Susceptibility of influenza viruses circulating in Western Saudi Arabia to neuraminidase inhibitors

Ahmed M. Tolah, BSc, MSc, Esam I. Azhar, MSc, PhD, Anwar M. Hashem, BSc, PhD.

ABSTRACT

Objectives: To investigate the sensitivity of circulating influenza viruses in Western Saudi Arabia to neuraminidase inhibitors (NAIs); mainly, zanamivir and oseltamivir.

Methods: Respiratory samples were collected from patients presenting with respiratory symptoms to King Abdulaziz University Hospital, Jeddah, Kingdom of Saudi Arabia (KSA) between September 2013 and October 2014. All samples were tested prospectively by real-time reverse-transcription polymerase chain reaction for influenza A and B viruses. Positive samples were then inoculated on Madin-Darby Canine Kidney (MDCK) cells and isolated viruses were examined for their sensitivity to NAIs using fluorescent neuraminidase inhibition assay.

Results: Out of 406 tested samples, 25 samples (6.2%) were positive for influenza A/pdmH1N1 virus, one sample (0.25%) was positive for influenza A/H3N2 virus, and 7 samples (1.7%) were positive for influenza B Yamagata-like virus. Screening of isolated influenza A and B viruses (9 out of 33) for their sensitivity to NAIs showed no significant resistance to available NAIs.

Conclusion: Our results show that circulating influenza viruses in Jeddah are still sensitive to NAIs.

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Current seasonal influenza vaccines are effective in reducing incidence and severity of influenza illnesses and complications. However, these vaccines mainly elicit strain-specific neutralizing antibodies against the viral hemagglutinin (HA) and neuraminidase (NA). Furthermore, the continuously changing nature of HA and NA, and the diversity of influenza viruses impose a challenge to vaccine developers and manufacturers.[1] Because of the considerable time, which is usually required to produce and distribute such vaccines, it is crucial to examine the effectiveness of currently available prophylactic and therapeutic anti-influenza drugs, which could play a key role in the control of seasonal epidemics and occasional pandemics of influenza. The reported high resistance levels of influenza A viruses to adamantane (amantadine and rimantidine), which are M2 ion channel blockers, since 2005 led to the recommendation against its use for the treatment and prophylaxis of influenza A viruses.[2] Moreover, while resistance to NA inhibitors (NAIs) (oseltamivir and zanamivir), was being reported sporadically, resistance to oseltamivir increased significantly since 2007 and spread globally.[3] Interestingly, regardless of the stockpiling of NAIs and its extensive use during influenza A (H1N1) 2009 pandemic, several studies[4,5] have shown low level of resistance to NAIs among viruses isolated during or after the 2009 pandemic. Nonetheless, resistance to oseltamivir can emerge even in patients with no known treatment,[6,7] which undoubtedly underscores the importance of the continued monitoring for resistant strains via active surveillance programs. Unfortunately, there is no existing influenza surveillance program in the Kingdom of Saudi Arabia (KSA) and current epidemiological and virological influenza data are very limited. In addition, more than 4 million Muslims from all over the world visit Western Saudi Arabia during the religious mass gatherings (Umrah and Hajj), which could lead to the importation of resistant and highly pathogenic viruses, especially during influenza seasons. Indeed, influenza has been shown to be one of the main respiratory viruses that are transmitted during these seasons.[8] Therefore, the aim of this study was to establish and start investigating the sensitivity of circulating influenza strains to NAIs in KSA. Such information should increase our knowledge on the spread of antiviral resistance in KSA and ultimately contribute to the global information on the level of antiviral resistance of influenza viruses worldwide.

Methods. *Samples.* A total of 406 samples collected prospectively from patients presented with respiratory manifestations at King Abdulaziz University Hospital (KAUH), Jeddah, KSA between September 2013

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and October 2014 were screened for influenza A and B viruses. Samples used in this study included throat and nasal swabs, tracheal and nasopharyngeal aspirates, sputum, endotracheal tube aspirates, and bronchial alveolar lavage. Upon receiving, 140 µl from each sample were used for ribonucleic acid (RNA) extraction and the rest of the sample was immediately frozen at -80°C.

Ribonucleic acid extraction. Viral RNA was extracted from all clinical samples using QIAamp Viral RNA mini kit according to the manufacturer's instructions (Qiagen, USA). Extracted RNA was stored at -80°C until use.

Screening for influenza A and B viruses. Extracted RNA from each clinical specimen was initially screened for influenza A and B viruses by real-time reverse-transcription polymerase chain reaction (rRT-PCR) using InfA and InfB primers and probes sets (**Table 1**) according to Centers for Disease Control and Prevention (CDC) protocol.[9]

Influenza A subtyping. Extracted RNA of influenza A positive samples was used to determine influenza A subtype using primers and probes specific for H1 (AH1-F, AH1-R, and AH1-P), H3 (AH3-F, AH3-R and AH3-P), or pdmH1 (pdmH1-F, pdmH1-R and pdmH1-P) subtypes (**Table 1**).[9]

Table 1 - Influenza real-time reverse-transcription polymerase chain reaction primers and probes.

Primer name	Primer sequence (5'>3')
InfA-F	GACCRATCCTGTCACCTCTGAC
InfA-R	AGGGCATTYTGGACAAAKCGTCTA
InfA-P*	TGCAGTCCTCGCTCACTGGGCACG
InfB-F	TCCTCAAYTCACTCTTCGAGCG
InfB-R	CGGTGCTCTTGACCAAATTGG
InfB-P*	CCAATTCGAGCAGCTGAAACTGCGGTG
AH1-F	AACTACTACTGGACTCTRCTKGAA
AH1-R	CCATTGGTGCATTTGAGKTGATG
AH1-P**	TGAYCCAAAGCC"T"CTACTCAGTGCGAAAGC
AH3-F	AAGCATTCCYAATGACAAACC
AH3-R	ATTGCRCCRAATATGCCTCTAGT
AH3-P*	CAGGATCACATATGGGSCCTGTCCCAG
pdmH1-F	GTGCTATAAACACCAGCCTCCCATT
pdmH1-R	AGACGGGAYATTCCTCAATCCTG
pdmH1-P**	ATACATCCGA"T"CACMATTGGAAAATGTCC
BHA-188F	AGACCAGAGGGAAACTATGCCC
BHA-270R	TCCGGATGTAACAGGTCTGACTT
Probe-VIC2†	CAGACCAAAATGCACGGGGAAHATACC
Probe-YAM2*	CAGRCCAATGTGTGTGGGGAYCACACC

* Probes are labeled with 6-carboxyfluorescein (FAM) at the 5'-end and Blackhole Quencher 1 (BHQ-1) at the 3'-end, ** Probes are labeled with FAM at the 5'-end, quenched internally at a modified "T" residue with BHQ-1, and the 3'-end was modified by C3-spacer to prevent probe extension by Taq polymerase, †Probe is labeled with Yakima Yellow at the 5'-end and BHQ-1 at the 3'-end.

Influenza B lineage determination. The RNA from specimens that tested positive for influenza B was used to determine influenza B lineage by multiplex rRT-PCR assay using influenza B primers (BHA-188F and BHA-270R) and probes specific for Victoria (Probe-VIC2) and Yamagata (Probe-YAM2) lineages (**Table 1**) according to WHO protocol.[10]

Influenza virus isolation. Virus isolation was performed on 85-90% confluent Madin-Darby Canine Kidney (MDCK) cells (ATCC: CCL-34) as previously described.[11] Cells were observed daily for cytopathic effects (CPE). Cell supernatant was collected from infected cells upon CPE. Collected supernatant were re-inoculated to ensure inability of virus isolation when no CPE was observed. All supernatants were screened initially by hemagglutination assay[11] to verify viral isolation and confirmed by InfA and InfB rRT-PCR assays. All isolated viruses were aliquated, stored at -80°C, and used for NAIs sensitivity testing.

Neuraminidase inhibition assay. The NA enzymatic activity of each viral isolate was measured by a fluorescence-based assay using the fluorogenic substrate (2'-(4-methylumbelliferyl)-a-D-N-acetyl neuraminic acid) MUNANA (Sigma-Aldrich, St-Louis, MO, USA)[12] to determine the optimal virus dilution to be used in NA inhibition assay. Fluorescence was measured on Synergy HT microplate reader (BioTek, USA) using excitation wavelengths of 360 nm and emission of 460 nm. Curves were constructed by plotting the relative fluorescence units (RFU), namely, NA activity, against the dilution factor. The appropriate concentration of each virus was then determined by selecting a viral dilution which gives NA activity in the linear portion of the enzyme activity curve. Next, the 50% inhibitory concentration (IC_{50}) for each drug was determined by mixing 10 µl of each virus diluted according to the NA activity assay with increasing concentrations of the active form of oseltamivir phosphate, oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland), or zanamivir (GlaxoSmithKline, Stevenage, United Kingdom) starting from 0.01 to 10,000 nM. NA activity was then measured by MUNANA assay.[12] Curves were constructed by plotting NA activity (RFU) in the presence of drugs against drug concentration (nM) to determine IC_{50} values using nonlinear curve fit in GraphPad Prism version 5. The IC_{50} values for each drug were calculated as the drug concentration required to reduce the RFU of each virus by 50% compared with virus only control. Measurements were carried out in duplicates and the mean IC_{50} was reported.

Table 2 - Detected and isolated influenza viruses among 406 samples.

Tested samples	n	Positive samples (isolated)			
		Inf-A		Inf-B*	Total
		A/pdmH1	A/H3		
Throat and nasal swabs	309	11 (0)	1 (0)	2 (2)	**14 (2)**
Tracheal aspirates	31	5 (0)	0	0	**5 (0)**
Nasopharyngeal aspirates	34	2 (2)	0	5 (5)	**7 (7)**
Sputum	24	3 (0)	0	0	**3 (0)**
Endo tracheal tube	6	3 (0)	0	0	**3 (0)**
Bronchial alveolar lavage	2	1 (0)	0	0	**1 (0)**
Total	**406**	**25 (2)**	**1 (0)**	**7 (7)**	**33 (9)**

All detected influenza B viruses were from Yamagata lineage.

Table 3 - Summary of oseltamivir and zanamivir inhibitory concentration 50% (IC_{50}) values.

Isolated virus	Oseltamivir IC_{50} (nM)	Zanamivir IC_{50} (nM)
A/Jeddah/1/2014(pdmH1N1)	2.4	2.3
A/Jeddah/2/2014(pdmH1N1)	2.1	2.1
B/Jeddah/1/2013	2.1	7.3
B/Jeddah/2/2013	2.2	2.6
B/Jeddah/3/2013	2.2	4.9
B/Jeddah/4/2013	3.2	5.1
B/Jeddah/5/2013	2.2	2.4
B/Jeddah/6/2014	1.2	0.8
B/Jeddah/7/2014	0.8	0.7

pdmH1N1 - influenza A virus, nM - nanomolar

Results. Of the 406 samples collected from KAUH between September 2013 and October 2014, 33 samples (8.1%) were positive for influenza A and B viruses by rRT-PCR. Twenty-six samples (6.4%) were positive for influenza A and 7 samples (1.7%) were positive for influenza B (**Table 2**). Further analysis showed that almost all of the influenza A viruses are pdmH1N1 viruses (25/26) with only one H3 virus, and all detected influenza B viruses belong to the Yamagata lineage as determined by rRT-PCR (**Table 2**). All clinical samples that tested positive for influenza A or B by rRT-PCR were then inoculated on MDCK cells. Samples were passaged up to 3 times in MDCK cells to ensure virus isolation. Of the 26 influenza A positive samples, only 2 samples (both were pdmH1N1 viruses) showed CPE upon inoculation in MDCK cells. The remaining 24 samples did not show any CPE even after the third passage in MDCK cells and the viruses were redeemed unisolated (**Table 2**). All the 7 samples that tested positive for influenza B showed CPE in MDCK cells upon inoculation (**Table 2**). Isolation of influenza viruses in cell culture was confirmed by hemagglutination assay and rRT-PCR. Isolated viruses were then tested for their sensitivity to oseltamivir and zanamivir using standard fluorescent NA inhibition assay. The NA inhibition assay showed that all isolated viruses are associated with low IC_{50} values (**Table 3**). Specifically, IC_{50} values for oseltamivir and zanamivir against the 2 isolated influenza A/pdmH1N1 viruses ranged from 2.1 to 2.4 nM. Similarly, all isolated influenza B viruses were associated with low oseltamivir IC_{50} values ranging from 0.8 to 3.2 nM and zanamivir from 0.7 to 7.3 nM. While the number of isolated influenza viruses in this study is very limited, which might hamper the analysis of the drug susceptibility data and the identification of any resistant viruses, our results provide a snapshot of the sensitivity of circulating influenza viruses in KSA to NAIs, and shows that circulating influenza viruses in Jeddah, KSA are sensitive to NAIs.

Discussion. Influenza antivirals represent an important strategy for influenza prophylaxis and treatment. Although resistance to M2 blockers seem to be widespread all over the world, NAIs still represent a viable option for influenza treatment.[13] However, resistance to these antivirals can emerge not only upon extensive use of these drugs, but also in patients who never received such treatment.[6,7] These resistant

strains can spread globally,[3,4] which highlights the importance of the continued monitoring via active influenza surveillance programs. Such programs should be an integral part of any existing public health system to monitor circulating influenza strains, as well as the occurrence of any resistance using appropriate laboratory methods. This is of particular importance in KSA, especially that millions of Muslims from all over the world visit the Kingdom every year to perform Hajj and Umrah. Mass gathering in the holy places (Makkah and Al-Madinah) during a very short period of time could play a major role in the introduction of new influenza viruses to KSA, as well as other countries. Unfortunately, there is no such program in KSA, which could pose several public health concerns.

While a very limited number of reports investigated the prevalence of influenza viruses in KSA, most of these studies have mainly focused on Hajj season. In a recent report,[14] screening of 1600 pilgrims upon their arrival to 2010 Hajj season showed that 120 individuals (7.5%) had influenza A viruses and only 9 individuals out of the 120 had pdmH1N1 virus.[14] Importantly, it showed that pilgrims cannot only introduce influenza viruses to KSA, but they could also import them back to their home countries.[8] However, none of these reports have really examined the susceptibility of circulating influenza viruses to NAIs in KSA. Here, we tried to establish and investigate the susceptibility of circulating influenza viruses to NAIs using cell-free NA inhibition assay. Our data showed that all the isolated viruses (2 influenza A/pdmH1N1 viruses and 7 influenza B viruses) were associated with low oseltamivir and zanamivir IC_{50} values suggesting their susceptibility to NAIs, which is consistent with the current low worldwide rate of NAI resistance.

Study limitation. The lack of previous data on drug susceptibility from KSA and the limited number of viral isolates in this study may represent a limitation in our report and may not provide a clear picture on drug resistance in KSA.

In conclusion, our report shows that circulating influenza viruses in Jeddah are still sensitive to NAIs and establishes baseline data for future studies in KSA. Most importantly, it highlights the importance of establishing a continued and active surveillance and monitoring program of circulating influenza viruses in KSA to ensure effective use of antivirals for the prophylaxis and treatment of influenza, especially that the rate of vaccination against influenza is very low among pilgrims and health care workers.[15]

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From the Department of Medical Laboratory Technology (Tolah, Azhar), Faculty of Applied Medical Sciences, the Special Infectious Agents Unit (Tolah, Azhar, Hashem), King Fahd Medical Research Center, and the Department of Medical Microbiology and Parasitology (Hashem), Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia. Address correspondence and re-prints request to: Dr. Anwar M. Hashem, Department of Medical Microbiology and Parasitology, Faculty of Medicine, Jeddah, Kingdom of Saudi Arabia. E-mail: amhashem@kau.edu.sa

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