogen detected. This is in contrast with findings from other studies done in the country which revealed the common pathogens detected were viruses (16,27). These viruses included Respiratory Syncytial virus, Rhinovirus, Parainfluenza and Adenovirus. The findings suggest that there is different distribution and changing of aetiological agents of ARI periodically.

5.1 Aetiological cause of Acute Respiratory Infection among patient who tested negative for both human influenza and SARS-CoV-2

5.1.1 Respiratory viral pathogen detected

The study found that rhinovirus 38.7%, enterovirus 29.0%, and RSV 12.9% were the commonest viruses detected. Contrary to the studies done in Kenya and Tanzania, rhinovirus,

RSV, and adenovirus were the predominant viruses reported (16,27,53). These findings could be attributed to seasonal patterns or climatic changes and diagnostic testing used (54,55).
Most viral detection was found between 5 to 17 years, 26.3%, and children below five years, 20.5%, compared to adult and elderly patients. Similar findings have been reported in a study done in Mexico, where children aged 0 to 9 years had the highest rates of positive cases, 62.5% (11). Several studies have shown consistent findings that younger age groups are more susceptible than adults and are at high risk of developing serious illnesses (4,9,10). This highlights that children are more susceptible, possibly due to immature immune systems, higher social interactions, and less acquired immunity. Our study was conducted between October and March, when most of the regions were the rainy seasons. This aligns with previous studies suggesting that rainfall season increases exposure to crowded circumstances, increasing the risk of viral pathogen transmission (16,56).

We found almost 79% of rhinovirus detection were from subspecies human rhinovirus A, followed by human rhinovirus B and rhinovirus C similar findings reported by *Ogunbayo et al* (57). Enterovirus D68 subtype were detected in four patients. Other literature shows that the enterovirus D68 was linked with more severe and fatal respiratory illnesses than other enterovirus genotypes (57, 58). In order to contribute to epidemiological investigations, it is crucial to identify the virus strain-specific in order to determine the viral pathogenicity.

5.1.2 Respiratory bacteria detected

In the current study, the common detected bacteria were *S. pneumoniae* 78.2% as the cause of ARI and were more common in children under five years (74.7%). These findings are similar to several studies done in South-East Asia and Tanzania which revealed that *S. pneumoniae* was common in children younger than five years (16,59). The detection of *S. pneumoniae* in children from this study differ from the study done in the United States, which showed adults over 65 years were more affected (60). Other studies reported that colonization with *S. pneumoniae* and *Staphylococcus aureus*, increases the risk of ARI and the disease severity leading to high mortality rate (61,62). The high proportion of *S. pneumoniae* in children in the current study could be caused by a decrease in children vaccination rates during the COVID-19 pandemic or

by an increase in antibiotic resistance to drugs used to treat pneumococcal infections (63). The WHO and several studies observed a similar decline in vaccine coverage in 2020 compared to 2019 before pneumococcal conjugate vaccine (PCV) vaccination levels plateaued in 2021 and 2022 (64–66).

The detected serotypes in our study (19A, 14, 19F, 6B, 3 and 11), are among the serotype included in the Prevenar 13 vaccines similar to the findings reported in a study done in South-East Asia (59). The study found that the most invasive serotypes, 19A serotype were detected from children under five years followed by 14 serotype and serotype 19F. The findings are similar to the studies done in UK, USA, Gambia, South Africa and Sweden (67–71). Our study shows that regardless of the introduction of PCV vaccine the invasive serotypes are not yet eliminated from children under five years. We found serotype 3 in children under five years, the findings differ from the study done in UK, England, Brazil and Portugal which revealed that this serotype was detected in health adults, older children and older adults (72–75). The introduction of effective vaccines like PCV13 is increasingly beneficial and lifesaving for Tanzania, where it significantly reduces child mortality rates due to pneumococcal infections. The detected serotypes 6/13 (46.2%) are among serotypes included in the Prevenar 13 vaccine indicating the protection coverage level to children under five years. This findings highlighting that still invasive serotypes are still circulating in the community hence the vaccination coverage should be maintained at high level. In our study we found that 8.7% of *Streptococcus pneumoniae* were nontypeable, the findings were high compared to the study done in Gambia which revealed a proportion of 6% (76) but lower than other study done in the same country which found to be 10% (77). The detection of these non typeable serotype could be due to the fact that Prevenar 13 vaccines has been related to serotypeable that do not express their capsule (76).

Furthermore, other rare pathogens including *Bordetella pertussis, Coxiella burnetii, Nocardia farcinica and Moraxella catarrhalis* were also detected in this study. While vaccines have helped to reduce the impact of *Bordetella pertussis* (the bacteria causing whooping cough), recent detection remind us it’s still a serious respiratory threat (78). *Coxiella burnetii* and

Nocardia farcinica, although challenging to diagnose due to nonspecific symptoms, can lead to persistent illness if not managed effectively (79,80). *Moraxella catarrhalis*, reported to colonizes the upper respiratory tract at the stable state and the third leading cause of otitis media in children (81), acute exacerbations of chronic obstructive pulmonary disease (COPD), and acute bacterial rhinosinusitis (82). Several studies in India reported resistance to common antibiotics used for the treatment of this pathogen (82,83). These findings highlight and exhibit the need for expansion of influenza surveillance.

5.1.3 Respiratory fungi detected

Additionally, *Aspergillus fumigatus* (7.8%) were detected in the current study. This species is found ubiquitously in the environment including homes and hospital facilities (84). Moreover, this fungi can withstand a variety of stressors and can develops adaptation mechanisms that allow it to resist adverse circumstances in the lungs (85). Several studies have reported that treating *Aspergillus fumigatus* in respiratory tracts is crucial due to limited options, drug interactions, and antifungal resistance and if not treated the disease can be severe, especially in immunocompromised patients (86,87). This finding should prompt further research to assess the growing threat posed by this mycopathogen and to assess the environmental prevalence.

5.2 The associated factors of ARI among patient who tested negative for human influenza and SARS-CoV-2

The study indicates that the age groups 64 years or less are at risk for ARIs. However, the likelihood of getting ARI due to multiple pathogens is high among individuals of the same age group. Several studies have also shown that younger age and middle-aged adults are at risk (4,9,10). Moreover, it was found that patients with cardiovascular disease had two to three times chance of having ARI caused by single pathogen and multiple pathogens respectively. Other studies have reported a link between patients with underlying medical condition as a risk factor for ARI (32,34,37,41) contrary to finding from this study. The observed findings might be due to small number of patients with underlying medical conditions included in the current study.

This study has strengths and limitations. The strength is that this study deployed next-generation sequencing of nasopharyngeal samples, which allows for broad and high-throughput analysis of

genetic material, assisting the detection of multiple pathogens simultaneously and its ability to identify rare or emerging pathogens, which enhances diagnostic accuracy. Data nationwide through sentinel sites will represent all patients with ARI in Tanzania. However, the study had some limitations, which included the limited time for data collection (6 months) and impaired seasonality analysis of the detected viruses. Other limitation including the NGS detect genome (DNA/RNA) and prone to contamination. Therefore, unable to differentiate true pathogens vs contaminants.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study revealed a high proportion of pathogens causing ARI that are not typically included in routine surveillance. The study detected several viruses, bacteria and fungi in patients with acute upper respiratory symptoms that was negative for human influenza and SARS-CoV-2. The predominant virus was human rhinovirus, S. pneumoniae was the predominant bacterial detected. The study further revealed that age played a significant role in susceptibility to respiratory infections, with children under five years old being more susceptible than middle-aged adults.

6.2 Recommendations

From the study findings the following are recommendations;

We recommend that the Ministry of Health and stakeholders expand respiratory illness surveillance strategies throughout the country to capacitate the detection of other agents of ARI, the detection of circulating variants/strains, and change or expand of diagnostic algorithm to include the detection of other ARI (Apart from the current surveillance which included only Influenza A&B and SARS-CoV-2 testing).

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APPENDICES

Appendix I: Approval for ethical clearance

UNITED REPUBLIC OF TANZANIA

MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES

OFFICE OF THE DIRECTOR – RESEARCH, PUBLICATIONS AND INNOVATION

Ref. No.DA.282/298/01.C/2003 Date: 15/01/2024

MUHAS-REC-01-2024-2003
PETER TOROKAA
MUHAS

RE: APPROVAL FOR ETHICAL CLEARANCE FOR A STUDY TITLED: AETIOLOGIC CAUSES OF ACUTE RESPIRATORY INFECTION IN HUMAN INFLUENZA AND SARS-CoV-2 NEGATIVE CASES IN TANZANIA

Reference is made to the above heading,

I am pleased to inform you that the Chairman has on behalf of the University Senate, approved ethical clearance of the above-mentioned study, on recommendations of the Senate Research and Publications Committee meeting accordance with MUHAS research policy and Tanzania regulations governing human and animal subjects research.

APPROVAL DATE: 15/01/2024
EXPIRATION DATE OF APPROVAL: 15/01/2025

STUDY DESCRIPTION:
Purpose:

The purpose of this study is to determine other aetiological causes of acute respiratory infection in human influenza and SARS-CoV-2-negative patients and associated factors.

The approved protocol and procedures for this study is attached and stamped with this letter, and can be found in the link provided:

https://irb.muhas.ac.tz/storage/Certificates/Certificate%20-%2015058.pdf and in the MUHAS archives.

The PI is required to:

1. Submit bi-annual progress reports and final report upon completion of the study.
2. Report to the IRB any unanticipated problem involving risks to subjects or others including adverse events where applicable.
3. Apply for renewal of approval of ethical clearance one (1) month prior its expiration if the study is not completed at the end of this ethical approval. You may not continue with any research activity beyond the expiration date without the approval of the IRB. Failure to receive approval for continuation before the expiration date will result in automatic termination of the approval for this study on the expiration date.
4. Obtain IRB amendment (s) approval for any changes to any aspect of this study before they can be implemented.
5. Data security is ultimately the responsibility of the investigator.
6. Apply for and obtain Data Transfer Agreement (DTA) from NIMR if data will be transferred to a foreign country.
7. Apply for and obtain Material Transfer Agreement (MTA) from NIMR, if research materials (samples) will be shipped to a foreign country.
8. Apply for and obtain permission to publish the results from National Institute for Medical Research. The application should be before submitting to a publisher and not concurrently through the email publications@nimr.or.tz.
9. Any researcher, who contravenes or fail to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III section 10 (2)
10. The PI is required to ensure that the findings of the study are disseminated to relevant stake holders.
11. PI is required to be versed with necessary laws and regulatory policies that govern research in Tanzania. Some guidance is available on our website https://drp.muhas.ac.tz/.



Dr. Kasusu Nyamuryekung'e
Ag. Chairperson, MUHAS Research and Ethics Committee

Appendix II: Ministry of Health Approval

THE UNITED REPUBLIC OF TANZANIA
MINISTRY OF HEALTH

Telegrams: "AFYA", DODOMA
Telephone: +255 26 2323267
(All letters should be addressed to
The Permanent Secretary)

Government city-Mtumba,
Health Road/Street,
P.O.Box 743,
40478 DODOMA.

In reply please quote:

Ref.No.BA.21/107/01/27

30 August, 2023

Peter Torokaa,
P.O. Box 65001,
DAR ES SALAAM

RE: APPROVAL TO USE ARCHIEVED SAMPLES FOR ACUTE RESPIRATORY INFECTION (ARI STUDY)

Reference is made to the heading above.

The National public health Laboratory received your letter dated 28th August, 2023 concerning your request to use Nasopharyngeal swab samples for analysis in the study titled **"Aetiologic Causes of acute Respiratory Infection in Negative Human Influenza and SARC-CoV-2 cases in Tanzania from January to December – 2023"** which will be funded by the CDC Tanzania through APHL

Regarding the important of this Study to Health sector in Tanzania with consideration to your statement of commitment on data privacy, The National Public Health Laboratory grants you a permission to continue with your activity including getting Ethical Approve. This permission will be valid from 1st September, 2023 to 30th December, 2023

We are wishing you best of lucks.

For: DIRECTOR
NATIONAL PUBLIC HEALTH LABORATORY
P. O. Box 9083
DAR ES SALAAM

Ambele Mwafulango
FOR: DIRECTOR NATIONAL PUBLIC HEALTH LABORATORY

Appendix III: Summary of Bacteria – Viral co-detection

		Pathogen 2																
Pathogen 1		**Rhinovirus**	**Enterovirus**	**Adenovirus**	**Respiratory syncytial virus**	**Human metapneumovirus**	**Influenza A virus**	**Parainfluenza virus-4**	**Human coronavirus NL63**	**SARS CoV-2**	**Streptococcus pneumoniae**	***Enterobacter cloacae***	***Staphylococcus aureus***	***Moraxella catarrhalis***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***	***Bordetella pertussis***	***Coxiella burnetii***	***Nocardia farcinica***
	Rhinovirus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Enterovirus	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Adenovirus	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Respiratory syncytial virus	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Human metapneumovirus	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Influenza A virus	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-
	Parainfluenza virus-4	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-
	Human coronavirus NL63	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-	-

	SARS CoV-2	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	-
	Streptococcus pneumoniae	**9**	**9**	**3**	4	2	1	0	0	1	-	-	-	-	-	-	-	-	-
	Enterobacter cloacae	**9**	**4**	**2**	1	2	0	0	0	1	**53**	-	-	-	-	-	-	-	-
	Staphylococcus aureus	**5**	**6**	**3**	0	2	0	0	0	0	**52**	**40**	-	-	-	-	-	-	-
	Moraxella catarrhalis	0	3	0	1	0	0	0	0	0	**21**	**12**	**10**	-	-	-	-	-	-
	Klebsiella pneumoniae	1	1	0	0	0	0	0	0	0	**17**	**17**	**17**	**3**	-	-	-	-	-
	Pseudomonas aeruginosa	3	3	0	0	0	0	0	0	0	**21**	**12**	**13**	**8**	**8**	-	-	-	-
	Bordetella pertussis	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	-	-	-
	Coxiella burnetii	1	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	-	-
	Nocardia farcinica	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	-

Appendix IV: Plagiarism summary report

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AETIOLOGIC CAUSES OF ACUTE RESPIRATORY INFECTION IN HUMAN INFLUENZA AND SARS-CoV-2 NEGATIVE CASES IN TANZANIA

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MSc Epidemiology and laboratory Management

Muhimbili University of Health and Allied Sciences

June 2024

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