Journal of Antivirals & Antiretrovirals - Open Access

JAA/Vol.1 Issue.1

Research Article | OPEN ACCESS Freely available online | doi:10.4172/jaa.1000008

Failure to Detect Oseltamivir Resistance by Rolling Circle Amplification and Sequencing Following Use in an Influenza A (H3N2) Outbreak at an Aged Care Facility

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Abstract

Background: The recent worldwide emergence of increasing oseltamivir resistance to influenza A H1N1 may have important implications for influenza prevention and control.

Objectives: To assess oseltamivir use, the emergence of resistance and the outcome of an influenza A H3N2 outbreak.

Patients/Methods: Following identification of an influenza outbreak through active surveillance, the investigators offered treatment and prophylaxis with oseltamivir to both Aged Care Facility (ACF) residents and staff. The investigators conducted genotypic sensitivity testing using sequencing and rolling circle amplfication for known oseltamivir resistance mutations.

Results: An influenza A H3N2 outbreak affecting an ACF with 90 residents and 79 staff was identified 6 days after the initial case. Oseltamivir prophylaxis was commenced on day 7. The overall attack rate was 10%, with 13 of 92 residents and 4 of 79 staff infected. There was no evidence of the development of genotypic resistance, even at low levels, to oseltamivir in patients tested whilst on treatment or prophylaxis.

Conclusions: There was no clinical or genotypic evidence of oseltamivir resistance, an important observation in the context of recent reported antiviral resistance in other influenza A subtypes.

Keywords: Human; Influenza; Oseltamivir; Resistance; Virus

Introduction

Influenza virus resistance to the neuraminidase inhibitors (NI) oseltamivir and zanamivir occurs less frequently than with the M2 ion channel inhibitors amantadine and rimantadine. Depending on the molecular techniques used, oseltamivir resistance rates of 5.5-18% in children and 1% in treated adults have been reported (Kiso, 2004; McKimm-Breschkin, 2005; Roche data on file; Whitley, 2001), although transmission of these strains appeared rare. However, recent global data indicates a change in NI resistance with influenza A (H1N1) strains. In the 2007/08 winter in the United States, 84 of 1,018 (8.3%) influenza A viruses (all influenza A (H1N1) strains), but none of 135 influenza B viruses, were found to have the H274Y neuraminidase gene mutation associated with high-level oseltamivir resistance (CDC, 2008). Similar resistance mutations have been detected in influenza A (H1N1) isolates collected in the last quarter of 2008; with very high rates globally (91%) (WHO, 2008). This resistance has generally occurred in the absence of oseltamivir treatment, and these viruses have been readily transmitted from person-to-person.

In influenza, antiviral drug resistance detection has required cell-culture methods to isolate viral strains, followed by genotyping or phenotyping analyses. An alternative genotyping method involves DNA sequencing of bulk RT-PCR products amplified from many extracted viral genomes (or quasispecies) derived from patient samples (McKimm-Breschkin, 2003). Potential problems with sequencing include reduced sensitivity for low levels of resistant viral quasispecies, relatively high cost, and difficulty with high throughput and rapid processing. More sensitive methods include the use of padlock probes that allow parallel, high-throughput single nucleotide polymorphism (SNP) genotyping at increased scales. Padlock probes are linear oligonucleotides that comprise two target-specific end-sequences and a linking segment, typically carrying sequences used for identification and detection. The end-sequences hybridize head-to-tail to the target DNA, forming a nick between the ends. This

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Received October 15, 2009; **Accepted** November 04, 2009; **Published** November 05, 2009

Citation: Rosewell A, Wang B, Saksena NK, MacIntyre CR, Lindley R, et al. (2009) Failure to Detect Oseltamivir Resistance by Rolling Circle Amplification and Sequencing Following Use in an Influenza A (H3N2) Outbreak at an Aged Care Facility. J Antivir Antiretrovir 1: 056-061. doi:10.4172/jaa.1000008

nick can be recognized and sealed by a high fidelity DNA ligase, only upon perfect hybridization to a target template. The subsequent detection by rolling circle amplification (RCA) provides a high throughput, isothermal method of target signal amplification and rapid detection (Nilsson, 1994; Lizardi, 1998).

Influenza outbreaks in aged care facilities (ACF) are common and can be severe, with high attack rates in staff and residents (Bush, 2004; Mitchell, 2006). They can result in major disruption and staff absenteeism, but timely diagnosis is uncommon (Mitchell, 2006; Monto, 2004; van der Sande, 2006). As a result, the use of effective antivirals for controlling influenza outbreaks is often delayed, thus hampering outbreak control (Bush, 2004; van der Sande, 2006). Guidelines for the prevention and control of influenza outbreaks in ACFs recommend treatment and post-exposure prophylaxis with antiviral drugs (Fiore, 2007; CDNA, 2005). Randomised, controlled clinical trials in healthy adults demonstrate that neuraminidase inhibitors are efficacious in both treatment and prevention (Treanor, 2000; Welliver, 2001). Although the efficacy of post-exposure prophylaxis with oseltamivir is yet to be established in ACF influenza outbreaks where the population are the frail elderly, observational studies suggest that treatment and post-exposure prophylaxis is effective in controlling influenza outbreaks (Bush, 2004; van der Sande, 2006; Guy, 2004; Parker, 2001). Oseltamivir treatment must be given within 48 hours from symptom onset for efficacy; effective prophylaxis also requires timely administration. Early management of outbreaks may be hampered by the size and conditions of the ACF, the health and age of residents, and the difficulties of rapidly contacting multiple primary care doctors. It has been established that early intervention in confirmed influenza outbreaks results in better outbreak control (Monto, 2004), and that active surveillance can be highly effective at identifying influenza outbreaks prospectively (Loeb, 2000), but as yet active surveillance has not been adopted as common practice in ACFs.

The investigators describe the course and outcome of an influenza A (H3N2) outbreak in an ACF detected by active surveillance and timely point-of-care (POC) testing where oseltamivir was used early; and report oseltamivir resistance in this cohort of exposed, institutionalised elderly.

Materials and Methods

Active surveillance

To enable the early identification of influenza outbreaks, mechanisms for active surveillance and rapid diagnosis of influenza were established in 16 ACFs in and around Sydney, Australia. In early winter 2006, the study team commenced active telephone contact at least 3 times per week with a designated nurse within each ACF for early identification of ILI in staff and residents. ILI was defined as acute onset of fever ≥38ºC, with newly acquired cough or any other respiratory symptoms. An influenza outbreak was defined as either two cases of ILI in staff and/or residents over a three day period, with at least one testing positive for influenza by POC test or other laboratory tests; or, three cases of ILI meeting the same criteria and at least one positive laboratory test. Clinical and demographic information was obtained from any ill staff or residents. An influenza outbreak was identified in one facility in the study and was investigated. This study was conducted in accordance with protocols approved by institutional review boards for human research at the University of Sydney and The Children's Hospital at Westmead, and was registered with the Australian Clinical Trials Registry (ACTRN12606000278538).

Epidemiological investigation

Surveillance identified an influenza outbreak on the 6th of October 2006 (day 6 of the outbreak) in a 100 bed single-level facility with two wings (A and B) containing unisex 1-4 bedrooms. There were 92 residents and 79 staff at this time. Data from surveillance telephone calls and medical notes of consented residents were reviewed, and nursing and other staff were interviewed by a public health physician to identify additional cases. For this investigation, any new respiratory illness was considered if onset occurred between the 30th of September (retrospectively identified as day 0 of the outbreak as it was the date of onset of the first possible case) and the 26th of October 2006 (8 days after symptom onset of the last symptomatic case). Possible cases were defined as a person with a respiratory sign or symptom but with a temperature below 38ºC during the outbreak. Probable cases met the clinical case definition but did not have laboratory confirmation, and confirmed cases met the clinical case definition and had influenza virus detected by rapid antigen (POC) testing, direct immunofluorescence (DIF), nucleic acid testing (NAT) by reverse transcriptase polymerase chain reaction (PCR) or influenza-specific serology.

Resident influenza vaccination status was validated by reviewing medical notes and general practitioner records. Attack rates in residents were calculated by dividing the number of resident cases by the total number of residents on the first day of the outbreak (day 0).

Laboratory investigation

Nursing home staff were trained to administer a rapid antigen POC test (Quidel QuickVue™, Quidel Corporation, San Diego, CA, USA). Combined nose and throat swabs and blood were collected from all consenting staff and residents before receiving either antiviral treatment (if ill) or prophylaxis, and transported at 4ºC in viral transport medium to the laboratory. If influenza virus was detected in the initial respiratory tract specimen and the patient was treated with oseltamivir, then combined nose and throat swabs were collected every 3 days until the resolution of respiratory symptoms or until NAT became negative. This included initially asymptomatic patients given oseltamivir prophylaxis, but who switched to oseltamivir treatment if they developed symptoms whilst on prophylaxis. DIF using fluorescein-conjugated monoclonal antibodies (Chemicon International, Temecula, CA, USA) against influenza A and B was performed on acetone-fixed smears of deposits from the respiratory tract specimens if taken within 24 hours of symptom onset. NAT was performed on all specimens from symptomatic staff and residents except if influenza had already been detected by both POC and DIF. RNA was extracted from the remaining original clinical sample using the High Pure viral RNA kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. A nested reverse transcriptase polymerase chain reaction (RT-PCR) was carried out to detect influenza A and B (Druce, 2005). Subtyping was performed by sequencing of the hemagglutinin gene in one ill patient.

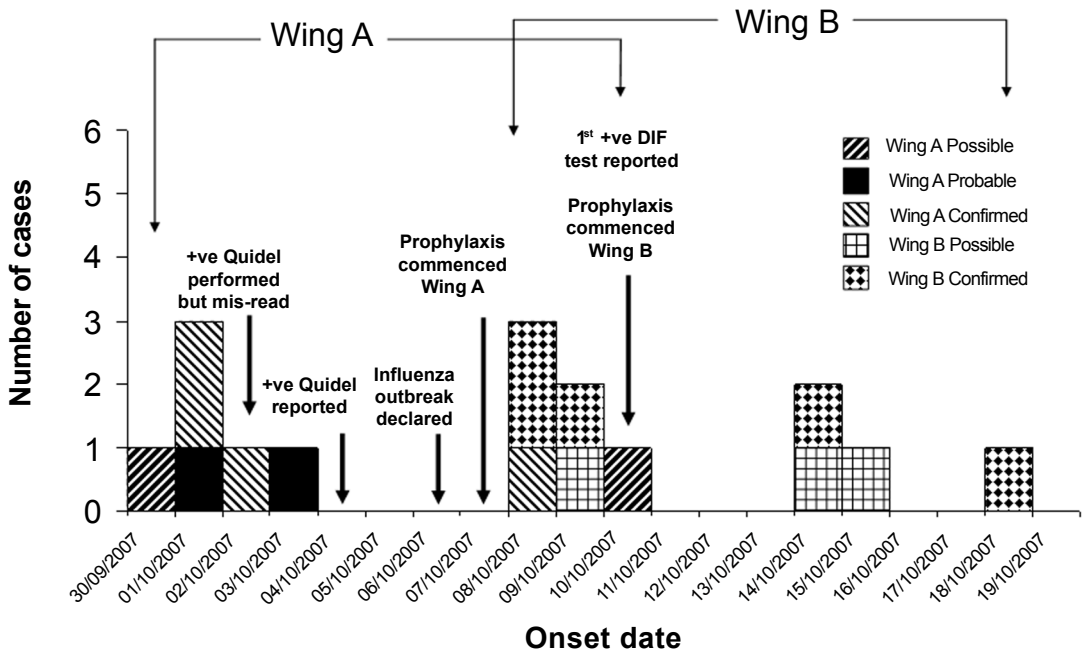

Figure 1: Epidemic Curve for Influenza A in an ACF, Australia, October, 2006.

RNA extracted from samples from eight patients was used for genotypic oseltamivir sensitivity testing. There were three samples from symptomatic patients (including the index case) prior to starting treatment; one of these had two further samples assessed as they had influenza A detected by RT-PCR on day 3 of therapy and day 6 (the day after completing therapy). Three samples from patients who developed respiratory symptoms whilst on oseltamivir prophylaxis and prior to switching to treatment were also assessed (McKimm-Breschkin, 2003). A one-step RT-PCR (QIAGEN OneStep RT-PCR Kit, Qiagen, Hilden, Germany) using primers spanning the full neuraminidase gene (N2) was conducted (sense N2A and antisense N2B primers were nucleotides 1-26 and 1420-1402 respectively from GenBank accession number EF512573, an influenza A/Wellington/1/2004(H3N2) strain), followed by a second round of PCR (sense N2C and antisense N2D primers were nucleotides 39-56 and 1361-1342 respectively from GenBank accession number EF512573) using the Pfu Ultra High-fidelity DNA Polymerase (Stratagene, La Jolla, CA, USA). These generated a segment that included sites for two potential N2 oseltamivir resistance mutations, Glu119Val and Arg292Lys. PCR products were purified using a Millipore vacuum apparatus and sequenced by the Big-Dye Terminator Cycle Sequencing Ready Reaction kit (ABI Prism, V3.0) on an ABI 377 automated sequencer. Neuraminidase sequences derived from each patient were analyzed using Sequencer 3.1 software. Sequences derived from both sense and reverse primers were aligned with a reference influenza A (H3N2) strain EF512573 and chromatograms were examined for known resistance mutations.

Sequencing based genotypic resistance can only achieve the detection of resistance mutations when it consists of more that 25% of the total population. To ensure that low-levels of resistance mutations can be detected, special padlock probes recognising the resistance mutations Glu119Val and Arg292Lys were designed (Steain, 2009). Using the PCR product as target template, the probes were ligated to the template followed by RCA of the probe signal. Standard testing using 10^{11} copies of the PCR template containing 119Val and 292Lys at various dilutions detected a strong signal when the resistance mutation was present at >0.5% (data not shown) when testing patient-derived templates with the same input copy, no significant increase of probe signal was detected indicating a lack of low levels of resistance mutations in the patient samples.

Results

Epidemiological investigation

On the morning of day 2 of the outbreak during a routine surveillance phone call, an ILI was reported in a resident. POC testing was performed on the ill resident later in the day, before hospital admission, but the positive result was mis-read by a facility nurse and then rectified 2 days later in consultation with another nurse. A second ILI case, reported from a staff member returning to work on day 6, was subsequently confirmed to have influenza by NAT. The reporting of a person with ILI with a positive POC test (after the two day delay) together with another ILI reported with onset within 3 days from the first was the trigger to declare an influenza outbreak on day 6. When the outbreak was declared, there were 3 other symptomatic individuals not identified by the routine surveillance activities, but subsequently identified through the outbreak investigation. The time taken for the outbreak to be identified from onset of the first retrospectively identified case was 6 days, when specific control measures were implemented. Oseltamivir prophylaxis was initiated the following day (day 7). The median time from onset of illness to case identification for staff was 4.5 days and 2 days for residents.

The epidemic curve is presented in the Figure 1, and shows how the infection spread from one wing to the next, and how quickly the outbreak was controlled in each wing. The overall attack rate in residents and staff, including all possible cases, was 10% (Table 1), with 13 of 92 residents (attack rate 14%) and 4 of 79 staff (attack rate 5%) with possible, probable or proven infection; 10 were laboratory-confirmed. Over 19 days,

	Residents (n=92)		Staff (n=79)*		Total (n=171)	
	n	(%)	n	(%)	n	(%)
Possible cases	3	(3)	2	(3)	5	(3)
Probable cases	2	(2)	0	(0)	2	(1)
Confirmed cases†	8	(9)	2	(3)	10	(6)
Total cases (suspect + confirmed)	13	(14)	4	(5)	17	(10)
Received influenza vaccination 2006	70	(76)	Insufficient data		-	
Received oseltamivir treatment‡	3	(3)	1	(1)	4	(2)
Received oseltamivir prophylaxis‡	49	(53)	14	(18)	63	(37)

* The average number of staff per day being on-duty during the outbreak period was 38

† Including one asymptomatic seroconversion

‡ one resident developed symptoms within one day of commencing prophylaxis, and received treatment course once diagnosed – counted as having received treatment.

Table 1: Cases Details and Prevention and Control Measures in an Outbreak of Influenza A in an ACF, Australia 2006.

five of 92 residents and 1 of 79 staff met the clinical case definition for ILI with onset between days 0 and 18. Seven other residents and three other staff members developed cough with or without low-grade fever. Influenza A virus infection was confirmed in 10 of the 17 cases. Of these 10 confirmed cases, two first tested positive for influenza A virus infection by POC testing, one by DIF, three by PCR and four who had negative respiratory samples during the outbreak and were subsequently confirmed by demonstrating seroconversion to influenza A. One of these four cases was a resident (Case 17) who received oseltamivir prophylaxis and seroconverted, despite being free from respiratory symptoms or fever 2 weeks before, during and 4 weeks after the outbreak (Table 1). Although all residents and staff without respiratory symptoms were offered oseltamivir prophylaxis, only some (overall 37%) accepted it (Table 1). There were no deaths reported during the outbreak, but 4 of the 5 resident cases in the first week were hospitalised.

Laboratory investigation

Influenza A was confirmed by laboratory testing in 10 cases of which two were by rapid antigen POC testing, one by DIF, three by NAT and four by influenza antibody seroconversion. Sequencing of the hemagglutinin gene from one patient showed that the virus was influenza A/New York/55/2004 (H3N2)-like, a strain well matched to the 2006 southern hemisphere vaccine.

PCR amplification and sequencing the neuraminidase region in 8 samples demonstrated that all were N2. No known oseltamivir resistance mutations were detected on sequence analysis. Rolling circle amplification using N2 oseltamivir resistance-specific padlock probes targeting E119V and R292K and performed on the original clinical specimens did not detect low levels of these resistance mutations.

Discussion

Early intervention in confirmed influenza outbreaks and high antiviral coverage results in better outbreak control (Monto, 2004; Rubin, 2008) even a 1-day delay in prophylaxis may allow infection of many more residents (Drinka, 2003). Active surveillance where there is regular routine contact with the ACF and includes zero-case reporting instead of awaiting passive reporting, complemented by early detection with POC testing results in better outbreak control using antiviral prophylaxis (Drinka, 2003; Thomas, 2003). The implementation of prevention and control measures, including oseltamivir prophylaxis for asymptomatic residents and staff within seven days of onset of illness of the first case, led to an attack rate in residents of 14% with no deaths. Reported attack rates in influenza outbreaks in ACFs generally range between 20-40%; attack rates as high as 70% have been reported (Bradley, 1999). In this investigation, the outbreak was curtailed rapidly following use of oseltamivir when started early, even with only 58% of residents and 20% of staff receiving antivirals. Treatment of active cases of influenza alone may also have an impact on outbreak control, given this can reduce viral shedding by up to 50% (Treanor, 2000).

The development of antiviral resistance has the potential to occur following widespread use of oseltamivir. In this study of elderly ACF residents and otherwise healthy staff, the investigators did not find genotypic evidence of resistance in any patient with influenza A (H3N2) infection prior to, or whilst receiving, oseltamivir. In the northern hemisphere winter of 2007-2008, oseltamivir resistant influenza A (H1N1) virus carrying the H274Y mutation has been readily isolated from 1 month to 53 year olds, with the majority of resistant viruses isolated from adults. This increase in prevalence and transmission of oseltamivir resistant viruses does not appear to be driven by selective drug pressure, as few of the cases are known to have been treated with oseltamivir (ECDC, 2008). The spread of oseltamivir resistant influenza A (H3N2) has not been observed.

Although only about 1% of clinical isolates from immunocompetent patients in oseltamivir treatment studies have reduced sensitivity (McKimm-Breschkin, 2005), one might expect that resistance develops more readily in the elderly given their prolonged viral shedding, atypical clinical presentation and possible reduced treatment compliance or drug absorption. The atypical presentation of influenza in the frail elderly may promote the development of resistance as people with influenza, but not fitting the typical picture, may only receive prophylaxis dosing of oseltamivir at half the treatment dose. The failure to detect oseltamivir resistance in an elderly population is encouraging but continued surveillance for resistance is clearly warranted, and the potential development of resistance following widespread oseltamivir use remains a public health concern.

Addendum

A Rosewell*§ - analysis and interpretation of data, critical writing

B Wang** - analysis and interpretation of data, critical writing

NK Saksena** - analysis and interpretation of data, critical writing

CR MacIntyre* - concept and design, critical writing, final approval

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C Chiu* - concept and design, analysis and interpretation of data, critical writing

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R Booy* - concept and design, critical writing, final approval

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Acknowledgements

The investigators would like to thank the nursing staff at the aged care facility for their ongoing involvement in the active surveillance activities and assistance during the outbreak, and Linda Donovan for assistance with the laboratory testing. Alexander Rosewell is a Master of Applied Epidemiology Scholar at the Australian National University, a program funded by the Australian Government Department of Health and Ageing.

Sponsor's role

The investigators acknowledge Roche Pharmaceuticals Pty Ltd Australia, Quidel Corporation, San Diego, California, USA, and the Moran Health Care Group for their ongoing collaboration.

Conflict of Interest

Financial

Richard Lindley is supported by infrastructure grants from NSW Health and the Commonwealth of Australia. This work was funded by the Australian Research Council Linkage Grant scheme (LP0668279) in partnership with the Moran Health Care Group.

Personal

Richard Lindley is funded by the Moran Foundation for Older Australians, a Foundation of The University of Sydney, established by the current owners of the Moran Health Care Group. He has received payment from CSL Limited in his role as Chairman of influenza vaccine study Data Monitoring Committees. No other authors have a conflict of interest.

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