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RESPIRATORY INFECTIONS (F ARNOLD, SECTION EDITOR)

Comparative Study of Molecular Approaches for the Detection of Influenza Virus from Patient Samples Using Real-time PCR: Prospective Disease Burden Study in Kerala (India) from 2010 to 2016

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Abstract

Purpose of Review Acute respiratory infections caused by influenza virus are a major cause of viral respiratory diseases globally. Surveillance of circulating subtypes and estimation of disease burden is of utmost clinical importance. Molecular surveillance and proper disease burden estimates are scarce in India although clinical influenza infections are on the rise. Our study aims to delineate the prevalent influenza subtypes in a South Indian population from cases requiring hospital visits. Using real-time polymerase chain reaction (RT-PCR), 2154 throat/nasopharyngeal swabs from patients attending Government Medical College, Thiruvananthapuram, Kerala, India, with suspected influenza-like illness, were tested for the presence of different influenza subtypes.

Research Findings Forty-three percent of specimens were positive for the influenza virus. Among these, prevalence of influenza A(H3N2), influenza B, and H1N1pdm09 was 26.7, 6.3, and 10%, respectively. Nominal co-infections were detected. An easy to use commercial kit was used for the majority of the study after proper evaluation for sensitivity and specificity against a gold standard protocol.

Summary Specific diagnosis using molecular tools caters to the urgency, and a precise measure of the disease burden and management actions are needed, especially in developing countries like India. Infection rate estimation using a sensitive RT-PCR assay signified that influenza was highly prevalent in the region. The study data generated will help understand the epidemiology of influenza in India as well as generate information for global influenza surveillance and disease burden.

Keywords Acute respiratory infections · Influenza virus · Influenza disease burden

Introduction

Influenza A and B viruses belong to the family *Orthomyxoviridae* with a genome makeup of a single-stranded, negative-sense RNA. They cause acute respiratory infections and form a major chunk of the global disease burden for viral respiratory diseases [1••, 2••, 3••]. Influenza A viruses are further categorized into subtypes H1N1 and H3N2 on the basis of surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA). While antigenically distinct B strains are currently circulating globally, these strains are not too genetically different from the A subtype. Influenza B viruses are currently grouped into two lineages, Victoria and Yamagata, but are not subtyped any further. Influenza A viruses circulate among a diverse range of host species [3••]. The WHO Global Influenza Surveillance Network has greatly contributed to the knowledge about circulating influenza viruses, including the emergence of novel strains [4••, 5••, 6••]. A limited number of studies on public health surveillance of influenza had been reported from India, but credible data on influenza disease burden from the southern region is still missing. Several outbreaks in Pune, Himachal Pradesh, Delhi, Kashmir, and Kolkata have been investigated [7••, 8••, 9••, 10••]. To date, the requisite data to estimate influenza-associated disease burden is scant or absent in most developing countries (http://

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www.who.int/influenza/resources/publications/manual_burden_of_disease/en/). In this report, we summarize the surveillance data on influenza from 2010 to 2016, in hospital attending/admitted patients from Thiruvananthapuram in southern India.

Traditional methods to detect influenza virus from clinical samples include virus culture, isolation, and characterization by immunoassay, which usually take 3–7 days [11••, 12••, 13]. Later, developed rapid point-of-care tests are simple to use and enable rapid testing within a few minutes, but generally has low sensitivity compared to viral culture or molecular techniques like polymerase chain reaction [14••, 15•, 16••, 17•, 18••]. Other diagnostic methods employ real-time (RT) PCR and multiplex RT-PCR combined with chip-based detection [19••, 20•, 21••, 22••]. However, the drawback with established RT-PCR protocols is that individual influenza subtypes have to be detected in separate reactions increasing cost and effort in disease diagnosis [23••, 24••, 25••, 26••].

There is a need for reliable disease burden estimates especially from developing countries to provide a better understanding of the impact of influenza in vulnerable communities or subpopulations. Use of molecular tests like RT-PCR is important for surveillance in order to accurately identify which influenza subtypes are circulating and the rate of co-infections with other seasonally co-occurring viral/bacterial pathogens or other influenza subtypes. Molecular tests also help clinicians reliably confirm the highly virulent 2009 influenza H1N1 virus (H1N1pdm09) from patients without the need of prolonged exposure and also help in better and quick management of such patients.

In light of the above facts, the present report investigates the influenza virus infection as the cause of an influenza-like illness (ILI) and severe acute respiratory infection (SARI)-like illness, which is a clinical syndrome characterized by symptoms such respiratory distress, fever, etc. Respiratory samples were taken from southern India to perform diagnosis of influenza as the cause of respiratory syndrome using influenza A(H1N1pdm09 and H3N2) and B (Victoria and Yamagata) specific real-time PCR. We also compared an easy to use commercial kit with the established CDC (Centre for Disease Control, USA) RT-PCR-based assay to assess the efficacy of the commercial kit which does not require handling of multiple reagents thus preventing chances of cross-contamination in a routine diagnostic laboratory setting.

Material and Methods

Sample Collection and Transport

A total of 2154 acute phase throat/nasopharyngeal swab samples were collected from patients suspected with influenza virus infection as the cause of ILI- and/or SARI-like illness, presenting between 3 and 7 days of onset of fever (with case definition of sudden onset of fever > 38 °C, cough, or sore throat as per WHO) at the Government Medical College in Thiruvananthapuram, Kerala, India [5••]. Informed consent was taken from patients prior to enrollment. Samples were received at the laboratory from the hospital in viral transport medium (Himedia, India) at 4 °C and in standard triple packaging. All the samples were processed in a high-containment facility.

Samples from 2010 to 2012 were assayed using the CDC protocol during which the commercial kit was also simultaneously tested for sensitivity and specificity. Both influenza-positive and influenza-negative clinical samples pretested with CDC protocol were included.

Viral RNA Extraction

Viral RNA was extracted from throat and nasal secretions using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for use with the CDC-based protocol.

Spin Star nucleic acid kit (ADT Biotech, Malaysia) was used to extract total nucleic acid from the clinical samples for influenza typing using the commercial kit (Real Star Influenza RT-PCR Kit, Altona diagnostics GmbH, Germany). Viscous samples were pretreated with kit-provided mucolytic agent prior to extraction.

CDC-Based Assay

The assay employs a one-step approach for reverse transcribing the viral RNA and then utilizing a multiplex RT-PCR approach using a mixture of various primer-probe sets to detect various influenza subtypes. The primer-probe mixtures used were Inf A for universal detection of seasonal A influenza viruses A(H3N2), and primer/probe set specifically to detect highly virulent A(H1N1) pdm09. The fourth primer/probe set targeted the human RNase P gene and served as an internal control [25••].

The RT-PCR assays were performed using the AgPath-ID one-step RT-PCR kit (Applied Biosystems). Briefly, 5 μl of purified RNA was reverse transcribed and amplified in a 25 μl reaction mixture containing 12.5 μl of 2XRT-PCR buffer (Applied Biosystems), 1 μl of 25XRT-PCR enzyme mix (Applied Biosystems), 300 nM forward primer, 300 nM reverse primer, and 75 nM probe [26]. RT-PCR was performed in a ABI 7500 real-time PCR system (Applied Biosystems) and analyzed by SDS software v2.0.1 (Applied Biosystems). The thermal cycling conditions comprised a 10-min reverse transcription step at 45 °C followed by a 10-min initial PCR activation step at 95 °C, and 40 cycles of 95 °C for 15 s and 55 °C for 45 s each.

Commercial RT-PCR Kit

The RT-PCR assays for single tube detection and differentiation of seasonal human influenza A (A(H1N1)pdm09, A(H3N2)) and B (Victoria and Yamagata) strains from clinical samples was done using the Real Star Influenza RT-PCR Kit 3.0 (Altona diagnostics GmbH, Germany) following manufacturer's instructions. Ten microliters of extracted eluent was used as a template for PCR amplification. All assays also detected the amplification of an internal control that did not interfere with target amplification, to check for possible PCR inhibitors. All RT-PCR reactions were performed on the Rotor gene 5 plex HRM real-time platform (QIAGEN, Germany) (Fig.1a, b, c, d).

Sensitivity and Specificity Testing of the Commercial Kit Compared to CDC Protocol

We compared the clinical sensitivity and specificity of the easy to use preassembled commercial kit as compared to those of the robust CDC protocol for detection of influenza virus using the RT-PCR assay. We randomly selected 10% of samples (from 2010 to 2012) diagnosed either positive or negative for influenza using the CDC protocol and retested the samples using the commercial kit and the CDC protocol simultaneously.

Data Analysis

Statistical analysis was carried out using Epitools (Epitools epidemiological calculators, Aus Vet Animal Health Services; http://epitoolsausvet.com.au) and concordance between the two tests was determined using concordance and kappa of agreement.

Results

Viral Surveillance

A total of 2154 throat and nasal swab samples were received with acute respiratory infections during October 2010 to December 2016. Using either of the real-time PCR-based methods, 43.2% were positive for influenza virus when tested. Among these, 136 (6.3%) samples were positive for influenza B (IFB), 576 (26.7%) samples were positive for A(H3N2), and 219 (10%) samples were positive for A(H1N1) pdm09. Among the positive cases, 652 (70%) were males and 279 (30%) were females. The clinical presentation in influenza-positive population included fever, cough, sore throat, nasal catarrh, headache, shortness of breath, and vomiting (Table 1).

The A(H3N2), IFB, and A(H1N1) pdm09 detection threshold cycle (C_t) mean and standard deviation values were 27.39

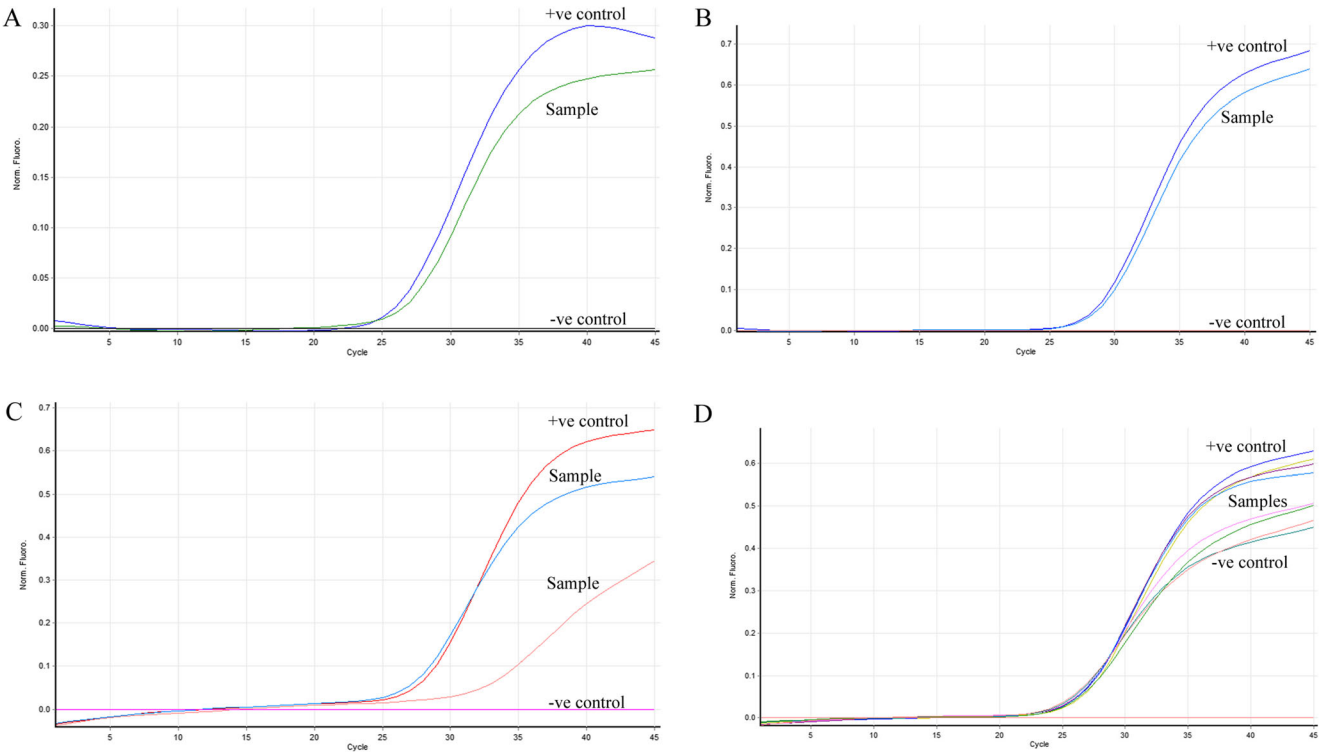

Fig. 1 Amplification plots for the real-time PCR based detection of **a** A(H3N2), **b** IFB, **c** A(H1N1) pdm09, and **d** internal control (for checking extraction efficiency and amplification inhibition)

Table 1 Demographic profile and clinical manifestations of subjects

Characteristics	No. (%)			
	A(H3N2) ($N = 576$)	IFB ($N = 136$)	A(H1N1)pdm09 ($N = 219$)	No virus ($N = 1223$)
Sex				
Male	345 (60)	95 (70)	137 (63)	769 (63)
Female	231 (40)	41 (30)	82 (37)	436 (36)
Symptoms				
Fever (oral)	441 (76)	125 (92)	198 (90)	947 (77)
Sore throat	514 (89)	116 (85)	184 (84)	1001 (82)
Cough (dry)	315 (55)	103 (75)	179 (82)	847 (69)
Headache	279 (48)	74 (54)	92 (42)	491 (40)
Muscle and joint pain	381 (66)	114 (83)	167 (76)	940 (77)
Severe malaise	342 (59)	97 (71)	146 (67)	916 (75)
Shortness in breath	264 (46)	84 (62)	98 (45)	412 (34)
Nasal catarrh	124 (22)	67 (49)	82 (37)	348 (28)
Vomiting	63 (11)	31 (23)	74 (34)	371 (30)

± 3.61, 34.22 ± 2.67, and 28.87 ± 1.77, respectively (Table 2; Fig. 1a, b, c, d). Acceptable mean C_t (23.34 ± 0.45) was obtained from the kit internal controls of the two real-time PCR methods used in this study. Apparently, the viral load detected from throat/nasopharyngeal swab was clearly much lower for IFB compared to that for A(H3N2) and (A(H1N1) pdm09.

Analysis of the data revealed co-infections of A(H3N2) and A(H1N1) pdm09 in only eight cases (3.6%) and even lower co-infection of IFB with A(H1N1) pdm09 (3 cases; 1.3%). There were no observed co-infections for A(H3N2) and IFB (Table 1). This observation suggests that these pathogens probably do not co-infect frequently and are probably mutually exclusive of each other. Also, co-infections do not contribute significantly to the overall disease burden.

Comparison of the Two Real-time RT-PCR Assays

The concordance and kappa coefficients for detection of individual viral pathogens compared to those for CDC protocol were as follows: A(H3N2) 100.0%, 1.000; IFB 100.0%, 1.000; A(H1N1) pdm09 100.0%, 1.000, respectively (Table 3). Sensitivity and specificity of CDC protocol were

Table 2 Number of patients positive for seasonal influenza A(H3N2), A(H1N1)pdm09, IFB, and co-infection

Year wise	No. of cases positive (%), mean and standard deviation(SD) of C_t (cycle threshold) value						
	A(H3N2)		IFB		A(H1N1)pdm09		Internal control
	Positive (%)	Target C_t mean or SD	Positive (%)	Target C_t mean or SD	Positive (%)	Target C_t mean or SD	Reference C_t mean or SD
2010 (Oct–Dec)	43/337 (12.7)	26.04 ± 2.06	11/337 (3.2)	34.88 ± 4.10	19/337 (5.6)	26.27 ± 2.05	23.56 ± 0.09
2011	267/522 (51.1)	23.97 ± 2.08	53/522 (10.1)	35.24 ± 2.45	93/522 (17.8)	27.23 ± 2.64	23.47 ± 0.12
2012	91/316 (28.7)	32.26 ± 1.09	17/316 (5.3)	33.58 ± 2.13	15/316 (4.7)	28.29 ± 1.09	23.65 ± 0.11
2013	22/93 (23.6)	22.74 ± 3.12	4/93 (4.3)	32.92 ± 1.01	3/93 (3.2)	29.12 ± 3.21	23.69 ± 0.06
2014	13/77 (16.8)	30.77 ± 2.06	14/77 (18.1)	29.21 ± 3.51	5/77 (6.49)	31.47 ± 3.27	23.68 ± 0.09
2015	67/404 (16.5)	29.93 ± 1.09	19/404 (4.7)	36.62 ± 2.82	48/404 (11.8)	30.21 ± 2.12	22.76 ± 0.05
2016	73/405 (18)	26.04 ± 3.04	18/405 (4.4)	37.12 ± 1.89	36/405 (8.8)	29.52 ± 3.21	22.61 ± 0.08
Overall	576/2154 (26.7)	27.39 ± 3.61	136/2154 (6.3)	34.22 ± 2.67	219/2154 (10.1)	28.87 ± 1.77	23.34 ± 0.45
Co-infection	A(H1N1)pdm09 versus A(H3N2) 8/219(3.6) A(H1N1)pdm09 versus IFB 3/219 (1.3)						

Table 3 Comparison and evaluation of CDC real-time RT-PCR assay and commercial RT-PCR kit

	Cases positive (%), mean and standard deviation(SD) of C_t (cycle threshold) value						
	A(H3N2)		IFB		A(H1N1)pdm09		Internal control
	Positive (%)	Target C_t Mean/SD	Positive (%)	Target C_t Mean/SD	Positive (%)	Target C_t Mean/SD	Reference C_t Mean/SD
Overall	CDC real-time RT-PCR assay						
	40/40 (100)	27.39 ± 3.61	8/8 (100)	34.22 ± 2.67	13/13 (100)	28.87 ± 1.77	23.34 ± 0.45
	Commercial RT-PCR kit						
	40/40 (100)	28.11 ± 3.42	8/8 (100)	35.82 ± 3.92	13/13 (100)	29.58 ± 1.92	24.34 ± 0.51
	CDC real-time RT-PCR assay versus commercial RT-PCR kit						
	+/+	+/−	−/+	−/−	Concordance (%)		Kappa
Overall	61	0	0	61	100.00		1.000
A(H3N2)	40	0	0	40	100.00		1.000
IFB	8	0	0	8	100.00		1.000
A(H1N1) pdm09	13	0	0	13	100.00		1.000
	CDC real-time RT-PCR assay versus commercial RT-PCR kit as gold standard				Commercial RT-PCR kit versus CDC real-time RT-PCR assay as gold standard		
	Sensitivity (%)		Specificity (%)		Sensitivity (%)	Specificity (%)	
Overall	100		100		100	100	
A(H3N2)	100		100		100	100	
IFB	100		100		100	100	
A(H1N1) pdm09	100		100		100	100	

overall 100.0 and 100%, respectively, and for individual pathogens were A(H3N2) 100.0, 100.0%; IFB 100.0, 100%; A(H1N1) pdm09 100.0, 100.0%, respectively, when commercial kit detection was considered as the gold standard (Table 3). On the other hand, sensitivity and specificity of commercial kit were overall 100 and 100.0%, respectively, when CDC protocol was taken as the gold standard. Individual pathogens then showed the following sensitivity and specificity, respectively: A(H3N2) 100.0, 100.0%; IFB 100%, 100.0%; A(H1N1) pdm09 100.0, 100.0%. Thus, the overall concordance between both the methods was 100% and kappa correlation was 1.000. Of note, we also observed that the C_t values for detection of each of the pathogens from the same samples done simultaneously by the two different methods were highly comparable, further reinstating that even at the quantitative levels, these two methods matched each other perfectly.

Discussion

In Asian countries, respiratory infections caused by the influenza virus have been generally ignored by the healthcare facilities. Information about influenza strains circulating in the Indian subcontinent stands majorly unknown due to lack of systemic studies. The available information on epidemiological and clinical features of influenza virus is entirely from research studies alone. Such studies are important to keep track of antigenic shifts in influenza virus which can lead to major endemics and economic losses [27, 28•]. With the rapid increase in number of influenza cases each year across the globe, and advent of more virulent strains and continued viral persistence in India and neighboring countries, there is an urgent need to systematically track the global dispersion of this virus in humans using molecular means.

A(H3N2) emerged as the etiology for the bulk of the cases positive for influenza. Contemporarily, this trend is also seen globally as reported from other studies reported from the USA and several other countries where A(H3N2) was detected in 26 to 30% of all influenza cases [29•].

Co-infections of different influenza viruses are rarely reported and reports focus solely on co-infections among different subtypes but not antigenic variants of the same subtype strains [30•, 31••, 32••]. The rate of co-infection determined in this study was 1.2% ($n = 931$) among all three tested pathogens. This rate is also agreeable with reported literature showing 0 to 3% co-infections among influenza subtypes [33•, 34•]. Although influenza co-infections are rare, we have shown that they occur during the first stage of a pandemic while seasonal strains co-circulated. This co-circulation poses a risk for further genetic reassortment in influenza strains, which could result in development and spread of new strains with pandemic potential.

Molecular methods, including one-step PCR and RT-PCR, have provided a convenient and sensitive approach for the

diagnosis of influenza virus within a reasonable turnaround time [18••, 35••, 36••]. Our study reinstates the importance of clear diagnosis and overall surveillance of the highly adaptive and evolving influenza virus using molecular methods to aid healthcare providers to adapt to the ever-changing clinical manifestations of antigenically mutated strains.

The molecular testing of influenza patients helped the clinicians in timely diagnosis and treatment of these patients during the study. The RT-PCR test has higher sensitivity and specificity; hence, it is considered to be the gold standard for true estimation of disease burden, as compared to the any other commercial antigen-based or other tests. Therefore, continued surveillance of the circulating influenza viruses in India will help implement influenza control and also serve as cues for determining priority populations for possible vaccination.

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Compliance with Ethical Standards

Conflict of Interest Seetha Dayaker, Heera R. Pillai, Vineetha P. Thulasi, Devakikutty Jayalekshmi, and Radhakrishnan R. Nair declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors. Informed consent was taken from patients prior to enrollment.

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