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Rapid detection tests for distinguishing between influenza virus type A and B among children less than 5 years, using direct immunofluorescence assay and RT-PCR techniques

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Abstract

Detection of influenza virus surveillance was world concerned for long time, because of series public health caused by the virus. In this study, rapid and sensitive methods of direct immunofluorescence assay (DFA) and the real time quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to detect and distinguish between influenza virus type A and B in children, with influenza like illness symptoms, of age ranged between 2 months and 5 years old in two children hospitals in Baghdad city. Eight out of 100 (8%) children under investigation were found positive for influenza virus type A by using DFA while 13 (13%) was detected by RT–PCR. Whilst influenza B was completely absent in all individuals tested. In summary, influenza virus type A was moderately present (8%-13%) and absence of type B in population tested in Baghdad city by using serological and molecular assays.

Keywords: key word, Influenza virus A and B, , DFA and RT-PCR

1. Introduction

One of the most important respiratory pathogens responsible for acute pneumonia worldwide is influenza virus type A and B. Only influenza virus type A has been responsible for pandemic influenza symptoms which known as H1N1 (Sonja *et al* .2008). Infection with Influenza viruses vary from mild to severe, life threatening syndromes and its associated with divers clinical symptoms ranging from high fever > 38C°, cough, sour throat, runny nose and weakness. It may develop complications due to secondary bacterial infection or fatal complications as cute pneumonia and bronchitis (Cate 1987). However influenza symptoms share the same syndromes with other respiratory viral or other infectious in patients known as "influenza-like illness" (ILI) (Call *et al*. 2005). Influenza can be classified into three major types A, B and C according to antigen variation in their nucleoprotein (Mahony 2008) ,and the C type is much lesser extent. Influenza virus type A can be arranged into different subtypes based on the antigen variation of hemagglutination and Neuraminidase proteins. These subtypes have been reported by World Health Organization (WHO) (WHO 2007). H1N1 the pandemic types which caused Swine Flu 2009; H5N1 caused Bird Flu 2004, H3N2 caused Hong Kong Flu 1968. In Northern Iraq, H5N1 was found in Erbil and Sulymanyia and it was confirmed with a fatal human case of infection, highly pathogenic H5N1 in poultry were reported in this area too. In addition, H1N1 caused epidemic during 2009 and 2013 (WHO 2010;WHO 2013) . Therefore the threat of influenza viruses demand rapid and sensitive tools for detection in short time specially during pandemic periods. However, still immunofluorescence antibody staining and molecular technique such as conventional RT-PCR and qRT-PCR are most sensitive and rapid detection for respiratory viruses like influenza (WHO 2009). The aim of this study was to develop such assays for detection of influenza virus genotype A and B in children under 5 years old by using DFA and Reverse transcription chain reaction RT-PCR as routine laboratory assays.

2. Materials and methods

One hundred of outpatient (infants) with influenza like illness (ILI) symptoms was tested for influenza virus, in two main children hospitals in Baghdad during the influenza season 2013-2014. Children with influenza like illness symptoms were defined with physical examination by the consultant physicians, chest X ray and signed

symptoms as running nose, fever, wheezing chest were cases of choice, together with detailed history such as sex, age for all patients recruited in this study.

2.1 Virus identification by direct immunofluorescence assay (DFA)

Specimens were processed to direct antigen detection according to manufacturer's directions of the kit D³ FastPoint™ (Quidel, USA). 3ml of Nasal and throat clinical specimens were washed and suspended with 1x PBS several times, 2 drops of murine monoclonal antibodies which directly labeled with R-phycoerythin and fluorescein isothiocyanate (FITC) against influenza A/B was added to viral antigen in the washed epithelia cell. Stained cells incubated at 35° to 37°C for 5-minutes, suspended pellets were rinsed with 1X PBS, and suspended with Re-Suspension Buffer, cells loaded onto a specimen slide channel examined using a fluorescence microscope.

2.2 RNA Extraction

RNA extraction was done by using QIAamp Viral RNA Mini spin protocol (Qiagen, Hilden, Germany) was used for extraction assay. All samples were extracted according to the manufacturer's instructions. Briefly, 140µl of sample was extracted and loaded onto QIAamp Mini spin column and the highly- quality RNA were eluted in 60 µL of buffer and stored at −70°C.

2.3 Taqman primers and probes of Real Time RT-PCR

Invetrogen SuperScript™ III Platinum® one step kit was used to detect influenza virus type A and B according to the manufacture procedure of CDC (CDC, 2008). Universal primers and Black hole quencher probes were used for influenza A, F primer '5GAC CRA TCC TGT CAC CTC TGAC3', R primer AGG GCA TTY TGG ACA AAK CGT CTA and probe sequence of influenza A was '5-FAM- TGC AGT CCT CGC TCA CTG GGC GAA -BHQ1-3'.Influenza B, F primer CGG TGC TCT TGA CCA AAT TGG, R primer, TCC TCA AYT CAC TCT TCG AGC G and Probe '5-FAM CCA ATT CGA GCA GCT GAA ACT GCG GTG-BHQI-3'.Cocktail of master mix, Tag polymerase , primers and probes prepared by mixing 12.5µl of 2xPCR master mix and equal volume 0.5 µl of Primers, probes and platium® *Taq polymerase* to get total volume 20 µl. 5µl of extracted RNA PCR was performed using Applied Biosystem 7500 (Applied Biosystem, Singapore) as follow: for 45 cycles , Reverse transcriptase 50C 30 mints, *Taq* inhibitors activation 95C 2 mints, PCR amplification 95C ,15 sec and data collection was at 55C for 30 sec.

3. Results

3.1 Influenza like illness symptoms

Children who were enrolled in this study was between 3-5 days of fever duration temperature of 38-39°C
The clinical symptoms of ILI associated with influenza virus was (80%) for fever, (72%) sore throat, (84%) cough, (76%) rhinorrhea, (78%) wheezing chest Table 1. P value for symptoms were positively correlated with flu like illness $P<0.01$, as well as the correlation coefficient $r = 0.97$. The positive predictive value of influenza syndrome PPV was range from (0.1- 0.13) for fever, sour throat, cough, rhinorrhea and wheezing chest compare with ILI .The negative predictive value range from (0.94- 0.98) for the same symptom respectively. Prevalence of laboratory-confirmed cases using clinical criteria of influenza-like illness was 13% by using both DFA and RT- PCR. The specimens in this study were obtained from nasal and throat, and they were sufficient and consider reliable for detection influenza virus. Because swab technique considered as best choice for many medical laboratories concerned with influenza surveillance during influenza season infection among children.

3.2 Detection of Influenza virus genotype A & B

Results of influenza virus A and B by using D³ FastPoint L-DFA found that 8 of 100 clinical specimens were positive of influenza A virus Figure 1. While genotype B disappeared in all the tested specimens Table 2. The quality of the sample was assessed by observing the number of respiratory epithelial cells present as well as repeating poor quality samples. The sensitivity and the specificity of the test estimated according to sensitivity and specificity formula described by Rajul *et al.* (2008), and were shown to be 66% sensitivity and 96% specificity.

3.3 Detection of influenza genotype A&B by RT-PCR.

Detection by RT-PCR showed that 13 children of 100 were infected with influenza virus genotype A. The detection for influenza virus genotype B was found negative in the entire tested specimens. The threshold cycle CT

values for Influenza genotype A were between (19.6- 35.9), which considered with range of strong to moderate of viral amount in the sample Figure 2. The influenza virus A was found in children with ILI age between two months and two years old, with rate of infection 2 months to 1 year 5%, 1- 2 years 7%, 2-5 years 1%, Table 3.

4. Discussion

In general, the ILI clinical symptoms of influenza in children are similar to other respiratory pathogens cause respiratory infection during the influenza activity season. Laboratory-confirmed influenza illness with simple clinical symptoms using rapid influenza test, would have identified 74% - 88% of the influenza infection among children **Matthew *et al.* (2010) and CDC (2009)**. Never the less, using rapid diagnostic test DFA in this study was able to detect 8 of influenza A positive infections. Many studies show that cough, high fever and sour throat are likely to be common symptoms for influenza virus infection Monto *et al*. (2000). The influenza like illness in this study showed well correlation with influenza activity during the infection season. This result is in agreement with Monto *et al.* (2000) and Jose *et al.* (2005) which determined that the symptoms and clinical observation might be good indictors for diagnosis influenza infection than the other illness due to respiratory viruses. The prevalence of influenza virus among children under five in this study was in agreement with Samransamruajkit *et al.(* 2008) and Muyembe *et al*. (2012) in Japan and Democratic Republic of Conge 12.5%, and 15% respectively. The specificity and sensitivity of using DFA for influenza virus diagnosis of this technique is in agreement with James *et al.* (2003) as has been demonstrated that the sensitivity range was from 39% -76% for adults and 56 - 66% for infants, while the specificity range was 94%-99% by using (DFA, direct fluorescent antibody) kit and other immunoassay methods for the detection of influenza. specificity and sensitivity effected by other factors such as specificity of the reagents, patients age, certain genotypes and the level of experience of those performing, reading, and interpreting the test Weinberg & Walker (2006). It has been argued that the sensitivity of those tests in general is higher in children compared to adults because, children shed more viruses and for longer period than adults (WHO 2005). Using DFA in this study appeared to be mostly reliable except in a few cases there was some false positive or negative and this may be due to clarity of (unclear) fluorescence of cells or accumulation of stain debris after insufficient washing by buffer. Gavin & Thomson (2003) attributed the reason for the false negative results of some specimens due to the lack of adequate numbers of respiratory epithelial cells in the specimen and non-specific florescence of cells, debris which can produce false-negative result or false-positive, respectively. However, DFA is still recommended as a reliable method for diagnosis of influenza virus for inpatients and outpatients in many international medical laboratories and hospitals. Although none of the direct immunofluorescence assay - rapid influenza A tests can differentiate genotype A virus subtypes or discriminate between those subtypes that commonly infect humans such as H3N2 and H1N1(CDC 2005). Rapid tests are usually useful for guiding treatment and patient management in a clinically relevant timeframe as approved by (CDC 2006). RT-PCR molecular technique considered one of the important tools that used for rapid and sensitive detection during the epidemic outbreak because of the primers and probes which designed specifically for differentiating novel influenza A viruses H1N1, and for typing influenza A and B virus, as well as subtyping H1, H2, H3, H5, H7, H9, N1 (human and animal), N2 or N7 of influenza Jie *et al*. (2009). On the other hand CDC universal influenza genotype A primers and probes were used in our study seem to be reliable for detection influenza genotype A in ILI patients. These results are similar to previous studies using the same technique of real-time PCR] Bo *et al*. (2011). This efficient technique is based on a Taqman technology, in which a probe is designed to hybridize to an internal region of the PCR product so that the highest sensitivity and specificity can be achieved during the PCR amplification. Results of this part of the study also agree with Nathamon et *al*. (2010) as they identify influenza genotype A with the same sets of primers that we used as they derived from the most conserved region of influenza matrix protein (M) gene Bo, Shu *et al.* (2011). On the other hand, influenza virus genotype B was not detected by using RT-PCR, it may be due to the lower transmission rate of influenza virus genotype B which is consider slower than influenza virus genotype A. Genotype B is known of its ability to transmit from human to human only. In addition, the rate of mutation is 3-4 times lower than influenza virus genotype A Nobusawa & Sato (2006). Khanna *et al.* (2008) demonstrated that all influenza B infection outbreaks are only found as regional epidemics. So that may give another explanation to this observation especially no relevant data about influenza virus genotype B was reported in our region (Middle-East). Furthermore, the WHO annual reports about seasonal influenza in Iraq did not indicate any significant numbers of infections with this genotype, except a very small number of infections reported in some Asian countries (outside our region) (WHO 2006; WHO 2014). However, some related studies by Harvala *et al.* (2014) in Europe reported circulating influenza genotype B among children population under 5 years old of more than 5% infection per 100,000. Other explanation may be attributed to the duration of shedding of avian Influenza genotype A and B viruses in which is humans is not known yet. Therefore the estimated duration of viral shedding is based upon seasonal influenza virus A infection which is estimation 5% -10% in adult and 20%-30% in children while Influenza genotype B is only 5% (WHO 2014).

5. Conclusion

In brief conclusion was found that that the circulating genotypes among children with influenza like illness (ILI) symptoms in children less than five years old, was influenza virus genotype A. Influenza virus genotype B was not detected in this cohort of patients. DFA and RT-PCR found reliable methods fast, accurate and sensitive for diagnosis of influenza A virus surveillance. And it may need to be developed in the future for fast and accurate identification of any novel influenza virus subtypes

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Table 1. Clinical symptoms for children infected with influenza like illness collected from children > 5 years by using nasal and throat swabs.

Code of the samples	Age	Sex	Fever over 38c	Cough	Sore throat	Runny nose	Difficulty breathing	Wheezing
ILI-1	3 months	M	––	√	––	√	√	√
ILI-2	7 months	M	√	––	√	√	––	––
ILI-3	2 years	F	√	√	√	√	√	√
ILI-4	4 years	F	√	––	√	––	––	––
ILI-5	3 months	M	√	√	√	√	√	√
ILI-6	5 years	M	√	––	––	√	––	––
ILI-7	3 months	F	√	√	√	––	√	√
ILI-8	4 months	M	√	√	√	√	√	√
ILI-9	4 years	M	√	√	––	√	√	√
ILI-10	4 months	F	√	––	––	√	––	––
ILI-11	2,6 years	F	√	√	√	√	√	√
ILI-12	3 years	F	√	√	√	––	√	√
ILI-13	11 month	M	√	√	√	√	√	√
ILI-14	5 years	M	––	√	––	√	––	––
ILI-15	4 years	M	––	√	√	√	––	––
ILI-16	4 years	F	––	√	√	√	––	––

ILI-17	7 months	F	√	√	√	––	√	√	√
ILI-18	8 months	M	√	√	√	––	√	√	√
ILI-19	4 months	M	√	√	––	√	√	√	√
ILI-20	1 year	M	√	––	––	√	––	––	√
ILI-21	2 years	F	√	√	√	√	√	√	√
ILI-22	2 months	F	√	√	––	√	√	√	√
ILI-23	2 years	F	√	√	√	√	√	√	√
ILI-24	2 years	M	√	√	√	––	√	√	√
ILI-25	5 years	M	√	√	√	√	√	√	√
ILI-26	2 years	F	√	––	√	√	––	––	––
ILI-27	3 years	F	√	––	––	√	––	––	––
ILI-28	1,4 year	M	√	––	––	√	––	––	––
ILI-29	1 year	M	√	√	√	√	√	√	√
ILI-30	4 months	M	√	√	√	––	√	√	√
ILI-31	2 years	F	√	––	––	√	––	––	––
ILI-32	1 year	M	––	––	––	√	––	––	––
ILI-33	2 years	M	√	√	√	√	√	√	√
ILI-34	11 months	M	√	––	√	––	––	––	––
ILI-35	5 years	F	√	√	––	√	√	√	√

Code of the samples	**Age**	**Sex**	**Fever over 38c**	**Cough**	**Sore throat**	**Runny nose**	**Difficulty breathing**	**Wheezing**	**C X**
ILI-36	1 year	F	––	√	––	√	––	––	
ILI-37	1,3 year	F	––	√	√	√	√	√	
ILI-38	2 years	M	√	√	√	––	√	√	
ILI-39	1,4 year	F	––	√	––	√	√	√	
ILI-40	9 months	M	––	√	––	√	√	√	
ILI-41	5 years	M	√	√	√	––	√	√	

ILI-42	1 year	M	√	√	√	√	√	√	√
ILI-43	1,4 year	M	√	––	√	√	––	––	––
ILI-44	2 years	M	√	√	√	√	√	√	√
ILI-45	9 months	M	√	√	√	––	√	√	√
ILI-46	5 months	M	––	√	––	√	√	√	√
ILI-47	9 months	F	√	√	√	√	√	√	√
ILI-48	6 months	M	√	√	√	√	√	√	√
ILI-49	1,2 year	M	√	√	√	––	√	√	√
ILI-50	3 years	F	––	√	––	√	√	√	√
ILI-51	1,8 year	M	√	√	––	√	√	√	√
ILI-52	5 years	F	√	√	√	√	√	√	√
ILI-53	4 months	F	√	√	√	√	√	√	√
ILI-54	1,7 year	M	√	√	√	––	√	√	√
ILI-55	3,6 years	F	√	√	√	√	√	√	√
ILI-56	3 months	F	√	––	√	√	––	––	––
ILI-57	2 years	F	√	√	√	√	√	√	√
ILI-58	1,6 year	M	√	√	––	√	√	√	√
ILI-59	5 years	F	√	√	√	√	√	√	√
ILI-60	4 years	F	√	√	√	––	√	√	√
ILI-61	1,8 year	F	√	√	√	√	√	√	√
ILI-62	9 months	M	√	√	√	√	√	√	√
ILI-63	3 years	F	√	√	√	––	√	√	√
ILI-64	6 months	F	√	√	√	√	√	√	√
ILI-65	3 years	M	√	√	√	√	√	√	√
ILI-66	11 months	M	√	√	√	––	√	√	√
ILI-67	3 years	M	√	√	√	√	√	√	√
ILI-68	1,9 year	M	√	––	√	√	––	––	––
ILI-69	16 months	F	––	√	––	√	√	√	√

ILI-70	3,6 years	F	––	√	––	√	√	√	√
ILI-71	5 months	F	√	√	√	√	√	√	√
ILI-72	1,2 year	F	√	√	√	––	√	√	√
ILI-73	1.2 year	F	√	√	√	√	√	√	√
ILI-74	2 years	M	––	√	––	√	√	√	√
ILI-75	2 years	M	––	√	––	√	√	√	√

Code of the samples	Age	Sex	Fever over 38c	Cough	Sore throat	Runny nose	Difficulty breathing	Wheezing
ILI-76	2 months	M	√	√	√	√	√	√
ILI-77	5 years	F	√	√	√	√	––	––
ILI-78	5 months	M	√	––	√	––	––	––
ILI-79	3 years	F	––	––	––	√	––	––
ILI-80	1,6 year	F	––	√	√	√	√	√
ILI-81	1 year	M	√	√	√	√	√	√
ILI-82	5 years	F	√	√	√	––	√	√
ILI-83	2 years	F	√	√	√	––	√	√
ILI-84	7 months	M	√	√	√	√	√	√
ILI-85	7 months	M	––	√	––	√	√	√
ILI-86	3,6 years	M	√	√	√	√	√	√
ILI-87	5 months	M	√	√	√	√	√	√
ILI-88	2,6 years	M	√	√	√	––	√	√
ILI-89	2 years	F	√	√	√	√	√	√
ILI-90	1,6 year	M	√	√	√	√	√	√
ILI-91	1,7 year	M	√	√	√	––	√	√
ILI-92	2 years	M	√	√	√	√	√	√
ILI-93	3 years	F	√	––	√	√	––	––

ILI-94	3years	M	––	√	––	√	√	√
ILI-95	9 months	M	√	√	√	√	√	√
ILI-96	2,6 years	F	√	√	√	––	√	√
ILI-97	1,6 year	F	√	√	√	√	√	√
ILI-98	3 months	F	––	√	––	√	√	√
ILI-99	1,5 year	M	√	√	√	––	√	√
ILI-100	2 years	M	√	√	√	√	√	√

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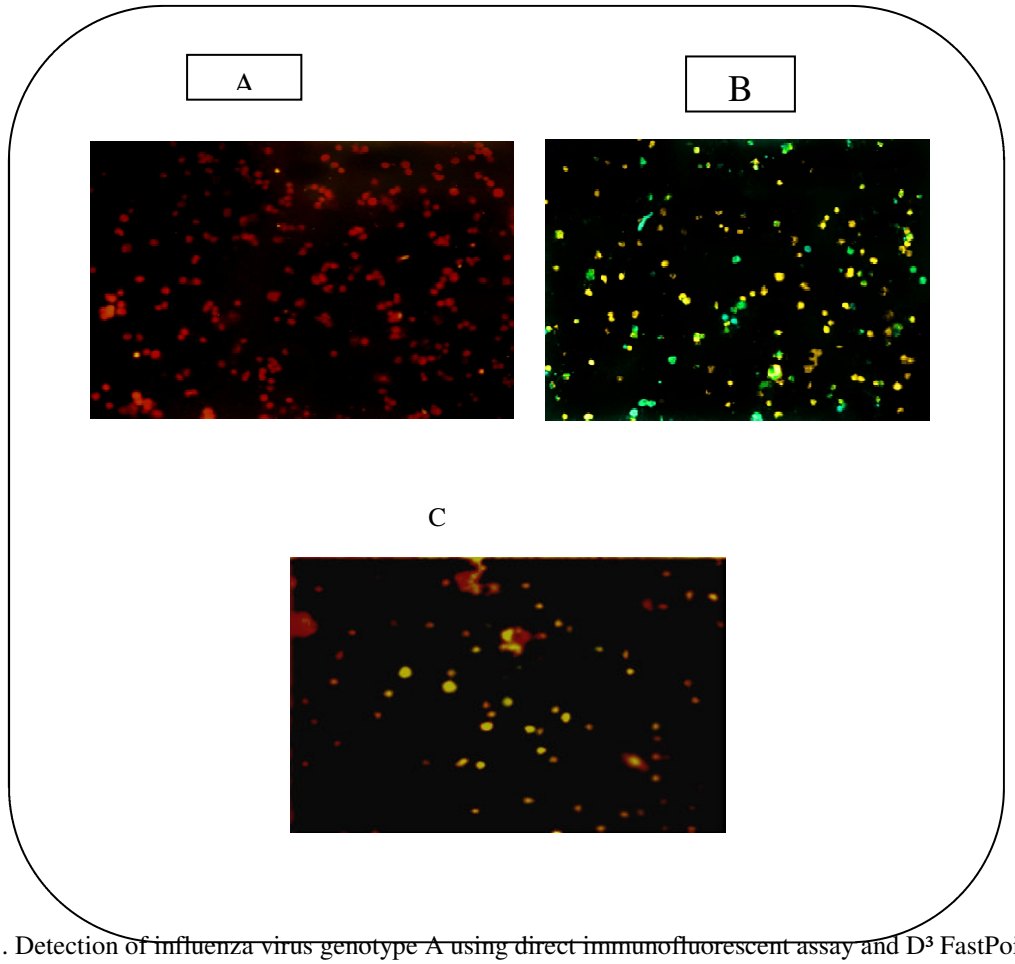

Figure 1. Detection of influenza virus genotype A using direct immunofluorescent assay and D³ FastPoint method. A florescence microscope (BX61 Olympus) with 200X magnification were used
A: negative control of influenza virus A and B;
B: positive control of influenza virus A and B, (as they appear in golden color);
C: positive result of influenza genotype A only.

Table 2. Detection of influenza virus genotypes A and B in children and infant infected with influenza like illness (ILI) by D³FastPoint.

Age	Total specimens evaluated (100)	Influenza A positive	Influenza B positive
>2months to 1 year	37	2	non
>1year to 2 years	35	6	non
>2 years to 5 years	28	0	non
Total	100	8	0

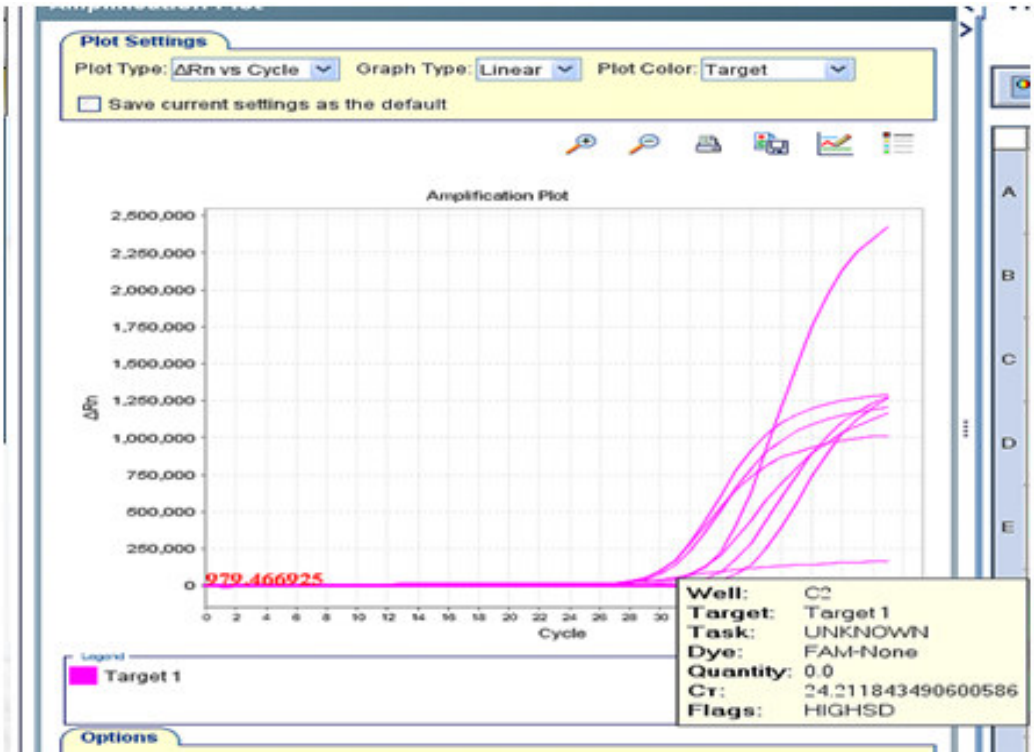

Figure 2. Profile of real-time RT-PCR amplification of influenza virus genotype A. CT value and thresholds of positive specimens from children between 2 months to 5 years old.

Table 3. Incidence of influenza virus genotype A and B in infant and young children.

Age	No. of specime	No. of Influenz virus A Infecti	No. of Influenza virus B infecti	Rate of infection of influenza A
>2months to 1 1 ye	37	5	non	5%
>1year to 2 years	35	7	non	7%
>2 years to 5 years	28	1	non	1%
Total	100	13	0	13%

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