Addis Ababa University

College of Health Science

School of Allied Health Sciences

Department of Medical Laboratory Sciences

Molecular Detection of Common Respiratory Viruses among Acute Respiratory Illness Cases in Selected Health Facilities of Ethiopia

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This is to certify that the thesis prepared by Adamu Tayachew Mekonnen, entitled with: **Molecular Detection of Common Respiratory Viruses Among Acute Respiratory Illness Cases In Selected Health Facilities In Ethiopia** and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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LISTS OF ABBREVIATIONS

ALRI----------Acute lower respiratory tract infections

ARI----------Acute Respiratory Illness

AURI----------Acute upper respiratory tract infections

CDC----------Centers for Disease Control and Prevention

CDNA----------Complementary Deoxyribonucleic Acid

DNA----------Deoxyribonucleic Acid

dNTP----------deoxynucleotide Triphosphate

DRERC----------Department of Research and Ethical Review Committee

EPHI----------Ethiopian Public Health Institute

EV----------Enteroviruses

FLUC----------Influenza virus C

FMOH----------Federal Minister of Health

HAdV----------Human Adenoviruses

HBoV----------Human bocavirus

HCoV----------Human coronaviruses

HMPV----------Human Metapneumoviruses

HPeV----------Human parechoviruses

ILI----------Influenza like Illness

NIL----------National Influenza laboratory

PIV----------Parainfluenza viruses

PI----------Principal Investigator

PP----------Primer Probe

RNA----------Ribonucleic Acid

RSV----------Respiratory syncytial virus

RT-PCR----------Reverse Transcriptase Polymerase chain Reaction

RV----------Rhino Virus

SARI----------Severe Acute Respiratory Illness

SERO----------Scientific and Ethical Review Office

VTM----------Viral transport media

WHO---------- World Health Organization

SUMMARY

Background: Acute respiratory illnesses (ARI) are among the major public health problems both in developed and developing countries. No any previous studies describing about the types and magnitude of viral etiologies responsible for acute respiratory infections in both severe acute respiratory illness (SARI) and influenza like illness (ILI) in Ethiopia except influenza viruses.

Objective: To assess common respiratory viruses among acute respiratory illness cases in selected health facilities in Ethiopia.

Method: Laboratory test by RT-PCR was conducted at National Influenza Reference Laboratory of Ethiopian Public Health Institute (EPHI) from 01, July, 2017 to 31, October, 2017 combination of retrospective and prospective study designs. Throat/throat and nasopharyngeal swab samples collected from 01, January, 2015 to 31, December 2016 from influenza sentinel surveillance sites were selected by systematic random sampling technique for detection of Parainfluenza viruses1-4 (PIV1-4), Human coronaviruses (HCoV), Human metapneumoviruses A/B (HMPV A/B); Rhinovirus (RV); Respiratory syncytial viruses A/B (RSV A/B); Human adenovirus (HAdV), Enterovirus(EV), Human parechovirus (HPeV),Human bocavirus (HBoV) and Influenza virus C *(*INF C*)*. Descriptive statistics was done using SPSS version 20.

Results: A total of 422 samples which consisted of 202 (47.9%) male were tested. Children under age of five years accounted for 47.6% (201) of the tested samples. Equal number of samples from outpatients and hospitalized cases were tested. Among the tested samples 55.5% (234/422) were positive for at least one respiratory virus and most frequently detected among children under five years of age (p=0.023). Respiratory viruses were co-detected in 18.2% (77) of the samples and 79.2% (61/77) were from SARI cases (p=0.007). The most frequently detected respiratory viruses were RV *(*18.7%), RSV A/B, (12.8%), HAdV (11.4%) and PIV1-4 (7.3%). HCoV(6.2%),EV(6.2%), HPeV(5.7%, HMPV A/B(4.5%), HBoV (3.8%). INF C (0.2%) were also detected

Conclusion and recommendation: The study detected the circulation of wide range of respiratory viruses among samples from ILI and SARI cases especially among under five children. Large scale study has to be done to better understand the seasonal variation, spectrum of illness and severity of ARI due to the different respiratory viruses.

Key words: Respiratory Virus, Multiplex RT-PCR, Acute respiratory illness, Influenza sentinel surveillance sites, Ethiopia

1. INTRODUCTION

1.1 Background

The World Health Organization (WHO) estimates that acute respiratory infections (ARIs) cause nearly four million deaths per year. It is the third largest cause of mortality in the world and the top killer in low- and middle-income countries. The mortality due to ARI is ten to fifty times higher in developing countries than developed countries. In 2010 alone,5.8 million deaths were due to ARI(1).

Worldwide, approximately 2.2 million children die due to acute respiratory infection (ARI) and about 40% of these deaths occur in Africa. Infections due to influenza virus, respiratory syncytial virus (RSV), Parainfluenza virus (PIV1-4), Human metapneumovirus (HMPV), Enterovirus, human Rhinovirus (HRV), Adenovirus, and Human corona virus (HCoV) account for a significant proportion of acute respiratory tract infections. However, ARIs are generally subject to presumptive treatment, and their causes are rarely sought(2).

Acute respiratory infections are classified as acute upper and lower respiratory infections depending on the part of the respiratory structure affected. The major AURI comprises nose, sinuses, middle ear, larynx and pharynx. Worldwide, about 85–88 % of ARI episodes are AURI while the remaining are Acute Lower Respiratory Infections(3,4).

The risk of infection is often related to age, preexisting medical conditions and immune competence and socioeconomic condition of individuals. Respiratory syncytial virus (RSV), for example, affects mainly the young, although less typical infections amongst adults are recognized(5).

Acute respiratory diseases can result in a spectrum of illnesses ranging from asymptomatic or mild infection to severe and fatal disease, depending on the causative pathogen, environmental, and host factors. Symptoms of ARI include fever, cough, and often sore throat, coryza, shortness of breath, wheezing, or difficulty breathing(6).

The vast majority of AURIs has a viral etiology and caused by Rhinoviruses, Respiratory syncytial viruses (RSVs), Parainfluenza and Influenza viruses, Human metapneumovirus, and

Adenoviruses, corona viruses and etc. Because most URI is self -limiting, their complications are more important than the infections. Acute viral infections predispose children to bacterial infections of the sinuses and middle ear, and aspiration of infected secretions and cells can result in ALRIs(7)

The ALRI include infection of trachea, bronchi and lungs. One of the most frequently associated diseases with ALRI is pneumonia which has both viral and bacterial roots. Viruses account for 40 to 50 percent of pneumonia hospitalizations for children in developing countries. RSV, Parainfluenza viruses, adenoviruses and influenza type A virus are the most significant causes of viral pneumonia(8).

Introductions of conjugate vaccines for the two leading bacterial causes of pneumonia, *Haemophilus influenzae* type B (Hib) and *Streptococcus pneumoniae*, into the Expanded Programme on Immunization (EPI) in Africa will likely, by reducing the numbers of pneumonias that they cause, also lead to a shifted set of priority pathogens targeted for pneumonia prevention(9).

As clinical presentations of viral respiratory tract infections are similar, making it difficult to distinguish between etiologic agents. Accurate and rapid identification of the etiological agent is important for patient management and control of respiratory disease outbreaks(10).

Confirmation of viral ARI by using viral cultures, direct immunofluorescence tests, and rapid antigen tests are too laborious or unreliable. Serological tests are unsuitable for the diagnostic evaluation of acute respiratory infections. Investigations using specific monoplex PCR for individual viruses are also too time consuming. Multiplex-PCR methods to detect pathogens in parallel in a single analysis is therefore increasingly gaining in importance(11). Direct immunofluorescence tests yield results within very few hours, but in many cases they lack sensitivity and specificity and are available for few viruses only(12).

In addition, the discovery of 6 new respiratory viruses since 2000 including Human metapneumovirus, the severe acute respiratory syndrome coronavirus, influenza virus strain H5N1, coronavirus strains NL63 and Hku1, and Human bocavirus has presented new challenges for comprehensive viral diagnostics. Multiplex PCR based systems provide potential solutions to this complex diagnostic problem(13).

1.2 Statement of the problem

Acute respiratory tract infections (ARTI) particularly lower respiratory tract infections (LRTI) are the leading cause of death among children under five years of age. ARI are the most common reason that most people access health services around the world and accounting for 20%-40% of all hospitalizations among children(1).

Acute respiratory tract infection (ARI) is the main cause of morbidity and mortality in both developing and developed countries. WHO recognized respiratory diseases as the second important cause of death for children less than five years in 2010.Viruses have already been recognized as the most common cause of acute respiratory tract infections in young children. According to WHO reports, viruses account for 30 to 67% of pneumonia mostly occurring in children < 1 year(8).

Pneumonia has consistently been estimated as the leading single cause of childhood mortality. As WHO bulletin Report 2008, Pneumonia etiology studies that incorporate viral studies show that respiratory syncytial virus is the leading viral cause, being identified in 15–40% of pneumonia or bronchiolitis cases admitted to hospitals in children in developing countries, followed by influenza A and B, parainfluenza, human metapneumovirus and adenovirus(14).A recent postmortem study of lung tissue samples from 96 Mexican children <2yrs who dead of pneumonia (Nested PCR) showed also that 62% of those were histopathologically diagnosed as viral pneumonia(of which 30% RSV) while 25% diagnosed as bacterial pneumonia(15).

A global meta-analysis study identified that in 2010 there were about 12 million episodes of hospital admissions for severe and 3 million for very severe ALRI. The study also further estimated that severe or very severe ALRI resulted in about 0·3 million deaths in hospitals in young children, of which 99% of these deaths were in developing countries, and in-hospital deaths were about 19% of the estimated total number of ALRI deaths in young children in 2010 while the rest dead outside hospitals(16).

In the United States alone, 100,000 infants are hospitalized yearly for respiratory syncytial virus (RSV). The great majority of respiratory infections are of viral origin and 10%–50% of patients

develop a secondary bacterial infection. Epidemics of influenza and RSV infections are associated with annual death of 36,000 and 11,000 respectively(17).

Other Studies in the USA showed that ARI had not only negative health impact but also causes a high economic burden for the country. The study result estimated the yearly cost for health seeking in health facilities with Otitis media, Pharyngitis,AURI,Sinusitis and Bronchitis(ARI) a total of 41.4milion prescriptions with estimated value of $1.3 bilion among which 55% of the prescribed antibiotics for ARI were used for infections unlikely to have a bacterial etiology(18).

Studies in China have shown that Acute respiratory infection (ARI) is the most common cause of hospital admission for children in Hong Kong, and viral etiologies have been shown to play an important role (19).

Data in Ethiopia have shown that upper respiratory tract infection and pneumonia are identified to be the highest cause of morbidity and mortality respectively. Acute respiratory tract infections are also recognized to be the second highest cause of hospital admission in Ethiopia just next to spontaneous delivery (20).

Despite this burden, many of the viral etiologies of ARI in Ethiopia are yet not known and data are limited to the influenza virus circulation. Hence this study aimed to fill the existing gap and lay a foundation for future and further research and to highlight the importance of multiplex RT-PCR during intervention measures to combat respiratory viral illnesses.

1.3. Significance of the study

- In Ethiopia, apart from influenza viruses there was no previous study about the types of respiratory viruses that cause ARI and their magnitude of circulation. Therefore this study assessed common respiratory viral agents and their circulation level among ARI cases in Ethiopia.
- This study also aimed to provide baseline information for further and large scale studies.

2. LITRATURE REVIEW

A global meta analysis and systematic review from 1995-2009 result showed that in 2005 an estimated 33·8 (95% CI 19·3–46·2) million new episodes of RSV-associated ALRI occurred worldwide in children younger than 5 years (22% of ALRI episodes), with at least 3·4 million episodes representing severe RSV-associated ALRI necessitating hospital admission. The study also estimated that 66 000–199 000 children younger than 5 years died from RSV-associated ALRI in the same year, with 99% of these deaths occurring in developing countries (21).

A SARI surveillance report from 8 sub Saharan African countries show Data were collected from 37 714 SARI cases, and 3091 (8.2%) tested positive for influenza virus. There were 1073 deaths (2.8%) reported, among which influenza virus was detected in 57 (5.3%). Case-fatality proportion (CFP) was higher among countries with systematic death reporting than among those with sporadic reporting. The influenza-associated CFP was 1.8% (57 of 3091), compared with 2.9% (1016 of 34 623) for influenza virus-negative cases (P <. 001). Among 834 deaths (77.7%) tested for other respiratory pathogens, rhinovirus [12.8%), adenovirus (64 [6.0%]), respiratory syncytial virus [5.6%], and Streptococcus pneumonia(5.3%) (22).

A recent study conducted in Brazil among 407 under five children with ARI between April 2008-2009 identified that HASV (37%), HAdV (25%), HBoV (19%),HMPV (10%) and Mychoplasma pneumonia (10%) were most prevalent respiratory viruses and a typical bacterial pneumonia. Based on the severity of the ARI, children with RSV were more likely to have moderate than mild disease (43.2% vs. 31.8%; p=0.019) compared to children without RSV infection, whilst children with Mpp infection were more likely to have moderate (12.4%) or severe (16.7%) than mild (6.4%) disease (mild vs. moderate, p=0.04: mild vs. severe,p=0.039) and concluded that children with RSV and Mpp infection were more likely to be hospitalized than children without these pathogens (23).

In 2005/2006 there was a study in Italy to evaluate the infectious agents associated with the first episode of severe acute wheezing among 85 infants aged <12 by taking nasopharyngeal swab and tested by real-time PCR and found 89.4% viral positivity among tested samples. The RSV (61.2%) found to be the dominant one followed by Influenza viruses (5.7%), hCoVs (5.7%),

hMPV (1.9%) and Bocavirus (1.9%). Fifteen (17.6) cases were infected with more than one respiratory virus (24).

A study from facility based surveillance in Philippines from Jan 2010 to March 2013 among 2,031 ILI cases 874 (43.0%) were positive for at least one of the respiratory viruses of from which Influenza virus11.1% (influenza A 5.76%, Influ B 51.2%, Influ C 0.08%) and respiratory syncytial virus 11.1% were equally predominantly detected (both were 25.7%) followed by human rhinovirus (7.5%) and hMPV (3.6%). Other viruses, including HAdV, PIV, herpesvirus, cytomegalovirus, and enteroviruses accounted for 197 (9.7%). Among the cases sampled, 1,637 (75.6%) were children aged <5 years (25).

A study done in China in 2010 among ILI cases show that 44.48% were positive for at least one respiratory virus. A single infection was identified in 369 (39.94%) patients and co-infection with two viruses was observed in 42 (4.55%). The most frequently detected agent was sFluB (9.74%), followed by RSV (7.14%), ADV (6.28%), sFluA (H3N2=4.87%) ,and HMPV (4.65%) in single infections. In co-infections, the most frequent combinations were sFluA (H3N2) plus RSV, followed by sFluA (H3N2) + PIV, sFluA (H3N2) + ADV, sFluB + RSV, PIV+ RSV, PIV +ADV, RSV +HMPV, and RSV +ADV (26).

Another study in China from 2011-2013 among SARI cases(throat swab) using multiplex RTPCR showed that 35.75 % (217/607) of cases had at least one virus, comprising 183 single infection cases (84.33 %), and 34 co- infection cases (15.67 %). The most frequently detected virus was influenza virus (30.47 %, 78/256), followed by PIVs (18.36 %, 47/256), RSV (16.02 %, 41/ 256) and AdV (14.84 %, 38/256) (27).

A study conducted from 2006-10 in a teaching hospital in South Korea identified that 63% of 5318 specimens were positive for at least one respiratory virus tested by mPCR.The infection rates were 15.8% for human rhinovirus, 14.4% for HRSV A, 9.7% forHRSV B, 10.1% for HAdV, 5.4% for influenza A virus, 1.7% for influenza B virus, 4.7% for metapneumovirus, 2.3% for human coronavirus OC43, 1.9% for human coronavirus 229E/NL63, 3.7% for human parainfluenza virus (HPIV)-1, 1.1% for HPIV-2, and 5.3% for HPIV-3. The co-infection analysis showed 17.1% of double infections and 1.8% of triple infections.HRSV was the most common

virus in children under 5 years of age, and the influenza A virus was the most prevalent virus in cases over 5 years of age (28).

A study in Iran among cases aged <17yrs with ARI problem admitted in a hospital identified viral etiologies from respiratory samples using PCR. From the study finding RV which accounted (22%) is the leading respiratory virus. The other viruses constituted Enterovirus(2%), RSV (4 %),Inf (2%), HMPV (0%),HPIV(6%), AdV (8%), HCoV (12%) and HBoV (6%) (29).

A prospective study conducted in Burkinafaso to 209 children aged <3yrs among whom 73 children were outpatients and the rest were admissions showed that 73.2% carried at least one virus and Co-infections were detected in 9.4%samples which mainly involved Rhino Virus and Entero Viruses. From the study it is indicated that 72.1% of the inpatients carried at least one virus and 75.3% of the outpatients. The RT PCR result also showed rhinovirus (n=88; 59.1%), enterovirus (n=38; 25.5%), RSV (n=24; 16.1%), influenza (n=13, including one case of influenza C; 8.7%), and one case of hMPV(12).

A study conducted in Egypt between June 23, 2009 and December 31, 2013 ARI cases from oropharyngeal/nasopharyngeal samples by multiplex pcr identified that 36% of the 5768 tested had at leat 1 virus detected.from the study the viruses most frequently detected was RSV(11.6%), followed by influenza A(8.4%),adenovirus(6.2%),influenza B(5.5%),hMPV(4.6%),parainfluenza virus 1,2 and 3 together(4.2%).The largest proportion of RSV- positive patients occurred among children aged 1–11 months, while influenza was the dominant viral pathogen detected among patients >5 years of age. Also the study showed that most commonly detected co- infection was adenovirus and RSV (30).

A prospective study in Cameroon conducted in 2009 among ILI cases identified that at least 1 respiratory virus detected among 65.1% of tested specimens (365 of 561). Overall, influenza virus was the most commonly detected virus (28.2% of specimens), followed by human rhinovirus (17.8%); parainfluenza virus (PIV) types 1–4 (7.5%); enterovirus (5.9%); respiratory syncytial virus (RSV; 5.7%); human coronavirus (HCoV) OC43, 229E, NL63, and HKU1 (5.3%); and human metapneumovirus (HMPV; 5.0%). RSV (26 of 31 specimens [83.9%]), PIV (30 of 39 [76.9%]), and HRV (64 of 99 [64.6%]) were most common among children <5 years of age. Confections were found in 53 of 365 positive specimens (14.5%), and most (71.7%) were in

children <5 years of age. While influenza virus, enterovirus, RSV, and HMPV had a defined period of circulation, the other viruses were detected throughout the year (2).

A study held in South Africa from 2009-2010 among 8173 SARI cases of all age groups in 6 government hospitals reviled a result of at least 1 respiratory virus detected in 57% of the samples tested(40% single virus detected and 17% coinfections).Of 8173 patients from the study the most common viruses were RV (25%), RSV (14%), AdV (13%), and influenza A virus (5%). The study also indicated that Influenza virus, RSV, PIV type 3, and hMPV showed seasonal patterns. From the coinfections identified rhinovirus and adenovirus, rhinovirus and RSV, RSV and adenovirus, and adenovirus and enterovirus were frequently observed in a single cases among cases more with more than 1 respiratory virus (31).

A health facility based surveillance study result in Kenya among under 5yrs SARI cases show overall prevalence viral etiology of 46.8% of the cases tested of which RSV accounted for the highest etiologic fraction (16.2%), followed by adenoviruses (10.6%), parainfluenza viruses (7.0%), and influenza A and B combined (6.7%), and hMPV (6.3%).From the study Chylamdae pneumonia and Mychoplasmapneumonia (both known to cause atypical pneumonia) not detected in any of respiratory specimens by multiplex PCR (9).

Another study in Kenya among 6264 (both ILI and SARI) cases in two refugee camps from 2007-10 showed that 49.8% were positive for at least one viruscontributed from 46.1% of ILI and 51.3% SARI cases positivity.Viral positivity among the all oropahryngeal/nasopharyngeal samples were for HAdV(21.7%), HRSV(12.5%), HMPV(5.7%), HPIV(9.4%), influenza A(9.7%) and influenza B was 2.6%. The annual rate of SARI hospitalisation for 2007-2010 was 57 per 1000 children per year. Virus-positive hospitalisation rates were 14 for AdV; 9 for RSV; 6 for PIV; 4 for hMPV; 5 for influenza A; and 1 for influenza B. The rate of SARI hospitalisation was highest in children < 1 year old (156 per 1000 child-years) (32).

Published influenza surveillance study data in Ethiopia from 2008-2010 among acute respiratory illness cases (both ILI and SARI) indicated that influenza viruses were detected in 7% of the tested samples. The majority of the positive samples were due Influenza A (92%) virus while the rest were due Influenza B(8%) virus (33).

3. OBJECTIVE OF THE STUDY

3.1 General Objective

- To assess respiratory viruses among acute respiratory illness cases in selected health facilities of Ethiopia.

3.2 Specific Objectives

- To identify common viral respiratory etiologies among influenza like illness and severe acute respiratory illness cases using real time PCR.
- To assess the existence of respiratory virus co-infections among acute respiratory illness cases.

4. MATERIALS AND METHODS

4.1. Study design and period

This laboratory based prospective (laboratory analysis) and retrospective (for the data) study was conducted from 1, July to 31, October 2017 at National Influenza Reference Laboratory (NIL) of Ethiopian Public Health Institute (EPHI). Throat / throat and Nasopharyngeal swab samples collected for national influenza surveillance from 2015 to 2016 were analyzed by multiplex real time PCR to detect common respiratory viruses among ARI cases.

4.2. Study site

This study was conducted in selected health facilities found in three regions (Amhara, Southern nations nationalities and peoples region and Tigray region) and one city administration (Addis Ababa City). In total, samples collected from seven government health facilities (four hospitals and three health centers) were analyzed. The hospitals were served as SARI surveillance sites while the health centers were designated for ILI surveillance (Table 4.1).All the selected health facilities included as study site for this study were influenza sentinel surveillance sites and were selected purposely based on patient load, staff profile, and existence of the necessary infrastructure to support surveillance efforts (33).

4.3. Source population

The source population was all samples collected among acute respiratory illness cases from influenza sentinel surveillance sites from 01, January, 2015 to 31, December, 2016.

4.4. Study population

The study population was all samples collected among acute respiratory illness case from influenza sentinel surveillance sites from 01, January, 2015 to 31, December, 2016 with sufficient samples volume and completed data.

4.5. Sample size determination

Sample size calculation was performed using single population proportion formula and since there is no published study data about these respiratory viruses and there positivity rate in Ethiopia the value of p was 0.5.

$$\mathbf{n = z^2 P (1\text{-}P) / d^2}$$

Where n = sample size,

Z = Z statistic for a level of confidence (95% level of confidence; z=1.96),

P = Reasonable estimate which is 0.5 because no available data in this respect in Ethiopia), and

d = precision (in proportion of one; if 5%, *d* = 0.05).

$n = 1.96^2 * 0.5(1\text{-}0.5)/0.05^2$ n= 384

So by considering insufficient sample volumes and incomplete demographic data we calculated 10% contingency and the total minimum number of samples to be tested will be 422.

4.6. Sampling procedure

The total calculated Sample size was first equally allocated for ILI and SARI case samples in order to give equal chances. Then, to get the required samples for the study, 211 samples were selected from each case category using systematic random sampling technique as indicated below.

Sampling from ILL cases: The total number of samples received from ILI surveillance sites within the study period was 1370. The following procedure were used to select 211 samples, first the sampling interval was calculated by dividing the total number of samples by the targeted sample size for the group (1370/211) and the sampling interval calculated to be 6. Then, the first sample was selected between 1 and 6 by lottery method. Then after selecting the initial number by this lottery method every 6^{th} samples was selected until the desired samples obtained.

Sampling from SARI cases: The total number of samples received from SARI surveillance sites within the study period (within the two years) was 423. All these samples were registered in a serial order using standard registration book based on the study site. The sampling interval was calculated to be 2 to get 211 samples from SARI case category and the first sample was randomly selected between 1 or 2 using lottery method. Every 2nd sample was selected until the desired 211 number was reached. After these samples ID with demographic information were selected from the registration book with our prepared checklist we collect stored samples from storage boxes in freezer for analysis.

Table 4.1: Total Number of samples selected and analyzed by study sites and case category (2015- 2016)

Case Category	Name of the Facility	Total number of samples received	Number of samples Selected and tested
ILI	Shiromeda HC	814	125
	Kolfe HC	518	80
	Akaki HC	38	6
	Total ILI	**1370**	**211**
SARI	Yekatit HO	132	66
	Adari HO	58	29
	Felege Hiwot HO	132	66
	Mekele HO	101	50
	Total SARI	**423**	**211**
Total ILI and SARI		**1793**	**422**

4.7. Inclusion criteria

All samples collected from influenza sentinel surveillance sites from 01, January 2015 to 31, December 2016 with adequate volume and completed case based information were used for this study.

4.8. Study variables

4.8.1. Dependent variables

- Positivity rate of respiratory virus
- magnitude of multiple respiratory virus in the same sample

4.8.2. Independent variables

- Age group
- Gender
- Sentinel site
- Case category of a patient (ILI or SARI)

4.9. Data collection

A standardized checklist was used to collect socio demographic data of the patients, sample ID and any other relevant information for this study. This data was collected from existing completed case based formats and from archived registration book by the principal investigator using a prepared sample tracking sheet **(Annex 1.1)**

4.10. Laboratory Method

4.10.1. Nucleic Acid extraction

The left over samples from previous influenza virus detection that were selected from its long term storage freezer and extracted using Qiagen extraction procedure strictly following the standard operating procedure (SOP). This extraction was a manual and silica membrane based type of extraction.

The extraction process had a series of steps and involved lysis buffer, carrier RNA, absolute ethanol, two types of washing buffers and elution buffer. The sample was first lysed under the highly denaturing Buffer AVL to inactivate RNases and DNases and to ensure the NA to come out from the rest of the viral components and this was also facilitated by short vortex and centrifugation. Based on the number of samples processed measured volume of Carrier RNA is added to the Buffer AVL to facilitate the binding of viral NA to the membrane and to prevent possible degradation of the viral NA due to any residual RNase and DNase activity. Molecular grade absolute ethanol was added for isolation of intact viral NA. Viral NA from the lysate added and bound on to the QIAamp silica membrane binding columun in two step 1 minute centrifugation steps for each. The NA binds to the membrane and contaminants will be efficiently washed away in two steps using two different wash buffers (washing buffer 1 and 2). High quality NA will be then eluted by adding elusion buffer. The elusion buffer, in addition to function for detachment of the NA from the silica membrane, served as a preservative for the purified NA until the NA analyzed by RT PCR. The overall procedure of this extraction contains lysis, binding, washing and elusion process.(for detailed instruction see Annex 3 bellow)

Oropharyngeal/ Oropharyngeal + Nasopharyngeal swab sample

⇩ AVL +carrier RNA → Vortex and then centrifugation

Lysis of Sample

⇩ Addition lysate to the binding column → centrifugation

Binding in to silica membrane or column

⇩ Addition of wash buffer 1 and then 2 → centrifugation

Washing (two times) → and then Add buffer AVE

⇩

Elusion of NA (ready to use)

Table 4.2: Reagents and Chemicals with required volume for extraction of a sample taken among ARI cases in selected health facilities of Ethiopia 2015-2016.

Reagent	**volume added(ul)/sample**
Buffer AVL	896
Carrier RNA	9
Sample	224
Internal control	2.24
Absolute ethanol	896
Wash solution(AW1)	500
Wash solution (AW2)	500
Elusion buffer/AVE	96

4.10.2. Master Mix preparation and PCR Set up

Master mix was prepared by adding 12.5ul buffer, 1.5ul of primer probe mix and 1ul enzyme mix for each reaction and for specific pathogen group using 1.5ul DNase RNase free micro tubes in clean room (laminar flow). Of the prepared master mix 15ul was added to the appropriate 96 well PCR plat on the cold rack. The 10ul of the elute was added for the respective target pathogen and 25ul of final reaction volume was loaded to PCR. (For detailed instruction see Annex 4 bellow).

The viral RNA transcribed into cDNA using a specific primer mediated reverse transcription step followed immediately in the same tube by polymerase chain reaction for RNA viruses while the DNA of different pathogens amplified simultaneously in the same tube by polymerase chain reaction. The presence of specific pathogen sequences in the reaction was detected by an increase in fluorescence observed from the relevant dual-labeled probe, and was reported as a cycle threshold value (Ct) by the real time thermo cycler. Depending on the presence of exponential amplification the result interpreted as negative, invalid or positive**.**The master mix was prepared using the fast track diagnostics protocol.

4.10.3. Result interpretation

For samples to be considered as positive; all the following criteria should be fulfilled:

- Internal control and positive control cycle threshold(CT) value should be less than 33
- No amplification detected for negative control.
- Standard amplification curve should be observed for the sample

But the above two are fulfilled and when standard amplification curve was not observed for the samples the result was interpreted as negative.

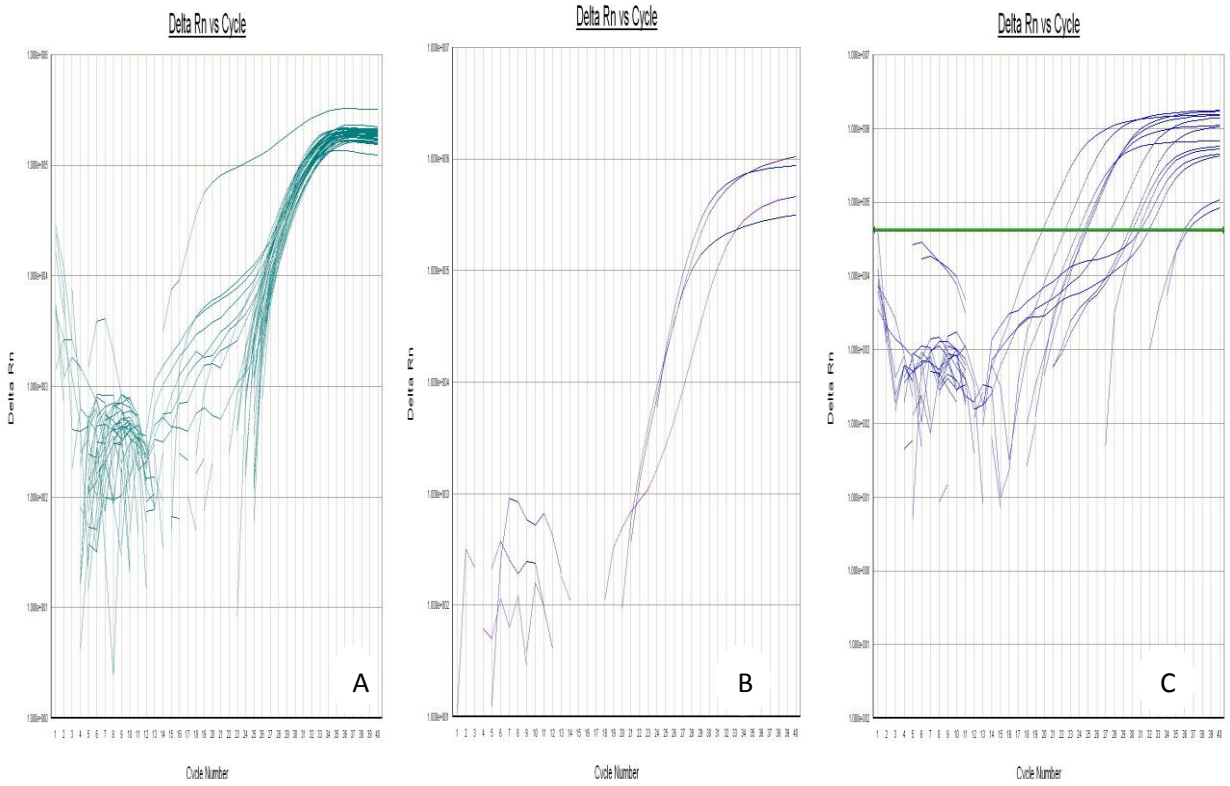

Figure 1: Standard amplification curves for A) Internal control B) four Positive controls: Respiratory syncytial virus taken from a single reaction PCR well, Adenovirus, Enterovirus and Human Parechoviruse. C) Positive samples for Respiratory syncytial virus.

4.11. Quality assurance

- SOPs for sample extraction, master mix preparation and multiplex RT- PCR set up adapted and strictly followed. Small no of samples extracted and analyzed at a time in order to prevent the problem of contamination.
- The RT-PCR set up was always checked by other laboratory personnel for correctness of its time; cycle no, temperature, run mode, target test and other crucial components before the start up of the PCR run.
- An internal control, Negative Control and a Positive Control were done for every run to verify that the sample collection and extraction, and the master mix preparation steps were performed correctly.
- Result interpretation and recording was checked by other lab personnel in the laboratory.

4.12. Data entry and analysis

All patient demographic data and laboratory results entered into Excel spread sheet 2007 and was analyzed using IBM SPSS Software Version 20.0. Using actual number of cases and proportions the categorical variables such as sex, ILI/SARI case classification, referring health facility, type of virus and co-infection were presented using percentage. Binary logistic regression was used to see the relation of viral acquisition and independent variables. Chi-square test was used to compare the difference of magnitude of viral positivity among the independent variables. $P \leq 0.05$ was considered statistically significant.

Data quality was maintained by recording result of each sample from the RT-PCR analysis after the end of every run using the printed PCR plate/worksheet and rechecking it before and after the data entered to the excel spread sheet and SPSS software

4.13. Operational definitions

Influenza like illness (ILI)	A person, child or adult with Sudden onset of fever > 38^0C AND Cough or sore throat in the absence of other diagnosis.
Severe acute respiratory illness(SARI)	Cases that have fever > 38 or reported history of fever and cough or sore throat and Shortness of breath or difficulty breathing with or without clinical or radiographic findings of pneumonia AND requiring hospitalization OR any person who died of an unexplained respiratory illness
Multiple detection	Detection of more than one respiratory virus in a single sample.

5. ETHICAL CONSIDERATIONS

The study research protocol was reviewed and approved by the departmental research and ethics review committee (DRERC) of the medical laboratory sciences, school of allied health sciences, College of Health Sciences; Addis Ababa University. Permission was obtained from Ethiopian Public Health Institute. The privacy and confidentiality of each individual participant from whom samples had been collected was ensured. Personal name and identifier never be used at any stage of the research project.

6. RESULTS

6.1. Demographic Characteristics

A total of 422 samples were tested which consisted of 52.1% (220/422) females and 47.9% (202/422) males. The age group ranged from age of 7 days to 80 years with median and mean age of 6 yrs and 14.5yr respectively. Under five children accounted for 47.6% (201/422) of the total tested samples. Equal numbers of ILI and SARI samples (211 from each category) were tested. All samples were tested for RSV A/B, RV, HCoV-OC43, HCoV-NL63, HCoV-HKU1, HCoV-229E, PIV1-4, HAdV, HBoV, HPeV, EV, HMPV A/B and FLU C virus. Relatively larger numbers of samples were tested from Shiromeda health center (from ILI), Yekatit 12 hospital and Felegehiwot hospitals (from SARI) as total samples collected during 2015-2016 were larger compared to other study sites.

Table 6.1: Demographic data and tested samples from selected sites among ARI cases in Ethiopia, 2015-16

Facility	Female							Male							Grand Total
	Age group in Years						Female Total	Age group in Years						Male Total	
	<1	1-4	5-14	15-24	25-59	≥60		<1	1-4	5-14	15-24	25-59	≤60		
ADR	6	6	0	0	2	0	14	4	5	3	1	2	0	15	29
AKA	1	0	1	2	0	0	4	1	1	0	0	0	0	2	6
FHW	13	6	1	3	2	0	25	21	10	4	0	3	3	41	66
KOL	1	9	9	14	16	3	52	2	6	5	7	8	0	28	80
MKL	2	14	1	0	1	0	18	18	11	1	1	1	0	32	50
SHM	0	3	18	12	37	6	76	1	1	14	10	14	3	49	125
YEK	20	6	3	0	2	0	31	18	15	1	0	1	0	35	66
Total	**43**	**44**	**33**	**31**	**60**	**9**	**220**	**65**	**49**	**28**	**19**	**35**	**6**	**202**	**422**

Key: ADR=Adari Hospital, AKA=Akaki health center, FHW=Felegehiwot hospital, KOL=kolfie health center, MKL=Mekle hospital, SHM= Shiromeda Health center, Yekatit 12 hospital

6.2. Respiratory virus distribution

From a total tested samples one or more viruses was identified in 55.5% (234/422) samples which consisted of 62.9% (127/202) positive samples from males and 48.6% (107/220) female sample. RV was the most frequently detected respiratory virus which accounted for 18.7% (79) positivity followed by RSV A/B 12.8% (54), HAdV, 11.4% (48) and PIV 1-4, 7.3% (31) **(Table 6.2)**.

Respiratory viruses were detected in all age groups with different frequencies. Among the total tested samples positive test results in children under 1 years of age were 67.6% (73), 1-4yrs, 71.0% (66) and children 5-14years of age were 54.1% (33). In total 67.1% (139/201) samples were from under five children and from the binary logistic regression analysis it was 2.44 (95% AOR= 2.44 (1.13-5.27), p =0.023) times more likely to have at least one respiratory virus compared to children 15-25 years old (Table 6.6). The number of positive samples and the positivity proportion within the age group in cases aged 15-24 years, 25-60 years and 60 years were 40.0% (20), 37.9% (36) and 6(49.0%) respectively**.**

At least one respiratory virus was detected in 43.6 % (92) of ILI samples and 67.3% (142) of SARI samples and the overall positivity difference seems big even thought the adjusted odds ratio from binary logistic regression analysis output showed insignificant association (AOR 95% CI=1.40 (0.80-2.24), p=0.24) (Table 6.6).The number of positive samples and proportions among the tested ILI case samples were RV 11.8% (25), HPIV1-4 9.5% (20), HCoV 8.1% (17), HMPV A/B 7.1% (15) and these were the most frequently detected respiratory viruses detected from ILI samples. HRV, HRSV A/B, HAdV were detected in 25.6% (54), 22.7% (48) and 18.0% (38) of SARI samples respectively (Table 6.3).

Table 6.3: Respiratory virus distribution with age, sex and case category in selected health facilities of Ethiopia, 2015-2016:

Demog-raphic Data	RESPIRATORY VIRUS N(%)										Positivity (%)
	RV	RSV	HAdV	PIV 1-4	EV	HPeV	HCov	HBOV	HMPV	INFC	
Sex											
Female (N=220)	33	25	19	15 **	11	16	12[a]	10	9	0	107
	(15.0)	(11.4)	(8.6)	(6.8)	(5.0)	(7.3)	(5.4)	(4.5)	(4.1)	(0.0)	(48.6)
Male (N=202)	46	29	29	16	15	8	14	6	10	1	127
	(22.8)	(14.3)	(14.4)	(7.9)	(7.4)	(4.0)	(6.9)	(3.0)	(5.0)	(0.5)	(62.9)
Age in years											
<1 (N=108)	27	27	18	4*	7	15	5[b]	6	2	0	73
	(25.0)	(25.0)	(16.7)	(3.7)	(6.5)	(13.9)	(4.6)	(5.5)	(1.9)	0	(67.6)
1-4 (N=93)	20	22	20	8	10	9	3	6	5	0	66
	(21.5)	(23.6)	(21.5)	(8.6)	(10.8)	9.7)	(3.2)	(6.4)	(5.4)	0	(71.0)
5-14 (N=61)	14	1	4	7*	3	0	2	2	5	1	33
	(22.9)	(1.6)	(6.6)	(11.5)	(4.9)	(0.0)	(3.3)	(3.3)	(8.2)	(1.6)	(54.1)
15-24 (N=50)	6	0	4	5	1	0	5	1	4	0	20
	(12.0)	(0.0)	(8.0)	(10.0)	(2.0)	(0.0)	(10.0)	(2.0)	(8.0)	0	(40.0)
25-59 (N=95)	11	4	2	6	3	0	8[b]	1	2	0	35
	(11.6)	(4.2)	(2.1)	(6.3)	(3.1)	(0.0)	(8.3)	(1.0)	(2.1)	0	(36.8)
≥60 (N=15)	1	0	0	1	2	0	3	0	1	0	7
	(6.7%)	(0)	(0.0)	(6.7)	(13.3)	(0.0)	(20.0)	(0.0)	(6.7)	0	(46.7)
Case											
ILI (N=211)	25	7	10	20**	10	1	17[b]	1	15	1	92
	(11.8)	(3.3)	(4.7)	(9.5)	(4.7)	(0.5)	(8.1)	(0.5)	(7.1)	(0.5)	(43.6)
SARI (N=211)	54	47	38	11**	16	23	9[b]	15	4	0	142
	(25.6)	(22.3)	(18.0)	(5.2)	(7.6)	(10.9)	(4.3)	(7.1)	(1.9)	(0.0)	(67.3)
Over all positivity	79	54	48	31	26	24	26[a]	16	19	1	234
	(18.7)	(12.8)	(11.4)	(7.3)	(6.2)	(5.7)	(6.2)	(3.8)	(4.5)	0.2	(55.5)

**Two samples were found positive for both PIV2 and PIV4; numbers in brackets are percentages,

*One sample contained 1 HPIV2 and HPIV4; [a] Two cases carried two human corona subtypes

[b] one cases carried two human corona subtypes, N=number

The positivity rate across the study sites varies from 42% (28/66) of Felegehiwot Hospital to 92% (46/50) of Mekele hospital within the SARI sites while Kolfie health center and Shiromeda health center showed a positivity rate of 53.7% (43/80) and 39.2% (49/125) respectively. All the six samples from Akaki hospital were found negative for anyy of the tested viruses.

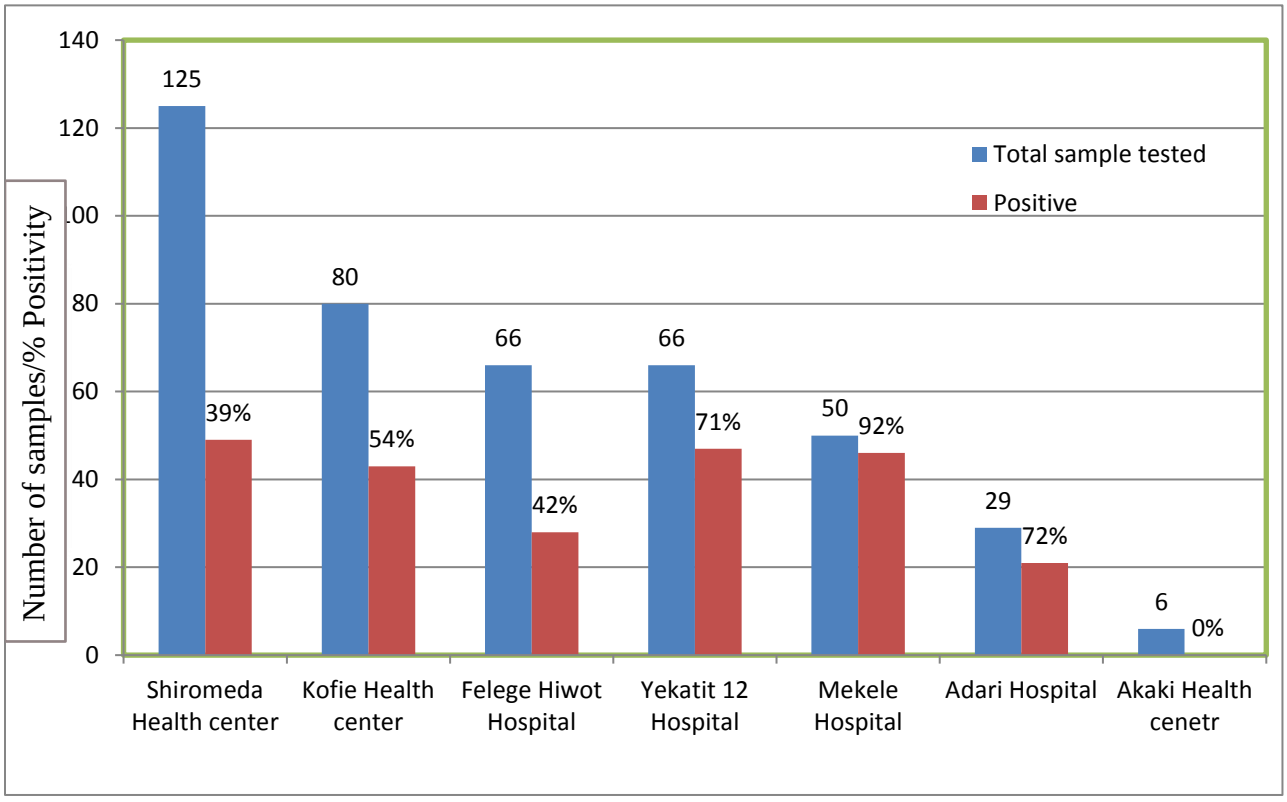

Figure 6.2: Respiratory virus positivity across the selected study health facilities of Ethiopia 2015-2016.

The study also identified the four types of Parainfluenza and human corona viruses among tested samples. PIV-3, 42.4 % (14/33) was dominantly detected within the group followed by PIV-4, 27.3% (9) and PIV-1, 18.2% (6). Among the human corona virus group HCoV-OC43 (39.4%, 11/28) and HCoV-229E (28.6%, 8/28) detected most frequently among the tested samples. HPIV2 and HPIV4 co-detected in two samples at the same time. HCoV-NL63 with HKU1 was detected in one sample and HCoV-OC43 with HCoV-229E in the other (**Figure 6.3**).

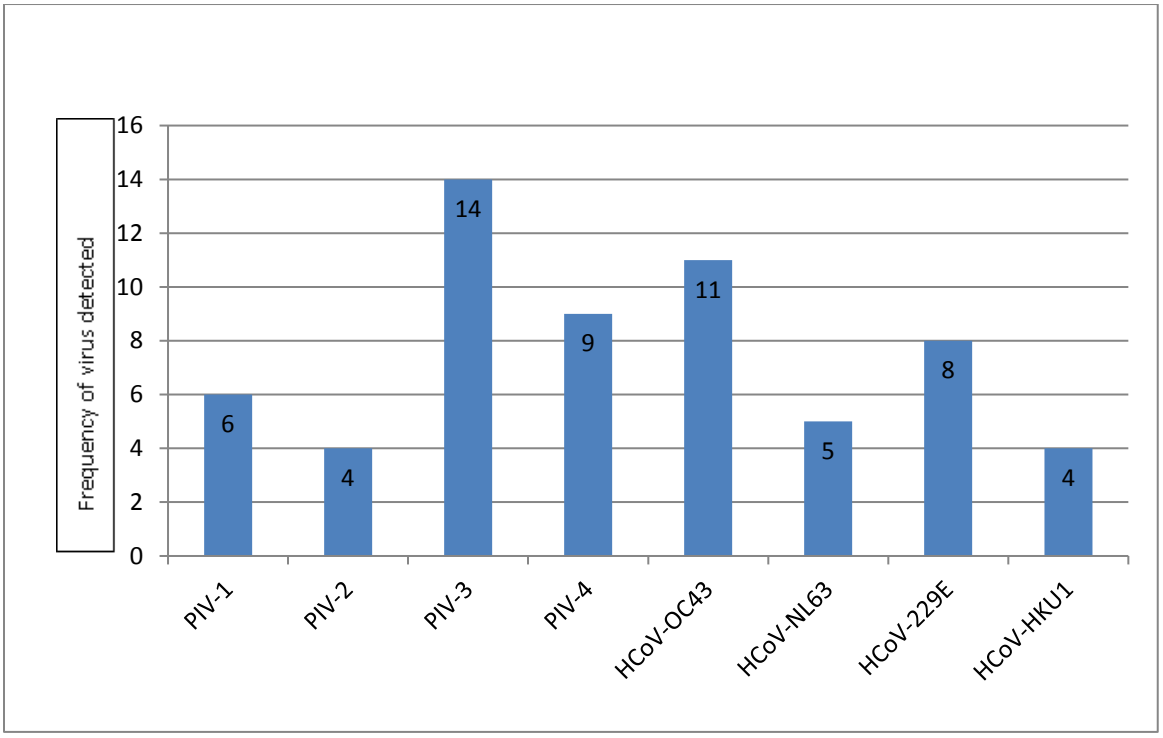

Figure 6.3: Distribution of Parainflunza viruses 1-4 and Human corona virus (OC43, NL63, 229E and HKU1) positive samples among ARI cases in selected health facilities of Ethiopia, 2015-2016.

The number of tested samples in each month varied from 24(July) up to 58(February).The overall respiratory virus positivity proportion was highest during the rainy season and declines in the spring but larger number of samples with a positive respiratory viruses were found in dry season (February and March) even though the positivity proportion was masked by also large number of negative samples during this month (Figure 6.2 A). RV and HAdV circulated throughout the year with peak numbers at March. RSV A/B was also circulated throughout the year and become peak at August and December **(Figure 6.4 B)**.

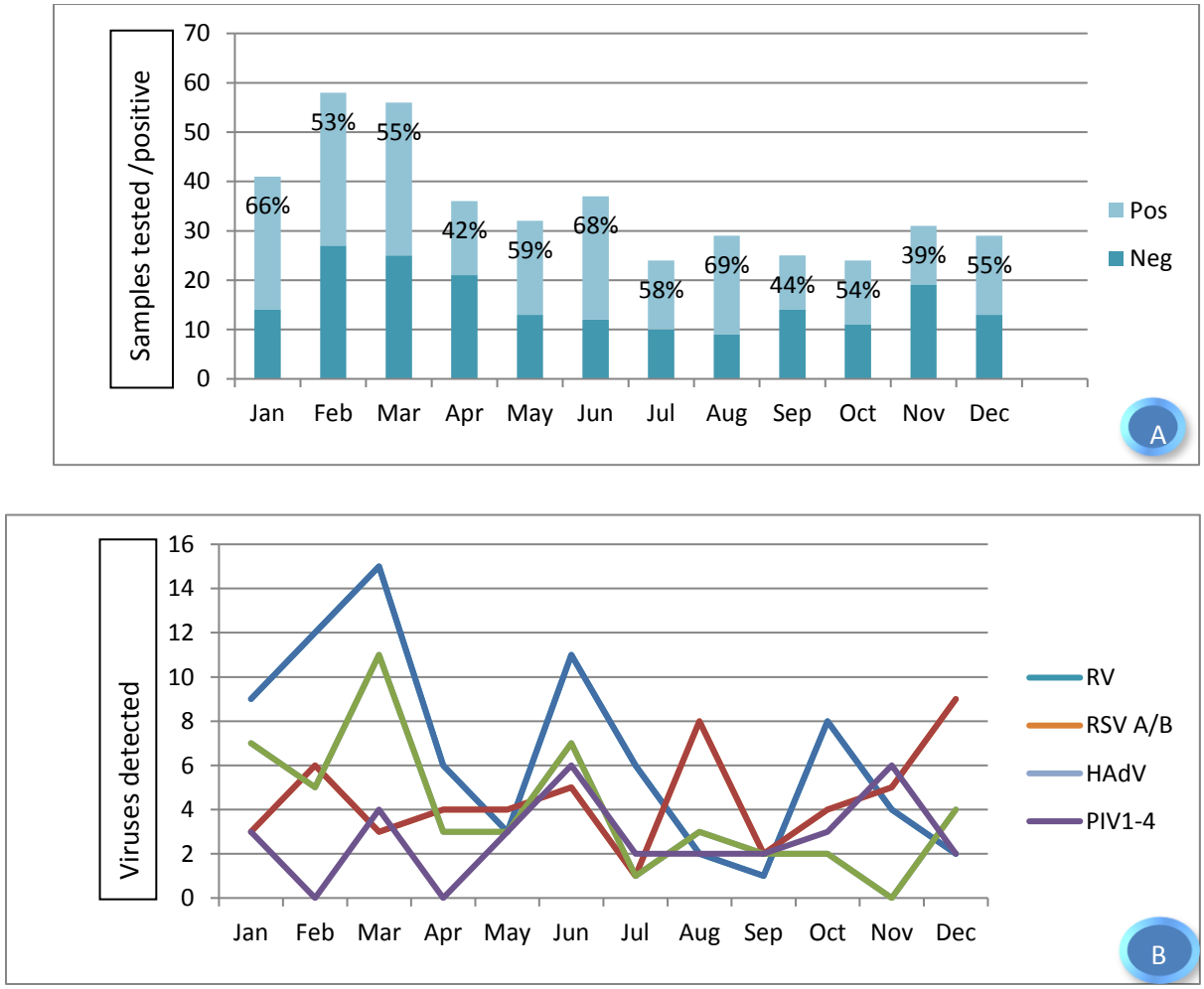

Figure 6.4: Monthly distribution of all tested samples and the positivity rate of respiratory viruses (A) and monthly distribution of Rhino virus, Respiratory syncytial virus, Human adenovirus and Parainfluenza virus (B) among acute respiratory illness cases in selected health facilities of Ethiopia, 2015-2016.

6.3. Multiple virus concurrently identified

From our study, we confirmed that 18.2% (77) samples possessed more than one respiratory virus and the most frequently co-detected virus with others was HAdV 68.7% (33/48) followed by RV, 39.2% (31/79) and RSV A/B, 53.7% (29/54). All HPeV positive samples 100.0% (24/24) were also positive for one or more other tested respiratory viruses. HCoV, 39.3% (11/28); PIV, 39.4% (13/33) and HMPV 26.3% (5/19) were detected simultaneously with one or more other respiratory viruses. Frequent viral combinations were observed between RSV/HPeV in 19 samples followed by RV/AdV which occurred among 18 samples **(Table 6.4)**.

Table 6.4: The number of samples with more than 1 respiratory viruses and combination of respiratory viruses within the samples, Ethiopia 2015-2016

Respiratory virus	RV	RSV	AdV	HBoV	PIV1-4	HCoV	EV	HMPV	HPeV	FLUC	Co-detection rate[b]
RV		2	**18**	**8**	4	4		1	1	1	39.2%(31/79)
RSV			5	1	2	1	3	0	**19**	0	53.7%(29/54)
HAdV				7	5	2	4	1	2	0	68.7%(33/48)
HBoV					2	1	1	0	0	0	68.7%(11/16)
PIV1-4					2*	1	2	2	1	0	39.4%(13/33)
HCoV						2**	1	1	2	0	39.3%(11/28)
EV								0	1	0	73.1%(19/26)
HMPV											26.3%(5/19)
HPeV											100.0%(24/24)
Single[a]											37.2%(157/422)
Multiple[a]											18.2%(77/422)
Total Pos											55.5%(234/422)

Key: a= from total samples tested , [b] =compared to each total Positive vurus
* HPIV2 with HPIV4 detected in two samples at the same time
**HCoV43 detected together with HCoV229 in one sample and HKU1 detected with HCoV229 in the other sample

Out of the tested 422 samples the samples identified to be positive were 55.5% (234) and from these positive samples 37.2% (157/422) were single infections while 18.2% (77/422) tested samples were positive for two or more respiratory viruses with 19.3% (39/202) among males and 17.3% (38/220) among females. Among the multiple respiratory viruses detected 14.9% (63/422) were two viruses while 2.8% (12/422) samples were identified to be positive for three respiratory viruses and 0.5 % (2/422) were positive for four tested respiratory viruses. These two samples were from under five children from SARI (Mekele hospital) site. The majority of the multiple viruses were found among under five children (76.6%, 59/77) while the other age groups accounted only for 23.4% (18/77). More than one respiratory virus was also found in 7.6% (16/211) ILI and 28.9% (61/211) of SARI samples **(Table 6.5).**

Table 6.5: Summary on positivity and multiple respiratory viruses with gender, age category and case Category in selected health facilities of Ethiopia, 2015-2016.

Demographic data	Respiratory Virus					Multiple respiratory sample
	Neg	Pos For 1 Virus	Pos For 2 Virus	Pos For 3 Virus	Pos For 4 Virus	
Sex						
Female (N=220, 52.1%)	113(51.4%)	69(28.2%)	31(17.3%)	6(2.7%)	1(0.5%)	38(17.3%)
Male (N=202, 47.9%)	75(37.1%)	88(41.6%)	32(16.3%)	6(4.0%)	1(1.0%)	39(19.3%)
Age						
<1 yrs(N=108)	35(32.4%)	43(39.8%)	22(20.4%)	8(7.4%)	0(0.0%)	30(27.8%)
1-4yrs(N=93)	27(29.3%)	37(38.9%)	23(24.7%)	4(4.3%)	2(2.1%)	29(31.2%)
5-14(N=61)	28(45.9%)	25(41.0%)	8(13.1%)	0(0.0%)	0(0.0%)	8(13.1%)
15-24(N=50)	30(60.0%)	14(28.0%)	6(12.0%)	0(0.0%)	0(0.0%)	6(12.0%)
25-59(N=95)	60(62.5%)	32(33.7%)	3(3.1%)	0(0.0%)	0(0.0%)	3(3.1%)
≥60yrs(N=15)	8(53.3%)	6(40.0%)	1(6.7%)	0(0.0%)	0(0.0%)	1(6.7%)
Case						
ILI (N=211)	119(56.4%)	76(36.0%)	15(7.1%)	1(0.5%)	0(0.0%)	16(7.6%)
SARI(N=211)	69(32.7%)	81(38.4%)	48(22.4%)	11(5.2%)	2(0.9%)	61(28.9%)
Total	188(44.5%)	157(37.2%)	63(14.9%)	12(2.8%)	2(0.5%)	77/422(18.2%)

From the binary logistic regression analysis; sex seems less important in the overall and multiple virus acquisition of respiratory viruses. Among the total multiple virus positive samples 74.0% (57/77) were found among under five children even though the adjusted odds ratio from binary logistic regression indicated that association was not statistically significant (p=0.32, and AOR 95%CI=1.70(0.60-4.84)). More over the odds of having multiple respiratory virus per sample in severe acute respiratory illness cases was 2.79 times (95% AOR=1.31-5.91, p= 0.007) more than samples from influenza like illness cases (**Table 6.6)**

Table 6.6: Association of Respiratory viruses distribution with independent variables among ARI cases tested from selected health facilities of Ethiopia, 2015-2016

Demographic data	Multiple viruses per sample					Total positivity				
	N (%)	COR (95%CI)	P-value	AOR (95%CI)	P-value	N (%)	COR (95%CI)	P -value	AOR (95%CI)	P -value
Sex										
Female (N=220)	38 (17.3)			Ref*		107 (48.6)				
Male (N=202)	39 (19.3)	1.15 (0.70-1.88)	0.59	NA	NA	-	1.79 (1.21-2.64)	0.003	1.48 (0.98-2.23)	0.061
Age in Years										
0-4 (N=201)	57 (28.5)	3.41 (1.38-8.45)	0.008	1.70 (0.60-4.84)	0.32	73 (69.1)	3.36 (1.77-6.39)	0.00	2.44 (1.13-5.27)	**0.023****
5-14 (N=61)	8 (13.1)	1.05 (0.34-3.26)	0.92	0.89 (0.28-2.81)	0.84	33 (54.1)	1.77 (0.83-3.77)	0.14	1.65 (0.77-3.55)	1.00
15-24 (N=50)	6(12.0)			Ref*		20 (40)				
25-59 (N=95)	5 (3.1)	0.39 (0.11-1.36)	0.14	0.36 (0.10-1.25)	0.11	35 (36.8)	0.86 (0.43-1.74)	0.67	0.84 (0.45-1.71)	0.64
≥60 (N=15)	1 (6.7)	0.48 (0.05-4.29)	0.51	0.40 (0.04-3.67)	0.42	7 (46.7)	1.50 (0.46-4.93)	0.50	1.50 (0.45-4.99)	0.51
Case category										
ILI	16(7.6)			Ref*		92(43.6)				
SARI	61(28.9)	4.96 (2.75-8.94)	0.00	2.79 (1.31-5.91)	**0.00****	142 (67.3)	2.66 (1.79-3.95)	0.00	1.40 (0.80-2.46)	0.24

** = Indicates *statistically association (p<0.05); Ref* =Reference, COR=crud odds ratio,AOR=Adjusted Odds ratio: Numbers in brackets are percentage;* NA: not applicable (the P value >0.05 for crude odds ratio and no need to calculate the P value and Adjusted odds ratio in the case of sex)

7. DISCUSSION

A total of 422 samples were tested during the year from 2015-16 for common respiratory viruses that can cause ARI including RV,RSV A/B, PIV 1-4,HCoV,HMPV,HPeV, EV,HAdV, HBoV and INF C viruses. Among the total tested samples 55.5% (234/422) were positive for at least one respiratory virus while the rest 44.5% (188/422) samples were negative for any of the tested virus. Our study finding was in agreement with findings done in Korea (63.0%) (28), South Africa (57.0%) (31), Kenya (49.8%)(32), Gabon (61.0%) (34) and China (55.7%)(35). However the positivity rate from our study was lower than study finding in Brazil (85.0%) and the reason might be due to the study subject(all inpatients), study season (during peak season) and type of sample they used (nasopharyngeal aspirate) (36). From our study, samples from under five children showed a larger positivity rate 69.1% (139/201). Similar overall viral positivity was reported in Egypt (67% positivity of 1-11 month) (30), Gabon (68.1% among under five children) (34), South Africa (76.5% for age of 0-1years) (31) and Australia 0-4 yrs children viral positivity of 67.2% from the influenza surveillance sites study result (5).

The ILI samples were found to be 43.6% (92/211) and this study result was similar with other studies in Philipins (43.0%) (25), Hong Kong (46.7%) (37), and China(44.5%) (38), positivity among ILI cases. The most frequently identified respiratory virus among the ILI cases was HRV 11.8% (25) followed by PIV1-4 9.5% (20), HPMV 7.1% (15), and HAdV 4.7%(10). This result was concordant with the study done in Cameroon (RV=17.8%, PIV1-4=7.5%,HMPV A/B=5.0%) (2). The number and positivity rate among SARI samples identified in this study was 67.3% (142) and the dominant viral etiologies detected were RV and RSV A/B which accounted 25.6% (54) and 22.3%(47) respectively. A study in Burkinafaso among SARI cases showed similar finding (73.2%) (12).

The respiratory virus positivity ranged from 42.4% (28/66) from Felegehiwot hospital to 92.0% (46/50) from Mekele hospital. The difference might be due to the sample type/number collected from each case and technical skill of sample collectors. The type of the sample collected in Mekele hospital was the combined throat and nasopharyngeal swab for each case and studies indicated that combined samples increase the positivity rate(39). The other possible reason for lower positivity for samples from Felegehiwot hospital might be related lack of appropriate case

detection, sample collection and sample storage skill of the sample collectors. The positivity of the samples from ILI sites were 0% (0/6) for Akaki, 39.2% (49/125) for Shiromeda and 53.7% (43/80) for Kolfie health centers.

From our overall study RV was most frequently detected virus which accounted for 18.7% (79/422) of the total samples tested. Similar positivity rate with study in Korea(20.8%) (28) ,Iran(22.0%) (29) and South Africa(25.0%) (31). Higher RV positivity was found among age groups less than 15years (0-1 yrs, 24.1%; 1-4 yrs, 26.9%, 5-14 yrs, 26.2%) than positivity rates among age $\geq$15yrs. SARI samples showed 25.6% (54) positivity which was similar with similar study in south Africa (40) while ILI positivity was 11.8% (25) with a similar finding in Cameron on ILI cases(17.8%)(2).

Our study identified that RSV was detected in 12.8% (54) of the tested samples and 90.7% (49/54) of RSV positive samples was from under five children. The RSV was also detected more frequently from SARI (22.3%, 47/211) cases than ILI (3.3%, 7/211).The overall HRSV positivity of our finding was in agreement with studies done in South Africa(14.0%) (31) Kenya (12.5%) (32), Gabon(13.5%) (34) and Southern China (13.7%) (41). However other study in Brazil showed 37.3% positivity which is higher than that of ours may be due to reason that the study participants were entirely under five children(42). In many studies RSV positivity was associated with acute lower respiratory illness in children with SARI (5,9,13,21,23,38).

HAdV was detected in 48 (11.4%) of the total tested samples which was similar with the study finding in South Africa (13.0%) (31), Southern China(12.0%) (41). This respiratory virus detected in all age categories with larger rate from under five children (18.9% ,38/20).The viral positivity among the SARI cases was higher than samples from ILI (38,18.0% vs 10, 4.7%). HAdV recognized as one of the etiology associated with acute respiratory illness (34,39).

As seen from the figure PIV1-4 was detected in 7.3% (31/422) of the total tested samples and PIV-3 accounted for 42.4% (14/33) of this positivity. Like our study result, PIV-3 dominance over the rest HPIVs subtypes was seen in other studies in Kenya, Korea, Brazil and USA(9,27,37,40-42). The overall PIV positivity finding was similarity with finding in Cameroon (7.5%) (2), Kenya (9.4%) (32) and America (6.8%) (42). PIV positivity was also observed in all age groups with small positivity difference.

HCoV was detected in 6.2% (26/422) of the 422 samples tested which had similar finding in Cameroon (5.3%) (2) and in Cheonana of korea (5.5%)(28). Like other studies in America, human coronaviruse -OC43, 2.6%(11/422) was detected more frequently than other corona viruses (43).This respiratory virus detected in all category of age group with a higher positivity rate among adults which is a consistent result with a study in Beijing (44). Among 26 HCoV positive samples 8.1% (17) were ILI cases while 4.3% (9) were from SARI cases.

Other respiratory viruses were detected in 6.2% (26) for EV, 5.7% (24) for HPeV, 4.5%(19) for HMPV, 3.8% (16) for HBoV and this result was fairly similar with other studies done in Cameroon, Kenya (2,32). We also detected influenza C virus from one ILI sample out of the 422 total samples tested, and this was obviously rarely detected respiratory virus among ARI cases and also indicated by a study in Burkina Faso(12).

Our finding showed a total of 55.5% (234) positive samples tested and 88(20.8%) of these samples were identified to had more than one respiratory virus from which 65/88(73.9%) was samples from under five children. Our result was consistent with the study findings in Cameroon (over all co- infection of 14.5%, children accounted for 71.7%)(2), Korea(18.9%)(28) and Kenya(27.0%)(9). Previous studies in Brazil showed multiple detections in $\geq$ 40% of the samples(36,40). Unlike single infections the age category of the cases and illness classification showed large difference in possessing of multiple viruses. Among the total 77 multiple respiratory virus positive samples the majority of multiple respiratory viruses were detected among SARI (15.9%, 67/211) samples than ILI (5.0%, 21/211) samples. This might indicate that multiple virus infection might cause some severe form of acute respiratory illness and needs further study to elucidate the association.

The presence of multiple viruses in SARI and under five children might be an indication; for the severity like recent study in Brazil on children under years of age; revealed that longer hospital stay and oxygen supplement was associated with presence of RV or RV and other co-detected virus (36). However, studies in Senegal (45), Cameroon (2) and China (46) indicated that there was no difference on the clinical characteristics or severity of the patient being infected with single or multiple viruses.

Of the 77 samples with ≥2 viruses RV was detected in the majority of the samples (46/94, 48.9%) and similar findings were reported in china (44). HAdV 68.7 % (33/48) and RSV 53.7% (29/54) were also the most frequently co-detected viruses identified with one or more other respiratory viruses. Our study finding was also similar with a study in South Africa where RV, AdV and RSV were the most frequently detected respiratory viruses(31). All HPeV positive samples were also positive for one or more respiratory virus from which 79.2% (19/24) HPeV were detected together with RSV. Studies done in Scotland (47) and Norway (48) indicated that other respiratory viruses were detected together 70% and 67% HPeV positive samples . This needs additional study to evaluate the role of HPeV in ARI and its interaction with other respiratory virus including RSV.

8. STRENGTH AND LIMITATION OF THE STUDY

8.1. Strength of the study

To our knowledge this is the first laboratory finding which showed wide range of respiratory virus that can cause acute respiratory illness. We used a molecular test (multiplex RT-PCR) which is known for its high sensitivity and specificity so that we were able to detect different respiratory viruses simultaneously from a limited volume of sample and within a short period of time. All the equipments we used for analysis of the laboratory test including RT-PCR machine, micro-pipettes, PCR work station/hood, bio safety cabinet were calibrated which otherwise could affect the quality of test results.

8.2.Limitation of the study

- The clinical characteristics and severity of ARI due to single or multiple respiratory viruses was not addressed.
- Small number of study sites was also one of the limitations of this study compared to the total population of the country to generalize.
- Due to small number of SARI and ILI samples and lack of uniform distribution under each month and these could affect the seasonal trend of the respiratory viruses analysis result.
- The samples we tested were collected for primarily Influenza virus detection not for this study.
- Samples we tested were not uniform. We used combined oropharyngeal and nasopharyngeal swabs from each case for one study site and only oropharyngeal swab samples for the rest of study sites.

9. CONCLUSION

There were wide ranges of respiratory viruses circulating in selected health facilities of Ethiopia among acute respiratory illness cases. The prevalence of respiratory viruses were also high particularly among under five children and sever acute respiratory illness cases. RV, RSV, HAdV, HPIV1-4 and Human corona viruses were the leading circulating respiratory viruses identified in the study. More than one respiratory virus per sample was also detected in a large proportion of samples. The majority of co detections were observed among samples from under five children and SARI cases.

10. *R*ECOMMENDATION

- As large number of respiratory virus was detected among ARI, infection control and case management strategies should be given a prior attention.
- Large scale study has to be done to assess the disease severity and spectrum of illness, seasonality, risk factors and transmission with respect to specific respiratory virus.
- Studies to the strain level of the respiratory viruses should be considered.
- Laboratory capacity should be established for confirmation of respiratory virus and to support clinical decision for better case management.

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12. ANNEXES

11.1. Annex 1: Sample tracking form

S.No	Lab ID	Age	Sex	Date of sample collection	Facility Name	Case category	remark

11.2. Annex 2: Steps of Sample extraction

1. Label the Micro centrifuge tube to specified patient ID
2. Add 896ul of buffer AVL to micro centrifuge tube
3. Add 9ul of carrier RNA to Micro centrifuge tube that already contains the AVL
4. Add 224ul sample to each micro centrifuge tube according to the patient ID
5. Mix each centrifuge with vortex and incubate for 10 minute at room temperature then centrifuge for 30 seconds
6. Add 896ul high grade absolute ethanol, mix and then short centrifugation(30 seconds)
7. Prepare, label and add 630ul mix to binding column and centrifuge it with 8000rpm/1 minute
8. Replace the collection tube with the new one and add the remaining mix and repeat step 7
9. Repeat step 8
10. Add 500ul AW1 and centrifuge by 800rpm for 1 minute
11. Add 500ul AW2 and centrifuge with 1400 rpm for 3 minute
12. The recommended step of centrifugation with 1400 rpm for 1 minute is important
13. Label new micro centrifuge tube and add 96ul elution buffer (AVE) that facilitates the RNA from binding column to the micro centrifuge tube,the purified RNA is now ready for PCR analysis.

Generally the extraction consistes of lysis,binding,washing and elusion processes with centrifugation and vortex in between steps.

11.3. Annex 3: Procedure for master mix Preparation

1. Thaw reagents for the reaction: that is primer probes(PP), the positive controls (PC) and 2x RT-PCR buffer , Fast-track master mix. The PC and the extracted NC have to be included in each run. Before use, the reagents have to be thawed completely, mixed (by short vortexing) and spun down briefly. The positive controls need to be thawed at room temperature for 20-30 minutes and vortexed thoroughly right before use. Make sure to keep 25x RT-PCR enzyme of Fast-track mastermix in a freezer or on a cooling block at all times.

2. Pipette the required amount of **2x RT-PCR buffer** in a 1.5ml tube. Do not immerge the whole tip into the liquid when pipetting 2x RT-PCR buffer to avoid waste of material and to obtain accurate volumes. Pipetting must be done very slowly to prevent air bubbles. Wipe the tip against the edge of the vessel to remove excess liquid outside the tip before dispensing.
3. Add correct volume of **PP** (see Table 5) to 2x RT-PCR buffer. Take care to change the tips after each pipetting step.
4. Pipette the required amount (see Table 5) of 25x **RT-PCR enzyme** with 2x RT-PCR buffer.
5. Make sure to refreeze the remaining volumes of PP, PC and 2x RT-PCR after usage.

Table 11.2: Shown are the amounts of reagents that were needed for 1, 15, 32 and 64 wells.

Name of Reagent	**Number of Reactions per run**			
	1	**15**	**32**	**64**
Buffer(µl)	12.5	187.5	400	800
PP mix(µl)	1.5	22.5	48	96
Enzyme(µl)	1	15	32	64
Total(µl)	**15**	**225**	**480**	**960**

11.4. Annex 4: Master Mix and sample addition procedure to a 96 well plate

1. Take a 96 well plate which is compatible with the ABI® 7500 FAST machine.
2. Pipette 15 µl of master mix (primer probe=PP) to the wells specified for each target group of pathogens.
3. Add 10 µl of the extracted samples, the extracted negative control and the positive control (which is not extracted; thaw at room temperature for 20-30 minutes and vortex thoroughly right before use). Each run must include a negative and a positive control.
4. Mix briefly by pipetting up and down.
5. Close the plate with the optical adhesive film.
6. Slightly vortex the plate and centrifuge briefly afterward.
7. Put the plate in the ABI® 7500 FAST machine plate holder.

11.5. Annex 5: Principle of the PCR test and PCR program

Principle of the method

The viral RNA is transcribed into cDNA using a specific primer mediated reverse transcription step followed immediately in the same tube by polymerase chain reaction. The DNA of different pathogens is amplified simultaneously in the same tube by polymerase chain reaction. The presence of specific pathogen sequences in the reaction is detected by an increase in fluorescence observed from the relevant dual-labeled probe, and is reported as a cycle threshold value (Ct) by the Real-Time thermocycler. The assay uses Equine arteritis virus (EAV) as an internal control (IC), which is introduced into each sample and the negative control at the lysis buffer stage of the extraction process.

PCR programme

Fast-track mastermix
42°C for 15 minutes hold
94°C for 3 minutes hold
40 cycles of: 94°C for 8 seconds
60°C for 34 seconds

RESULT INTERPRETATION

For samples to be considered as positive all the following criteria should be fulfilled:

- Internal control and positive control cycle threshold(CT) value should be than 33 and for
- No amplification detected for negative control.
- Standard amplification curve should be observed for the sample.

Declaration

I the undersigned candidate, declare that this thesis is my original work and has not been presented for a degree in this or any other university and all resources used for this thesis have been acknowledged.

Name of the student: Adamu Tayachew Mekonnen

Signature: _____

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Approval of the primary Advisor

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