PREVALENCE AND GENOTYPING OF HUMAN ADENOVIRUS AMONG UNDER-FIVE CHILDREN WITH ACUTE FEBRILE ILLNESSES IN KASULU DISTRICT, KIGOMA, TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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EXTENDED ABSTRACT

Respiratory tract infections are the most frequent presenting complaint accounting for up to 60% of children with acute febrile illnesses (AFI) seeking health care in Tanzania. The emergence of COVID-19 has increased the threats of respiratory viruses causing the rise of incident cases of upper respiratory tract infections (URTI) to 42.82% of cases from all the diseases and injuries in 2020. Human adenoviruses account for about 2% to 5% of all respiratory infections worldwide and are a common cause of respiratory tract infections in children, accounting for 5% to 10% of all lower respiratory tract infections in children. Viral etiologies of febrile illness cause major disease burden in tropical and subtropical countries. Despite the burden of acute respiratory infection on morbidity and mortality in children under the age of five in the world, there is a scarcity of data to evaluate the contribution and the epidemiological features of viral etiologies of acute febrile illnesses in Tanzania. The objective of this study was to determine the prevalence and genetic characteristics of human adenovirus (HAdV) circulating in Kasulu District, Kigoma, Tanzania. A total of 110 nasopharyngeal swabs were collected from under-five children with acute febrile illnesses from three health facilities. The collected samples were tested for HAdV using nested polymerase chain reaction (nPCR). Of the 110 patients, 6 patients were HAdV-positive and the detection rate was 5.45%. The prevalence of HAdV infection was higher among females 7.41% (4/56) than males 3.57% (2/54). The analysis of the nucleotide sequences showed that all six positive samples belonged to HAdV C type 2 (HAdV C2). Phylogenetic analysis showed that all nucleotide sequences from this study clustered with HAdV C2 strains from Germany (MH121114.1 and EU867472.1), China (MH322262.1), Kuwait (MF085403.1 and MF085391.1) and Argentina (JX173079.1). The findings from this study suggest that active HAdV type 2 circulate among children in Kasulu district in Tanzania. The results confirm the presence of HAdV

among under-five children with acute febrile illnesses. Further studies are required to investigate the molecular epidemiology of HAdV in the country for appropriate control of the HAdV-associated diseases in the region.

DECLARATION

I, MWINYI BENJAMIN MASALA, declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

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(MSc. OHMB Candidate)

The above declaration confirmed by;

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Prof. Gerald Misinzo Date

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Dr. Leonard E.G. Mboera Date

(Supervisor)

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DEDICATION

I dedicate this work to my parents Mr. and Mrs. Benjamin K. Masala

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ABBREVIATION AND SYMBOLS

%	percent sign
≤	less or equal to
≥	greater or equal to
°C	degree Celsius
µL	microlitter
ADV	Adenovirus
AFI	acute febrile illness
AIDS	acquired immunodeficiency syndrome
ARI	acute respiratory infection
BLAST	Basic Local Alignment Search Tool
bp	base pair
COVID-19	coronavirus disease 2019
DNA	deoxyribonucleic acid
g	gram
GIT	gastrointestinal tract
HAdV	human adenovirus
HIV	human immunodeficiency virus
ILI	influenza-like illnesses
LRTI	lower respiratory tract infection
MEGA	Molecular Evolutionary Genetics Analysis
mg	milligram
min	minutes
mM	millmolar
PCR	polymerase chain reaction

pH	hydrogen ion concentration
RNA	ribonucleic acid
rpm	revolution per minute
s	seconds
SACIDS	SACIDS Foundation for One Health
SARS-CoV-2	severe acute respiratory syndrome coronavirus-2
SUA	Sokoine University of Agriculture
UNICEF	United Nations Children's Fund
URT	United Republic of Tanzania
URTI	upper respiratory tract infection
UTI	urinary tract infection
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

In low and middle-income countries, acute febrile illness is the most common reason for seeking health care and infections leading to these acute febrile illnesses are responsible for the majority of childhood deaths (O'Mea*a et al*., 2015). Considering non-malaria etiologies of fever, multiple studies indicate respiratory viruses as important causes of childhood fever in East Africa (O'Mea*a et al*., 2015). For many years, health-care providers assumed that malaria was the major etiology of childhood febrile illness and empiric treatment guidelines for fever in malaria-endemic areas have emphasized antimalarial administration (O'Mea*a et al*., 2015). A number of non-malaria febrile illnesses including Rift Valley fever, dengue, chikungunya, leptospirosis, tick-borne relapsing fever and Q-fever have been reported in Tanzania (Chipwaz*a et al*., 2014).

Adenoviruses are non-enveloped, double-stranded DNA viruses belonging to the *Adenoviridae* family (Y*u et al*., 2020). The family *Adenoviridae* is divided into five genera, namely Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus and Ichtadenovirus. Traditionally human adenoviruses (HAdV) were classified by neutralization reactions, hemagglutination and hydrolysis with restriction enzymes in 51 serotypes and multiple types (Kenmo*e et al*., 2018). To date, classification is based on the partial (Hexon, penton base and fiber) or complete viral genome sequencing. A recent study by (Y*u et al*., 2020) reported that, 100 unique genotypes of HAdV have been identified, from species A to G, which cause respiratory tract, gastrointestinal tract and ocular infections in human. Human adenovirus is a common cause of morbidity and mortality among children, particularly in low- and middle - income countries (Kenmoe

et al., 2018), mainly acute respiratory infections (ARIs) (Brin*i et al*., 2020). Human adenoviruses account for about 2% to 5% of all respiratory infections worldwide (Akell*o et al.,* 2020) and are a common cause of respiratory tract infections in children, accounting for 5% to 10% of all lower respiratory tract infections in children (X*ie et al*., 2019). Human adenoviruses accounts for 6% of deaths in patients with severe respiratory tract infections in Africa (Kenmo*e et al.,* 2018). The children under the age of 5 years, close-quartered populations such as crowded communities, schools, military training camps and immune-compromised individuals are generally the susceptible populations (Chen*g et* al., 2016). Viral etiologies of febrile illness cause major disease burden in tropical and subtropical countries. The lack of diagnostic facilities in these countries leads to failure to estimate the true burden of such illnesses and generally the diseases are rarely reported (Crump *et al*., 2013; Chipwaz*a et al*., 2014).

1.2 Problem Statement and Justification

Respiratory tract infections are the second leading killer diseases in low-income countries (Troeg*er et al.,* 2018) and accounts for 12.9% of in-hospital deaths in Tanzania (Kishamaw*e et al.,* 2019). Despite the burden of acute respiratory infection on morbidity and mortality in children under the age of five in the world (Tazin*ya et a*l., 2018), there is limited data to evaluate the contribution and the epidemiological features of viral etiologies of acute febrile illnesses in Tanzania. Clinical overlap between diseases results in inappropriate antimicrobial therapy due to lack of laboratory tests for differential diagnosis of etiologies of febrile illnesses. The need of this study was therefore to unravel the contribution to the acute febrile illnesses and epidemiological features of human adenovirus (HAdV).

1.3 Research Questions

i. What is the frequency of occurrence of HAdV strains circulating in Kasulu District, Tanzania?

ii. What are the genotypes of HAdV strains circulating in Kasulu District, Tanzania?

1.4 Objectives

1.4.1 General objective

To determine the prevalence and genetic characteristics of human adenovirus recovered among under-five children in Kasulu District, Tanzania.

1.4.2 Specific objective

i. To determine prevalence of HAdV responsible for acute febrile illnesses among under-fives and

ii. To examine the molecular characteristics of the detected HAdV.

CHAPTER TWO

2.0 Prevalence of Human Adenovirus among Under-Five Children with Acute Febrile Illnesses in Kasulu District, Kigoma, Tanzania

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2.1 Abstract

Respiratory tract infections are the most frequent presenting complaint accounting for up to 60% of children with acute febrile illnesses (AFI) seeking health care in Tanzania. The emergence of COVID-19 has increased the threats of respiratory viruses causing the rise of incident cases of upper respiratory tract infections (URTI) to 42.82% of cases from all the diseases and injuries in 2020. There is a scarcity of data to evaluate the contribution and the epidemiological features of viral etiologies of acute febrile illnesses in Tanzania. The objective of this study was to determine the prevalence of human adenovirus (HAdV) circulating in Kasulu District, Kigoma, Tanzania. A total of 110 nasopharyngeal swabs were collected from under-five children with acute febrile illnesses from three health facilities. The collected samples were tested for HAdV using nested-polymerase chain reaction (nPCR). Of the 110 patients, 6 patients were positive for HAdV and the prevalence was 5.45%. The prevalence of HAdV infection was higher among females 7.41% (4/56) than males 3.57% (2/54). The analysis of the nucleotide sequences showed that all six positive samples belonged to HAdV C type 2 (HAdV C2). The findings from this study suggest that active HAdV type 2 circulate among children in Kasulu district in Tanzania. The results confirm the presence of HAdV among under-five children with acute febrile illnesses. Further studies are required to investigate the molecular epidemiology of HAdV in the country for appropriate control of the HAdV-associated diseases in the region.

2.2 Background

In low and middle-income countries, acute febrile illness is the most common reason for seeking health care and infections leading to these acute febrile illnesses are responsible for the majority of childhood deaths (O'Mear*a et al*., 2015). Considering non-malaria etiologies of fever, multiple studies indicate respiratory viruses as important causes of

childhood fever in East Africa (O'Mear*a et al*., 2015). For many years, health-care providers assumed that malaria was the major etiology of childhood febrile illness and empiric treatment guidelines for fever in malaria-endemic areas have emphasized antimalarial administration (O'Mear*a et al*., 2015). A number of non-malaria febrile illnesses including Rift Valley fever, dengue, chikungunya, leptospirosis, tick-borne relapsing fever and Q-fever have been reported in Tanzania (Chipwaz*a et al*., 2014).

Acute respiratory infections (ARIs) are the most frequent infectious disease in humans, and the great majority of them are of viral etiology (Luder*t et al*., 2017). Acute respiratory infections are a leading cause of morbidity and mortality in under-five children worldwide (Tazin*ya et al*., 2018). According to global burden of diseases study of 2016, nearly 3 million deaths resulted from lower respiratory infections making them the sixth leading cause of mortality for all ages and the leading cause of death among children younger than 5 years (Troeg*er et al*., 2018). Most recent statistics indicate that upper and lower respiratory tract infections account for 22% and 13% of morbidities in under-five children, respectively (UNICEF, 2019). In Tanzania, respiratory diseases account for 12.9% of all in-hospital deaths reported (Kishama*we et al*., 2019). Viral etiologies of febrile illness cause major disease burden in tropical and subtropical countries. The lack of diagnostic facilities in these countries leads to failure to estimate the true burden of such illnesses and generally the diseases are rarely reported (Crum*p et al*., 2013; Chipwaz*a et al*., 2014).

Despite the burden of acute respiratory infection on morbidity and mortality in children under the age of five in the world (Tazin*ya et a*l., 2018), there is limited data to evaluate the contribution and the epidemiological features of viral etiologies of acute febrile illnesses in Tanzania. Clinical overlap between diseases results in inappropriate

antimicrobial therapy due to lack of laboratory tests for differential diagnosis of etiologies of febrile illnesses. The need of this study was therefore to unravel the contribution of human adenovirus (HAdV) to the acute febrile illnesses.

2.3 Materials and Methods

2.3.1 Study location

This study was conducted in Kasulu district in western Tanzania. Kasulu is one of the six districts of Kigoma Region, Tanzania. It is bordered to the north by Burundi, to the east by Kibondo District, to the south by Uvinza District, to the west by Kigoma District and to the northwest by Buhigwe District. Kasulu population is 628 677 (299 506 males and 329 171 females) with a total of 85 572 households making an average family size of 7.3 (URT, 2013 Tanzania, 2002 Population and Housing Census). Kasulu district hosts Congolese and Burundian refugees at Nyarugusu refugee camp consisting of about 67 000 people (Figure 1).

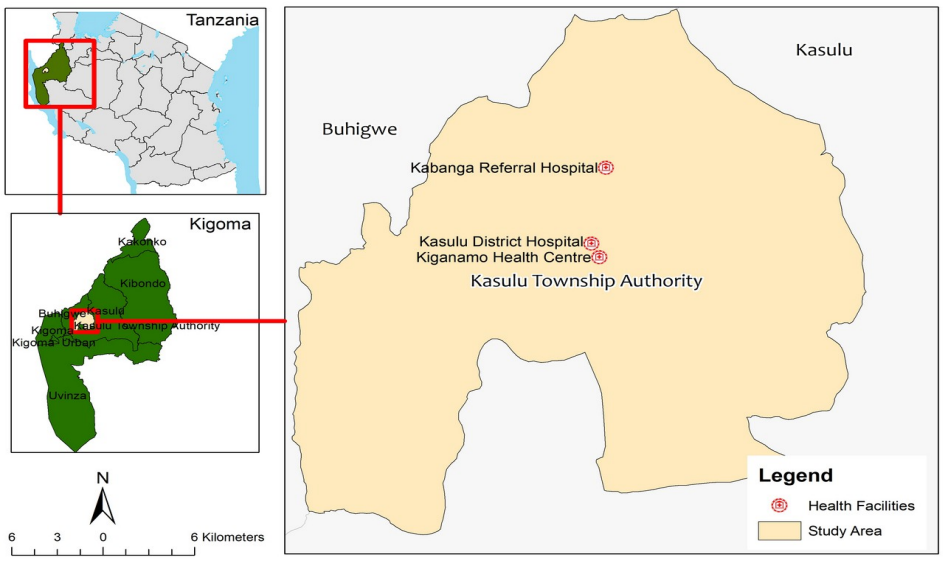

Figure 1: Kasulu District map showing health facilities where samples were collected

Source: This map was created using QGIS software version 3.16.8 downloaded from https://qgis.org/en/site/, accessed on 28th October, 2021.

2.3.2 Study design

This was a cross-sectional hospital-based study whereby nasopharyngeal swabs were collected from children with febrile illness visiting health care facilities at Kasulu District Hospital, Kiganamo Health Centre and Kabanga Referral hospital in October, 2020.

2.3.3 Sample size

The sample size was calculated by using the following formula (Martin *et al.,* 1987).

$$N = \frac{Z^2 p\,(1.0-p)}{d^2}$$

Where N = sample size

Z = standard normal deviate (1.96= 95% confidence level)

p = proportion in the target population estimate = 5% (Pond, 2005)

d = degree of accuracy desired, set at 5% or 0.05

$$N = \frac{(1.96)^2 \times 0.05 \times (1.0- 0.05)}{(0.05)^2} = 73$$

According to this formula the minimum sample size was 73, but a total of 110 nasopharyngeal swabs were collected from meeting the inclusion criteria. The nasopharyngeal swabs were collected more than the minimum sample size to account for any chances of specimen wastages and to increase precision of the study.

2.3.4 Inclusion criteria

The respiratory specimens were collected from under-five year children presenting with a fever (≥38°C) in the recent past (≤7days), cough or sore throat, sneezing and/or rhinorrhea. Any child who met the inclusion criteria qualified to be recruited into this study.

2.3.5 Exclusion criteria

Subjects with the following criteria were excluded from the study:

i. Children under 2 months of age,

ii. Unwillingness or refusal by the parent or guardian to participate in the study and

iii. Individuals with obvious exudative pharyngitis or tonsillitis.

2.3.6 Demographic and clinical information

Age, sex, fever, cough, sneezing, running nose, vomiting, health facility, onset of febrile illness, travel history, residence, history of recent influenza-like illnesses (ILI) and severe acute respiratory infections (SARI) were ascertained for all participants. Clinical parameters were documented.

2.3.7 Sample collection, transportation and storage

After obtaining a written informed consent, nasopharyngeal (NP) swabs were taken from enrolled subjects. Samples were collected by trained and assigned medical personnel at the health facilities, in accordance with standard operating procedures for collection and storage procedure for nasopharyngeal swabs for ILI and SARI investigation. The specimens were placed in a cryovials containing Trizol reagent and labeled using a unique study identification number. Trizol reagent was used for biological safety purpose because it inactivates the virus while preserving the nucleic acids making the specimens suitable for PCR analysis only. The samples were immediately stored in a cool box for short time storage and then frozen at -196 °C in liquid nitrogen. Frozen specimens in liquid nitrogen were transported to the laboratory at Sokoine University of Agriculture in Morogoro where they were stored at -80 °C in ultra-low temperature laboratory freezer.

2.4 DNA extraction and Virus Detection

DNA extraction was done using DNeasy Blood and Tissue Kit (cat# 69506) from Qiagen (Hilden, Germany), according to manufactures instructions. The hexone gene of human adenovirus was amplified using primer sequences (Table 1) as previously described by Okad*a et a*l. (2007). The outer PCR was performed in a 20µL reaction volume containing 5µL of extracted DNA, 4µL of FIREPol® Master Mix Ready to Load with 7.5 mM MgCl2 from Solis Biodyne (Teaduspargi 9, 50411 Tartu, Estonia), 1µL of forward primer (Adhex-GT1F), 1µL of reverse primer (Adhex-GT2R) and 9µL of nuclease free water. Cycling conditions consisted of initial denaturation at 95◦C for 5 min followed by 40 cycles of 95 ^{0}C for 30 s, 50 ^{0}C for 30 s and 72 ^{0}C for 1 min. Final extension at 72 ^{0}C for 10 min was performed to complete the extension. Nested PCR was performed with 2µL of 10-folds diluted outer PCR products, 4µL of FIREPol® Master Mix Ready to Load with 7.5 mM MgCl2 from Solis Biodyne (Teaduspargi 9, 50411 Tartu, Estonia), 1µL of forward primer (Adhex-GT3F), 1µL of reverse primer (Adhex-GT4R) and 12µl of nuclease free water in a 20µl reaction volume. Nested PCR was performed with identical cycling conditions to those of outer PCR using alternate primers (Adhex-GT3F and Adhex-GT4R), except for the annealing temperature step for the nested PCR which was 48 ºC.

Table 1: Primer sequences used for detection of human adenovirus

Primer name	Orientation	Primer sequence (5' to 3')	Amplicon size (bp)
Adhex-GT1F	Forward	CSGGNCAGGAYGCYTCGGRGTA	
Adhex-GT2R	Reverse	CACCCATGTTRCCWGTNCTGTT	986
Adhex-GT3F	Forward	AAYAARTTTAGRAAYCCCAC	863
Adhex-GT4R	Reverse	TTRTCYCTRAADSCAATGTARTT	

2.5 Gel-electrophoresis of PCR products

Gel-electrophoresis of nested-PCR products was done on agarose gel of 1.5% strength with 100 bp DNA ladder. The gel was transferred to the gel documentation system for visualization. The DNA fragments were observed as gray bands against a black background.

2.6 Sequencing for genotyping

Six amplicons from positive samples were sent to Macrogen Europe (Meibergdree 57, 1105 BA, Amsterdam the Netherland) for sequencing. The PCR products were purified and sequenced directly using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a genetic analyzer (ABI 3730xl System from Applied Biosystems). The raw sequence data were cleaned, edited and assembled by Geneious prime (version 2021.2.2) software to get consensus sequences. The obtained nucleotide sequences of human adenovirus were subjected to BLAST search to determine identity with other published Adenovirus strains available in PubMed database.

2.7 Data Management

Data collected from the clinical information of the participants were entered and analyzed using Microsoft Office-Excel 2010 (Microsoft, California, USA) and Epi Info version 7.0.8.0 (CDC, Atlanta, USA). Variables of sex, history of fever, vomiting, cough, sneezing were used to draw frequency tables in relationship to PCR results on human adenovirus.

2.8 Ethical Consideration

This study received ethical approval from the Medical Research Coordinating Committee of the National Institute for Medical Research (Ref. No. NIMR/HQ/R.8a/Vol.1/3237). The ethical approval was valid from 23rd October 2019 to 22nd October 2020.

2.9 Results

2.9.1 Clinical demographics

A total of 110 participants with febrile illness and acute respiratory infection were recruited into this study. Of these, 37 (33.64%) were from Kiganamo Health Centre, 39 (35.45%) from Kasulu District hospital and 34 (30.91%) from Kabanga Referral Hospital. Among the subjects, 56 (50.91%) were males and 54 (49.09%) were females (Table 2). Patient age ranged from 3 months to 4 years and 4 months old.

Table 2: Characteristics of 110 patients with human adenovirus infections

Characteristics		Overall	Not infected	Infected
Sex	Female	54 (49.09%)	50 (92.59%)	4 (7.41%)
	Male	56 (50.91%)	54 (96.43%)	2 (3.57%)
Facility	Kiganamo H/C	37 (33.64%)	34 (91.89%)	3 (8.11%)
	Kasulu D/H	39 (35.45%)	38 (97.44%)	1 (2.56%)
	Kabanga R/H	34 (30.91%)	32 (94.12%)	2 (5.88%)
Total	Patients	110	104 (94.55%)	6 (5.45%)

2.9.2 Prevalence of human adenovirus

Of the 110 patients, 6 patients were positive for HAdVand the detection rate was 5.45% basing on PCR results. The PCR results showed specific bands of approximately 900 bp. The electrophoresis gel image indicated that there was intact DNA that was suitable for nucleotide sequencing (Figure 2).

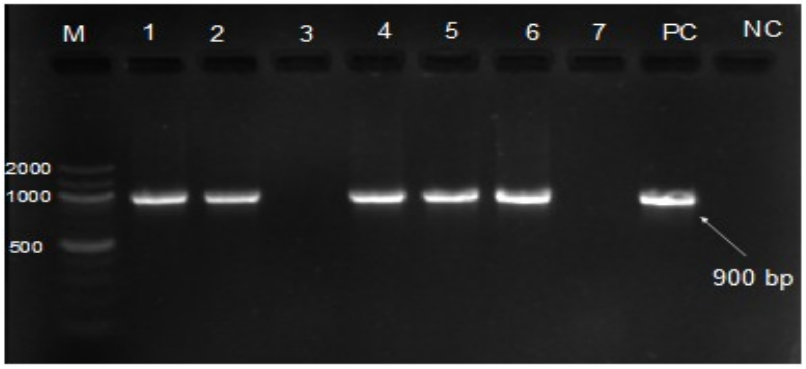

Figure 2: Gel-electrophoresis PCR results showing band for positive samples at 900 bp for human adenovirus. Lane M, 100-bp DNA ladder; Lanes 1–7, tested samples laboratory identification numbers; Lane PC, positive control and Lane NC, negative control

The prevalence of HAdV infection was higher in females 7.41% (4/56) than in males 3.57% (2/54). More HAdV infection cases were observed in patients from Kiganamo health center 8.11% (3/37) followed by those from Kabanga referral hospital 5.88% (2/34) while only 1 case out of 39 (2.56%) was observed in patients from Kasulu town hospital (Table 2).

2.9.3 Clinical manifestation among patients with HAdV infections

A total of 6 (5.88%) patients who presented with fever were positive for HAdV genome. Three (3.37%) patients among of those with running nose had HAdV infections. Patients without cough 10.00% (3/30) had higher detection rate of HAdV than those with cough 3.75% (3/80). Six percent (6/100) of patients who presented with sneezing tested positive for HAdV. Out of 20 patients who presented with vomiting 2 (10.00%) were HAdV-positive while 4.44% (4/90) of patients without vomiting were HAdV-positive basing on PCR detection of HAdV genome (Table 3).

Table 3: Clinical presentations among patients with HAdV infections

Clinical presentation	Response	Frequency	Infection	Infection rate
Fever	Yes	102	6	5.88
	No	8	0	0
Cough	Yes	80	3	3.75
	No	30	3	10
Sneezing	Yes	100	6	6
	No	10	0	0
Running nose	Yes	89	3	3.37
	No	21	3	14.29
Vomiting	Yes	20	2	10
	No	90	4	4.44

2.9.4 Genotype of human adenovirus

The obtained nucleotide sequences of human adenovirus were subjected to BLAST search to determine identity with other published Adenovirus strains available in PubMed database. The analysis of the nucleotide sequences showed that all six positive samples belonged to HAdV-C type 2 (HAdV-C 2).

2.10 Discussion

The present study aimed at determining the prevalence of human adenoviruses (HAdV) circulating in human populations in Kasulu district in Kigoma, Tanzania. This study confirmed the presence of HAdV among under-five children presenting with acute febrile illnesses seeking care from health facilities. The findings showed that, 5.45% of the tested subjects were positive for HAdV basing on nested- PCR results, and HAdV-C type 2 was detected from the tested samples. This prevalence of HAdV was consistent with prevalence of 5.8% for HAdV C reported earlier in Cameroon (Kenmoe *et al.*, 2018). A general HAdV prevalence of 3.5% and 2.4% in diarrhoeic and non-diarrhoeic children, respectively have been previously reported in Tanzania (Moyo *et al*., 2014). The exact prevalence and incidence of adenoviral infections are unknown; however it is estimated to be responsible for between 2% and 5% of all respiratory infections (Pond, 2005).

A slightly higher (7%) prevalence has been reported in Nairobi Kenya (Symekhier *et al*., 2009). A study in in Guangzhou, China, has reported HAdV prevalence of 6.3% among children between the ages of 5 and 10 years (Wang *et al.*, 2021).

This study shows the prevalence of HAdV 2 of 5.45% in under-five children. This is similar to the observation reported by Akello *et al.,* 2020, that HAdV2 was predominantly prevalent in young children in Switzerland. Most studies have indicated that HAdV is the major pathogen that causes lower respiratory tract infections in children aged 6 months to 5 years old (Xie *et al.,* 2019). In Tanzania, most of deaths due to respiratory diseases occur in children below five years old (Kishamawe *et al.,* 2019). Most recent statistics indicate that upper and lower respiratory tract infections accounts for 22% and 13% of morbidities in under-five children, respectively (UNICEF, 2019). Human adenovirus types 1, 2, 3 and 7 which are associated with respiratory infections are also known to be associated with diarrheal disease (Moyo *et al.,* 2014) leading to prolonged shedding of these HAdV species in feces. Therefore, the detected viruses under this study could also be associated with diarrhea. Further studies are needed to unravel this hypothesis.

There is limited published information regarding prevalence and molecular genotyping of HAdV species circulating in Tanzania and the East Africa in general. Globally, little attention is given non-influenza respiratory viruses in respiratory virus surveillance programs (Tan*g et al*., 2017). In Tanzania there is only one study which reported results for three non-influenza respiratory viruses; Human adenovirus, Respiratory syncytial virus and Human metapneumovirus (Umuhoz*a et al*., 2021).

2.11 Conclusion and Recommendations

2.11.1 Conclusion

This study intended to bring attention on the presence of viral etiologies of febrile illnesses in Kasulu district in Kigoma, Tanzania. The findings from this study have revealed the presence of human adenovirus type 2 circulating in Kasulu district with the prevalence of 5.45% and confirm the contribution of HAdV of to the acute febrile illnesses among children aged below 5 years old.

2.11.2 Recommendations

From this study, it is recommended that (i) there is a need to strengthen surveillance programs for non-influenza viral respiratory infections in Tanzania to provide evidence of their burden in time and space, and (ii) that viral etiologies should be considered in differential diagnosis of patients with febrile seeking for health care to avoid unnecessary prescription of antibiotics.

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CHAPTER THREE

3.0 Molecular characterization of human adenovirus associated with acute febrile illnesses in under-five children in Tanzania

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3.1 Abstract

Background: Human adenoviruses account for about 2% to 5% of all respiratory infections globally. There is limited data on molecular characterization of viral etiologies

of respiratory infections in Tanzania. The present study aimed at genotyping human adenovirus from under-five children at Kasulu District in Tanzania.

Methods: A total of 110 nasopharyngeal swabs were collected from under-five children with acute febrile illnesses from three health facilities. The collected samples were tested for HAdV using nested polymerase chain reaction (nPCR).

Results: Of the 110 patients, 6 patients were positive for human adenovirus and the detection rate was 5.45% basing on nested-PCR results. The analysis of the nucleotide sequences showed that all six positive samples belonged to HAdV C type 2 (HAdV C2). Phylogenetic analysis showed that all nucleotide sequences from this study clustered with HAdV C2 strains from Germany (MH121114.1 and EU867472.1), China (MH322262.1), Kuwait (MF085403.1 and MF085391.1) and Argentina (JX173079.1).

Conclusions: The results confirm the presence of HAdV among under-five children with acute febrile illnesses. Further studies are required to investigate the molecular epidemiology of HAdV in the country for appropriate control of the HAdV-associated diseases in the region.

Keywords: Human adenovirus, molecular characterization, febrile illnesses, nested-PCR, Sequencing, Phylogenetic analysis

3.2 Background

Adenoviruses are non-enveloped, double-stranded DNA viruses belonging to the *Adenoviridae* family (Y*u et al*., 2020). The family *Adenoviridae* is divided into five genera, namely Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus and Ichtadenovirus. Traditionally human adenoviruses (HAdV) were classified by neutralization reactions, hemagglutination and hydrolysis with restriction enzymes in 51 serotypes and multiple types (Kenmo*e et al*., 2018). To date, classification is based on the partial (Hexon, penton base and fiber) or complete viral genome sequencing. A recent

study by (Y*u et al*.,2020) reported that, 100 unique genotypes of HAdV have been identified, from species A to G, which cause respiratory tract, gastrointestinal tract and ocular infections in human. Human adenovirus is a common cause of morbidity and mortality among children, particularly in low - and middle - income countries (Kenmoe *et al.,* 2018), mainly acute respiratory infections (ARIs) (Brin*i et al*., 2020).

Different species or serotypes of HAdV are associated with different conditions, for example respiratory disease is mainly caused by species HAdV-B and C, conjunctivitis is caused by HAdV-B and D, gastroenteritis is caused by HAdV-F, (types 40, 41) and HAdV-G type 52). Human adenoviruses account for about 2% to 5% of all respiratory infections worldwide (Akell*o et al.,* 2020) and are a common cause of respiratory tract infections in children, accounting for 5% to 10% of all lower respiratory tract infections in children (X*ie et al*., 2019). Human adenoviruses accounts for 6% of deaths in patients with severe respiratory tract infections in Africa (Kenmo*e et al.*, 2018).

The children under the age of 5 years, close-quartered populations such as crowded communities, schools, military training camps and immune-compromised individuals are generally the susceptible populations (Chen*g et* al., 2016). Clinical manifestations of HAdV vary according to viral type characteristics, age and immune status of the host. While it generally causes mild clinical manifestations in healthy individuals (Bastug *et al*., 2021), more severe manifestations, including hemorrhagic cystitis, nephritis, pneumonia, hepatitis, enterocolitis and disseminated disease, are observed in immune compromised patients (Kneidinge*r et al.*, 2012).

Human adenovirus like other respiratory viruses spread via contact or aerosol transmission routes. Contact transmission can be through direct (contaminated hand) or indirect (fomites) virus transfer from infected person to susceptible individual (Kutter

et al.,2018). Droplets have short range of transmission, stay shortly in air and are generated when an infected person coughs, sneezes, talks or during procedures such as suctioning and endotracheal intubation (Kutte*r et al.*, 2018), while aerosols remain longer suspended in the air and have long range of transmission.

Viral etiologies of febrile illness cause major disease burden in tropical and subtropical countries. The lack of diagnostic facilities in these countries leads to failure to estimate the true burden of such illnesses and generally the diseases are rarely reported (Crump *et al*., 2013; Chipwaz*a et al*., 2014). Despite the burden of acute respiratory infection on morbidity and mortality in children under the age of five in the world (Tazin*y a et a*l., 2018), there is limited data to evaluate the contribution and the epidemiological features of viral etiologies of acute febrile illnesses in Tanzania. The lack of laboratory tests for differential diagnosis of etiologies of febrile illnesses results in inappropriate antimicrobial therapy. The need of this study was therefore to unravel the contribution of human adenovirus to the acute febrile illnesses and genotyping of (HAdV) recovered among under-five children in Kasulu District, Tanzania.

3.3 Methods

3.3.1 Study design

This was a cross-sectional hospital-based study whereby a total of 110 nasopharyngeal swabs were collected from children with febrile illness visiting three health facilities (Kasulu District Hospital, Kiganamo Health Centre and Kabanga Referral hospital) in Kasulu Township Authority. The respiratory specimens were collected from under-five year children presenting with a fever (≥38°C) in the recent past (≤7 days), cough or sore throat, sneezing and/or rhinorrhea. Any child who met the inclusion criteria qualified to be recruited into this study.

3.3.2 Study location

This study was conducted in Kasulu district in western Tanzania. Kasulu is one of the six districts of Kigoma Region, Tanzania. The population in Kasulu district is 628 677 (299 506 males and 329 171 females) with a total of 85 572 households making an average family size of 7.3 (URT, 2013 Tanzania, 2012 Population and Housing Census) (Figure 1).

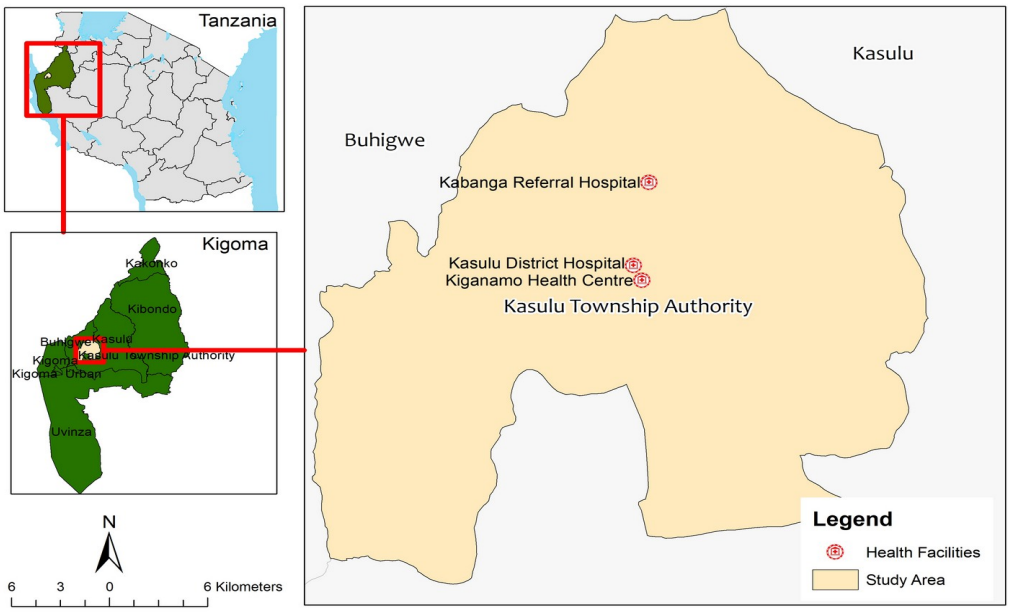

Figure 1: Kasulu District map showing health facilities where samples were collected

Source: This map was created using QGIS software version 3.16.8 downloaded from https://qgis.org/en/site/, accessed on 28th October, 2021.

3.3.3 Ethical consideration

This study received ethical approval from the Medical Research Coordinating Committee of the National Institute for Medical Research (Ref. No. NIMR/HQ/R.8a/Vol.1/3237). The ethical approval was valid from 23rd October 2019 to 22nd October 2020. After obtaining a written informed consent from the children's parent or guardian,

nasopharyngeal (NP) swabs were taken from enrolled subjects. Samples were collected by trained and assigned medical personnel at the health facilities, in accordance with standard operating procedures for collection and storage procedure for nasopharyngeal swabs for ILI and SARI investigation.

3.3.4 DNA extraction and virus detection

DNA extraction was done using DNeasy Blood and Tissue Kit (cat# 69506) from Qiagen (Hilden, Germany), according to manufactures instructions. The hexone gene of human adenovirus was amplified using primer sequences (Table 1) as previously described by Okad*a et a*l. (2007). The outer PCR was performed in a 20µL reaction volume containing 5µL of extracted DNA, 4µL of FIREPol® Master Mix Ready to Load with 7.5 mM MgCl2 from Solis Biodyne (Teaduspargi 9, 50411 Tartu, Estonia), 1µL of forward primer (Adhex-GT1F), 1µL of reverse primer (Adhex-GT2R) and 9µL of nuclease free water. Cycling conditions consisted of initial denaturation at 95◦C for 5 min followed by 40 cycles of 95 ^{0}C for 30 s, 50 ^{0}C for 30 s and 72 ^{0}C for 1 min. Final extension at 72 ^{0}C for 10 min was performed to complete the extension. Nested PCR was performed with 2µL of 10-folds diluted outer PCR products, 4µL of FIREPol® Master Mix Ready to Load with 7.5 mM MgCl2 from Solis Biodyne (Teaduspargi 9, 50411 Tartu, Estonia), 1µL of forward primer (Adhex-GT3F), 1µL of reverse primer (Adhex-GT4R) and 12µl of nuclease free water in a 20µl reaction volume. Nested PCR was performed with identical cycling conditions to those of outer PCR using alternate primers (Adhex-GT3F and Adhex-GT4R), except for the annealing temperature step for the nested PCR which was 48 ºC.

Table 1: Primer sequences used for detection of human adenovirus

Primer name	Orientation	Primer sequence (5' to 3')	Amplicon size (bp)

Adhex-GT1F	Forward	CSGGNCAGGAYGCYTCGGRGTA	
Adhex-GT2R	Reverse	CACCCATGTTRCCWGTNCTGTT	986
Adhex-GT3F	Forward	AAYAARTTTAGRAAYCCCAC	863
Adhex-GT4R	Reverse	TTRTCYCTRAADSCAATGTARTT	

Gel-electrophoresis of nested-PCR products was done on agarose gel of 1.5% strength with 100 bp DNA ladder. The gel was transferred to the gel documentation system for visualization. The DNA fragments were observed as gray bands against a black background.

3.3.5 Sequencing for genotyping

Six amplicons from positive samples were sent to Macrogen Europe (Meibergdree 57, 1105 BA, Amsterdam the Netherland) for sequencing. The PCR products were purified and sequenced directly using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a genetic analyzer (ABI 3730xl System from Applied Biosystems). The raw sequence data were cleaned, edited and assembled by Geneious prime (version 2021.2.2) software to get consensus sequences.

3.3.6 Phylogenetic analysis for HAdV genotyping

The obtained nucleotide sequences of human adenovirus were subjected to BLAST search to determine identity with other published Adenovirus strains available in PubMed database. Alignment of HAdV hexon gene partial nucleotide sequences from this study with selected reference HAdV hexon gene nucleotide sequences from GenBank was performed using MUSCLE in MEGA 11 software. The phylogenetic analysis was done using the UPGMA method (Sneath and Sokal, 1973), utilizing bootstrap test method with 1000 replicates which is included in MEGA 11 (Tamur*a et al*., 2021). The phylogenetic tree was generated based on the partial nucleotide sequences of HAdV hexone gene from

6 samples and 32 nucleotide sequences from references strains obtained from the GenBank.

3.4 Results

3.4.1 Detection of human adenovirus

Of the 110 patients, 6 patients were HAdV positive and the detection rate was 5.45% basing on PCR results. The PCR results showed specific bands of approximately 860 bp. The electrophoresis gel image indicated that there was intact DNA that was suitable for nucleotide sequencing (Figure 2).

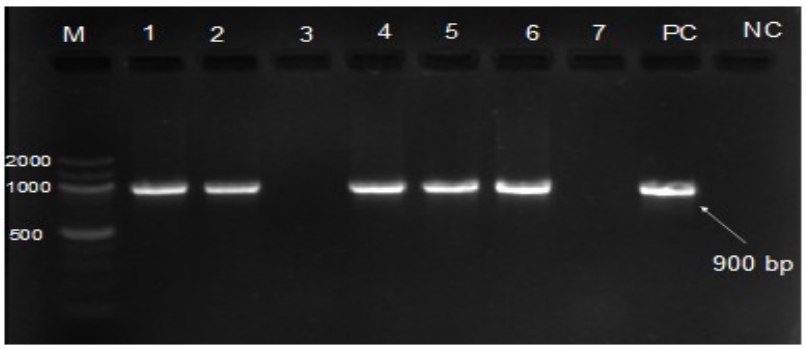

Figure 2: Gel-electrophoresis PCR results showing band for positive samples at 900 bp for human adenovirus. Lane M, 100-bp DNA ladder; Lanes 1–7, tested samples laboratory identification numbers; Lane PC, positive control and Lane NC, negative control

3.4.2 Phylogenetic analysis

The hexon gene partial nucleotide sequences of HAdV isolated from 6 samples were of 826 bp size. A BLAST search for hexon gene nucleotide sequences revealed the maximum sequence identity of 100% of HAdV-KS-162015-01-66-82 and HAdV-KB-2020-21 sequences with HAdV 2 isolate from Kuwait (MF085391.1) of 2017 and HAdV C2 isolate from China (MH322262.1) of 2020 while the identity with HAdV 2 strain from Germany (EU867472.1) of 2016 was 99.76%. The rest nucleotide sequences (HAdV-KG-

162020-00-76-29, HAdV-KG-162020-00-60-02, HAdV-KG-162020-00-22-23 and HAdV-KB-2020-24) showed identity of 99.88% with HAdV 2 isolate from Japan (LC504573.1) of 2019 and identity of 97.58% with HAdV C 2 isolate from China (MT990932.1) of 2021 (Table 2).

Table 2: Basic Local Alignment Search Tool (BLAST) results of HAdV 2 hexon gene partial nucleotide sequences from this study against sequences from GenBank

Description	Max score	Total score	Query coverage	Nucleotide identity	Accession
HAdV 2/Germany 2019	1526	1526	100%	100%	MH121114.1
HAdV 2/China 2019	1526	1526	100%	100%	MK041230.1
HAdV 2/Kuwait 2017	1526	1526	100%	100%	MF085391.1
HAdV 2/China 2020	1526	1526	100%	100%	MH322262.1
HAdV 2/Kuwait 2017	1526	1526	100%	100%	MF085382.1
HAdV C 2/China 2020	1520	1520	100%	99.88%	MH322415.1
HAdV 2/Japan 2019	1520	1520	100%	99.88%	LC504573.1
HAdV 2/Germany 2016	1515	1515	100%	99.76%	EU867472.1
HAdV 2/Germany 2005	1411	1411	100%	97.58%	AJ293904.1
HAdV C 2/China 2021	1411	1411	100%	97.58%	MT990932.1
HAdV C 2/China 2020	1406	1406	100%	97.46	MH322440.1

Source: Genbank database

Phylogenetic analysis shows that all HAdV hexon gene partial nucleotide sequences from this study clustered with HAdV C2 strain from Germany (MH121114.1) of 2019, HAdV C2 isolate from China (MH322262.1) of 2020, two HAdV C2 strains (MF085403.1 and MF085391.1) from Kuwait of 2017, HAdV C2 strain from Argentina (JX173079.1) of 2002 and HAdV 2 isolate from Germany (EU867472.1) of 2016. The phylogenetic tree was constructed using the Maximum likelihood method based on the Kimura 2-parameter model using MEGA 11 software (Figure 3).

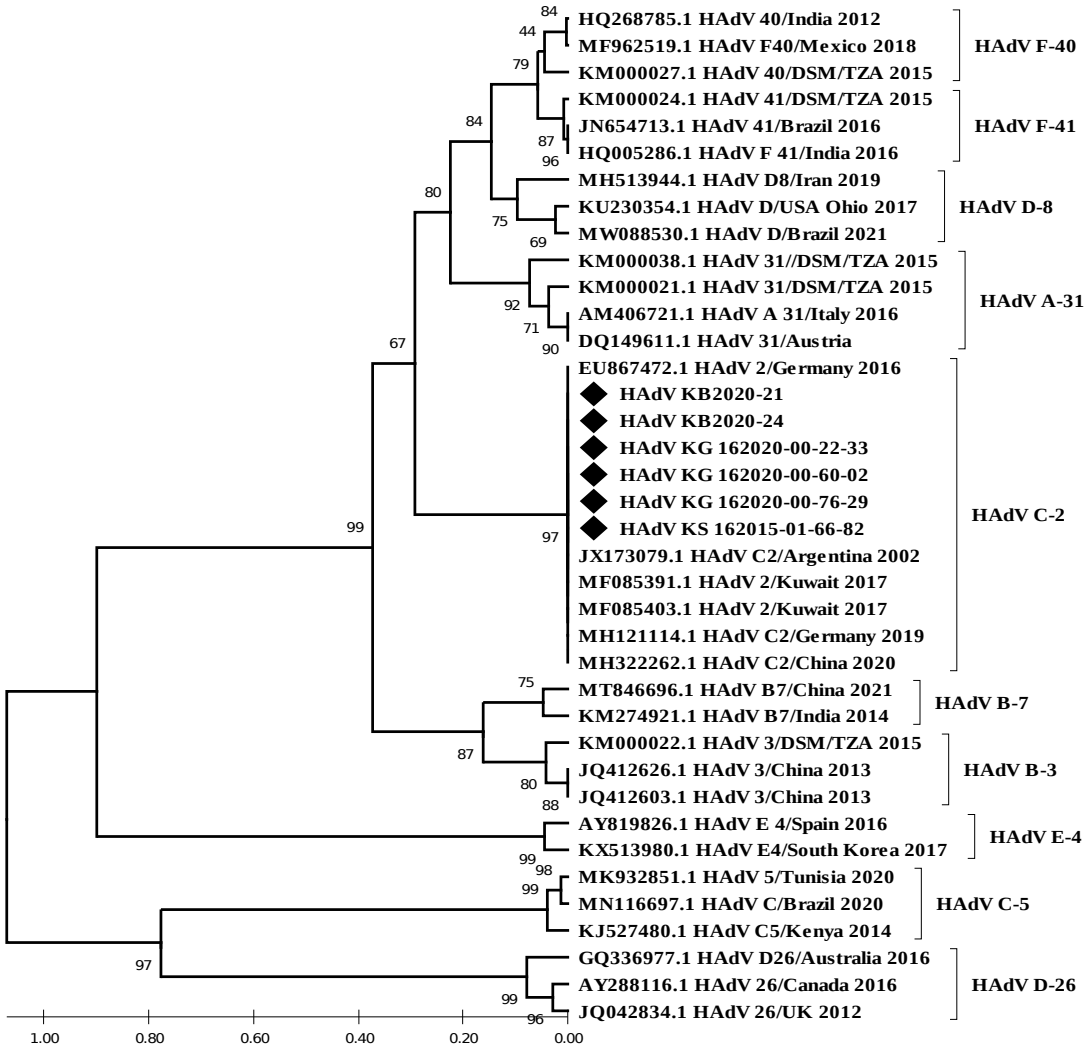

Figure 3: Phylogenetic tree of the hexon gene partial sequences of HAdV nucleotide sequences from this study and selected sequences from GenBank. ◆ Sign indicates nucleotide sequences from this study

3.5 Discussion

The present study aimed at determining the genotypes of human adenoviruses (HAdV) circulating in human populations in Kasulu district in Kigoma, Tanzania. This study confirmed the presence of HAdV among under-five children presenting with acute febrile illnesses seeking care from health facilities. The findings showed that, 5.45% of the tested subjects were positive by nested- PCR targeting HAdV hexon gene and HAdV C type 2 was detected from the tested samples. This study shows the detection rate of HAdV 2 of 5.45% in under-five children (basing on nested-PCR results). This is similar

to the observation reported by Akello *et al.*, 2020, that HAdV 2 was predominantly prevalent in young children in Switzerland.

Most studies have indicated that HAdV is the major pathogen that causes lower respiratory tract infections in children aged 6 months to 5 years old (Xie *et al.*, 2019). In Tanzania, most of deaths due to respiratory diseases occur in children below five years old (Kishamawe *et al.*, 2019). The recent statistics indicate that upper and lower respiratory tract infections accounts for 22% and 13% of morbidities in under-five children, respectively (UNICEF, 2019).

The identified HAdV species in this study show close relationship with isolates from other countries providing evidence that the distribution of HAdV 2 is not limited to certain geographical locations. The strains that clustered in the same group in the phylogenetic tree are genetically closely related and might have the same ancestral origin. This is important in molecular epidemiology as it helps to know the genetic characteristics, spatial and temporal distribution as well as transmission dynamics of the viruses.

Phylogenetic analysis shows that all HAdV hexon gene partial nucleotide sequences from this study clustered with HAdV C2 strains from Germany (MH121114.1 and EU867472.1), China (MH322262.1), Kuwait (MF085403.1 and MF085391.1) and Argentina (JX173079.1). The phylogenetic analysis also shows that, the HAdV type 2 isolates from the present study are clearly distinct from other species and types of HAdVs available from GenBank. The BLAST search did not give HAdV strains from neighboring countries (Kenya, Uganda, Burundi, Rwanda, Zambia, Malawi and Mozambique) which could cluster together with the HAdV C2 isolates from this study.

This might be due to inadequate studies concerning Human adenovirus and other non-influenza respiratory viruses conducted in the region. There is limited published information regarding molecular genotyping of HAdV species circulating in Tanzania and the East Africa region in general. Globally, non-influenza respiratory viruses have received less attention in respiratory virus surveillance programs (Tan*g et al*., 2017). In Tanzania there is only one study which reported results for three non-influenza respiratory viruses; Human adenovirus, Respiratory syncytial virus and Human metapneumovirus (Umuhoz*a et al*., 2021).

3.6 Conclusions and Recommendations

3.6.1 Conclusions

This study intended to bring attention on the presence of viral etiologies of febrile illnesses in Kasulu district in Kigoma, Tanzania. Acute febrile illnesses are common in children below 5 years of age and are predominantly caused by viruses. The findings from this study have revealed (i) the presence of human adenovirus type 2 circulating in Kasulu district and confirm the contribution of HAdV to the acute febrile illnesses among children aged below 5 years old and (ii) that HAdV C-2 strains exist in Tanzania.

3.6.2 Recommendations

From this study, it is recommended that (i) there is a need to strengthen surveillance programs for viral respiratory infections in Tanzania to provide evidence of their burden in time and space and (ii) molecular epidemiological studies are required to elucidate the transmission dynamics of HAdV in the country for appropriate control of the diseases associated with viral infections among under-five children.

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CHAPTER FOUR

4.0 General Discussion, Conclusions and Recommendations

4.1 General Discussion

The present study aimed at determining the prevalence and genotypes of human adenoviruses (HAdV) circulating in human populations in Kasulu district in Kigoma, Tanzania. This study confirmed the presence of HAdV among under-five children

presenting with acute febrile illnesses seeking care from health facilities. The findings showed that, 5.45% of the tested subjects were positive by nested- PCR targeting HAdV hexon gene and HAdV C type 2 was detected from the tested samples. This prevalence of HAdV was consistent with prevalence of 5.8% for HAdV C reported earlier in Cameroon (Kenmoe *et al.,* 2018). A general HAdV prevalence of 3.5% and 2.4% in diarrhoeic and non-diarrhoeic children, respectively have been previously reported in Tanzania (Moyo *et al*., 2014). The exact prevalence and incidence of adenoviral infections are unknown; however it is estimated to be responsible for between 2% and 5% of all respiratory infections (Pond, 2005). A slightly higher (7%) prevalence has been reported in Nairobi Kenya (Symekhie*r et al*., 2009). A study in in Guangzhou, China, has reported HAdV prevalence of 6.3% among children between the ages of 5 and 10 years (Wang *et al.,* 2021).

This study shows the prevalence of HAdV 2 of 5.45% in under-five children. This is similar to the observation reported by Akello *et al.,* 2020, that HAdV2 was predominantly prevalent in young children in Switzerland. Most studies have indicated that HAdV is the major pathogen that causes lower respiratory tract infections in children aged 6 months to 5 years old (Xie *et al.,* 2019). In Tanzania, most of deaths due to respiratory diseases occur in children below five years old (Kishamawe *et al.,* 2019). Most recent statistics indicate that upper and lower respiratory tract infections accounts for 22% and 13% of morbidities in under-five children, respectively (UNICEF, 2019). Human adenovirus types 1, 2, 3 and 7 which are associated with respiratory infections are also known to be associated with diarrheal disease (Moyo *et al.,* 2014) leading to prolonged shedding of these HAdV species in feces. Therefore, the detected viruses under this study could also be associated with diarrhea. Further studies are needed to unravel this hypothesis.

The identified HAdV species in this study show close relationship with isolates from other countries providing evidence that the distribution of HAdV 2 is not limited to certain geographical locations. The strains that clustered in the same group in the phylogenetic tree are genetically closely related and might have the same ancestral origin. This is important in molecular epidemiology as it helps to know the genetic characteristics, spatial and temporal distribution as well as transmission dynamics of the viruses.

Phylogenetic analysis shows that all HAdV hexon gene partial nucleotide sequences from this study clustered with HAdV C2 strain from Germany (MH121114.1) of 2019, HAdV C2 isolate from China (MH322262.1) of 2020, two HAdV C2 strains (MF085403.1 and MF085391.1) from Kuwait of 2017, HAdV C2 strain from Argentina (JX173079.1) of 2002 and HAdV 2 isolate from Germany (EU867472.1) of 2016. The phylogenetic analysis also shows that, the HAdV type 2 isolates from the present study are clearly distinct from other species and types of reference HAdVs selected from GenBank. The BLAST search did not give reference HAdV strains from neighboring countries (Kenya, Uganda, Burundi, Rwanda, Zambia, Malawi and Mozambique) which could cluster together with the HAdV C2 isolates from this study. This might be due to inadequate studies concerning Human adenovirus and other non-influenza respiratory viruses conducted in the region. There is limited published information regarding molecular genotyping of HAdV species circulating in Tanzania and the East Africa in general. Globally, non-influenza respiratory viruses have received less attention in respiratory virus surveillance programs (Tan*g et al*., 2017). In Tanzania there is only one study which reported results for three non-influenza respiratory viruses; Human adenovirus, Respiratory syncytial virus and Human metapneumovirus (Umuhoz*a et al*., 2021).

4.2 Conclusions

This study intended to bring attention on the presence of viral etiologies of febrile illnesses in Kasulu district in Kigoma, Tanzania. Acute febrile illnesses are common in children below 5 years of age and are predominantly caused by viruses. The findings from this study have revealed (i) the presence of active human adenovirus type 2 circulating in Kasulu district and confirm the contribution of HAdV to the acute febrile illnesses among children aged below 5 years old and (ii) that HAdV C-2 strains exist in Tanzania.

4.3 Recommendations

From this study, it is recommended that (i) there is a need to strengthen surveillance programs for viral respiratory infections in Tanzania to provide evidence of their burden in time and space, (ii) molecular epidemiological studies are required to elucidate the transmission dynamics of HAdV in the country for appropriate control of the diseases associated with viral infections among under-five children and (iii) that viral etiologies should be considered in differential diagnosis of patients with fever seeking for health care to avoid unnecessary prescription of antibiotics.

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