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Short communication

Genotypic and phenotypic resistance of pandemic A/H1N1 influenza viruses circulating in Germany

Susanne C. Duwe*, Marianne Wedde, Patricia Birkner, Brunhilde Schweiger

Robert Koch-Institut, National Reference Centre for Influenza, Nordufer 20, 13353 Berlin, Germany

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SUMMARY

In response to the rapid global spread of an antigenically novel A/H1N1 influenza virus in 2009, the World Heath Organization (WHO) recommended surveillance and monitoring for antiviral resistance of influenza viruses. We designed and evaluated pyrosequencing (PSQ)-based genotypic assays for high-throughput analysis of the susceptibility of pandemic A/H1N1 influenza viruses to neuraminidase (NA) inhibitors. A total of 1570 samples circulating in Germany between April 2009 and April 2010 were tested for determination of molecular markers of resistance to the NA inhibitors oseltamivir and zanamivir, and 635 of them were evaluated by phenotypic fluorescence-based assay with MUNANA substrate. Eight (0.5%) viruses were resistant to oseltamivir due to the H274Y NA substitution (N2 numbering). Six of these oseltamivir-resistant cases were treatment-related; four of them were selected in immunocompromised patients, two in patients suffered from chronic diseases. The two remaining oseltamivir-resistant viruses seem to have evolved in the absence of drug treatment and were isolated from immunocompetent healthy patients. All tested A/H1N1 pandemic viruses were sensitive to zanamivir. In addition, analysis of 1011 pandemic A/H1N1 virus samples by a PSQ-based assay according to the WHO protocol revealed the presence of mutation S31N in the M2 protein that conferred resistance to M2 ion channel inhibitors. Our data demonstrate a low incidence of oseltamivir-resistant pandemic A/H1N1 influenza variants isolated under drug selection pressure as well as community-acquired or naturally evolving viruses.

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Currently, two classes of antiviral inhibitors are licensed for treatment and prophylaxis of influenza infections in humans: neuraminidase inhibitors (NAI), represented by oseltamivir (Tamiflu™) and zanamivir (Relenza™) and the M2 protein inhibitors (M2I) amantadine (Symmetrel™) and rimantadine (Flumadine™) which belong to the group of adamantanes (Davies et al., 1964; Tsunoda et al., 1965; Govorkova et al., 2001; McKimm-Breschkin, 2002).

Antiviral resistance is caused by subtype- and inhibitor-specific point mutations within the target proteins neuraminidase (NA) and M2 (Hayden and Hay, 1992; McKimm-Breschkin, 2000). Amino acid substitutions H274Y and N294S (N2 numbering used throughout) of viral NA of subtype N1 (NA-N1) are known to confer resistance to oseltamivir (Moscona, 2005; Abed et al., 2006). In addition, the NA-N1 substitution E119V causes resistance to both NAIs (Abed et al., 2006; Deyde et al., 2010). In a few cases, mutations H126N or Q136K that confer resistance to zanamivir were observed (Sheu et al., 2008; Hurt et al., 2009). Resistance to adamantane-derivatives is associated with selective amino acid substitutions at positions 26, 27, 30, 31 or 34 of the M2 protein (Hay et al., 1986; Bean et al., 1989; Hayden and Hay, 1992). Due to the recent increase of resistance to adamantanes among A/H3N2 and A/H1N1 viruses (Deyde et al., 2007), the distribution of oseltamivir resistant seasonal A/H1N1 viruses since November 2007 (Lackenby et al., 2008a; Sheu et al., 2008; WHO, 2008; Meijer et al., 2009) and the occurrence of a pandemic A/H1N1 virus in April 2009 led to a recommendation of the World Health Organization (WHO) for surveillance and monitoring for antiviral resistance of influenza viruses (WHO, 2009a).

Pyrosequencing (PSQ) assays have proved to be an effective tool for detection of molecular markers of resistance of influenza viruses (Bright et al., 2005; Duwe and Schweiger, 2008; Deyde et al., 2009; Lackenby et al., 2008b). Due to the variability of the target sequences, these assays recently published for testing seasonal viruses required adaptation to pandemic A/H1N1. We designed and evaluated sensitive high-throughput assays for detection of the resistance-associated NA-N1 substitutions H274Y and N294S. A 229-bp fragment was amplified using a primer pair with a biotinylated sense primer. Specific antisense primers placed adjacent to the relevant nucleotides for subsequent PSQ analysis of the resistance-associated positions were designed (Table 1). Our PSQ analyses of resistant and wild-type NA gene fragments clearly distinguished between wild-type and mutant virus variants (Fig. 1). Cross-reactivity was neither detected with cDNA from seasonal influenza viruses (type A and B), nor with cDNA or DNA from other

* Corresponding author. Tel.: +49 30 187542283; fax: +49 30 187542328.
E-mail address: DuweS@rki.de (S.C. Duwe).

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Table 1
Primer sequences for amplification, pyrosequencing and cycle sequence analysis.

Project	Primer designation	Sequence
PSQ amplification	N1p_s739_Bio	5′-Bio-AGTGATGGACAGGCCTCATACAAG
	N1p_as968	5′-CCGAAAATCCCACTGCATATGTAT
PSQ analysis	N1p_Ss828	5′-CAGGAGCATTCCTCA
	N1p_Sas888	5′-CGATTCGAGCCATGC
Nested PCR cycle sequencing	N1p_CS_Os558	5′-TGGCATCAATTGGCTAACAA
	N1p_CS_Oas1133	5′-TTCGGATCCCAAATCATCTC
	N1p_CS_Is593	5′-GGGCAGTGGCTGTGTTAAGT
	N1p_CS_Ias709	5′-ATCATTAGGGCGTGGATTGT

Bio: biotinylated, s: sense, as: antisense, p: pandemic, S: pyrosequencing, CS: cycle sequencing, I: inner, O: outer.

viruses or bacteria associated with respiratory infections (data not shown).

The sensitivity of the pandemic A/H1N1-NA PSQ-PCR was evaluated by testing cDNA samples with concentrations consistent with quantitative PCR (qPCR) cycle threshold (ct) values between 25 and 39 and by using positive control plasmids prepared by site-directed mutagenesis. Reactions containing at least 100 cDNA copies ($ct \leq 35$) tested positive (data not shown). For specimens that did not contain sufficient virus RNA for PSQ-PCR ($ct > 35$, or cDNA concentration <100 cDNA copies per reaction), a more sensitive nested PCR and subsequent cycle sequencing analysis were used (Table 1).

Following the recommendations of the WHO for monitoring for antiviral resistance, a total of 1570 samples from A/H1N1 pandemic influenza cases confirmed by real time Reverse Transcriptase-PCR (qRT-PCR, Schulze et al., 2010) were examined. Genotypic analyses for detection of molecular resistance markers were performed on 1269 samples. A total of 635 virus isolates were analysed for phenotypic resistance (Table 2) and for 336 isolates genotypic as well as phenotypic susceptibility profiles were determined. All clinical respiratory samples were collected from patients presenting with influenza-like illness between April 2009 and April 2010 and were tested by the German National Reference Centre for Influenza in Berlin. With few exceptions specimens were pro-

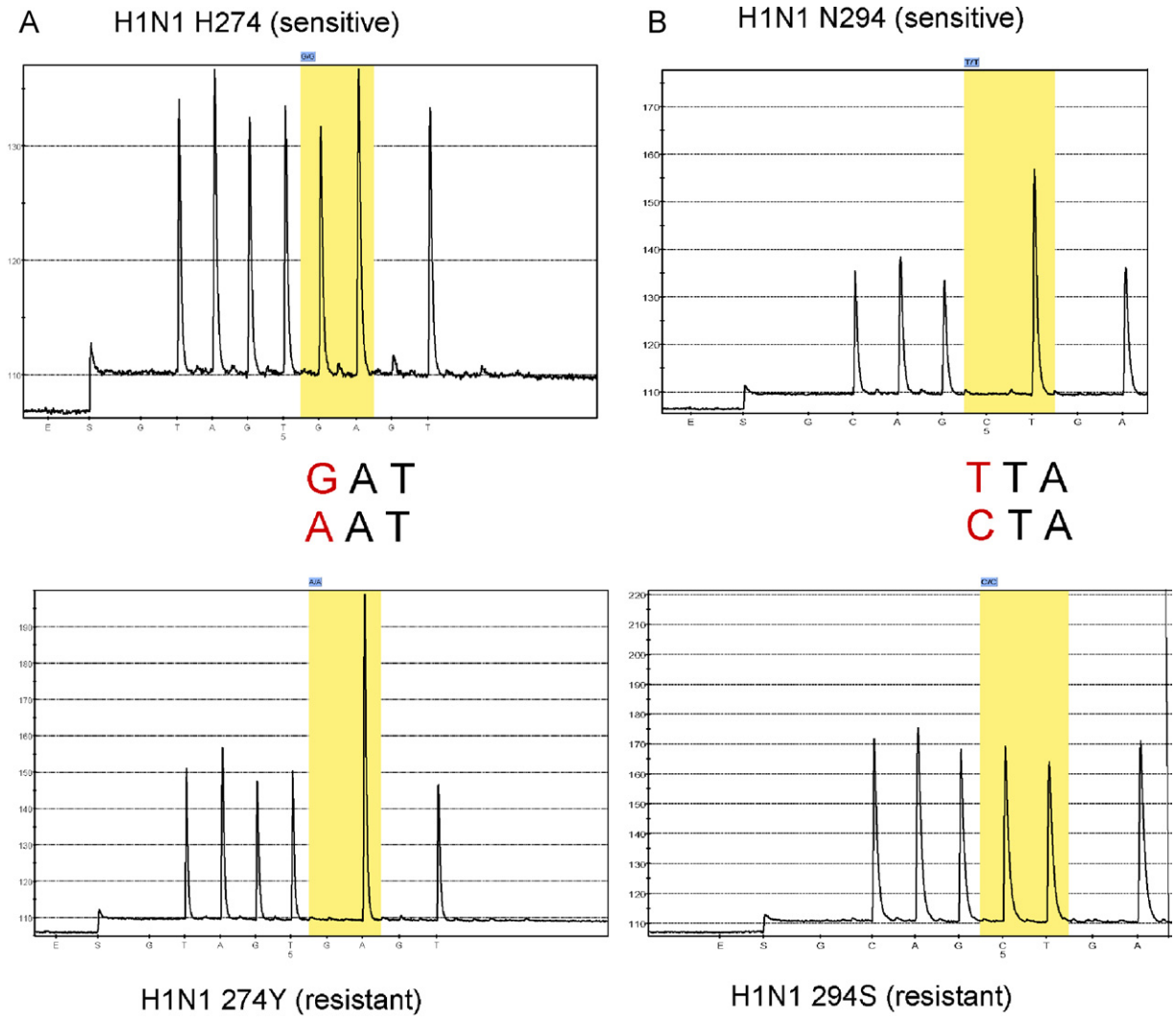

Fig. 1. PSQ analysis of resistance-associated mutations of NA by using antisense sequencing primers. Representative pyrograms are shown for codons 274 and 294, coding for sensitive and resistant NA. H = histidine, N = aspargine, S = serine, Y = tyrosine.

Table 2
Phenotypic susceptibility of pandemic A/H1N1 influenza viruses to neuraminidase inhibitors circulating in Germany (April 2009–April 2010).

Patient	Therapy-naive		Oseltamivir-treated	
		Patients 1 and 2	Patients 3 and 8	Patient 7 virus mixture
Oseltamivir IC_{50} (nM)				
n	630	2	2	1
Mean	1.81	600	400	200
SD	1.25	140	30	NA
Zanamivir IC_{50} (nM)				
n	630	2	2	1
Mean	0.51	1.30	0.85	3.5
SD	0.55	0.71	0.07	NA

The phenotypic susceptibility of influenza virus isolates to NA inhibitors was evaluated by using a fluorescence-based assay with MUNANA as substrate (Sigma–Aldrich). Briefly, equimolar amounts of NA-activity were incubated with inhibitor (1 h, 37 °C, 0–4000 nM final concentration) subsequently followed by an incubation period for 2 h, 37 °C, with 30 μl 100 μM MUNANA working solution. *n*: number of isolates, SD: standard deviation, IC_{50}: 50% inhibitory concentration, NA: not applicable.

vided by physicians working in the influenza surveillance scheme (Arbeitsgemeinschaft Influenza; Szecsenyi et al., 1995). The age of the patients ranged between 0 years and 82 years (mean ± standard deviation 20 ± 15 years). Susceptibility of viruses to oseltamivir and zanamivir was examined using a phenotypic fluorescence-based assay as described previously (Duwe and Schweiger, 2008). Briefly, the 50% inhibitory concentration (IC_{50}) for oseltamivir and zanamivir (final concentration ranged between 0 nM and 4000 nM) for pandemic A/H1N1 2009 virus isolates was determined in a fluorometric enzyme assay with 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma–Aldrich; 100 μM working solution) used as substrate (Potier et al., 1979).

In addition, the prevalence of resistance to adamantanes was determined by sequencing of 1011 pandemic virus M2 gene fragments using the WHO PSQ assay (WHO, 2009b). All of these fragments carried the S31N mutation, which is known to confer resistance to adamantanes.

Eight out of 1570 (0.5%) pandemic A/H1N1 viruses analysed for resistance to NAI were multidrug-resistant (characterized by M2-S31N substitution as well as the NA-H274Y mutation and/or IC_{50} oseltamivir >100 nM). In the first case, the virus was isolated from a respiratory sample of a 21-year-old man (patient 1) who developed an influenza-like illness (ILI) while on vacation abroad. Two days after onset of symptoms pandemic A/H1N1 infection was confirmed and sequencing analysis of the isolated virus showed presence of the NA-H274Y substitution. The second sample (patient 2) was obtained from a 9-year-old otherwise healthy boy, who developed ILI and bronchitis after attending a soccer match with approximately 300 other attendees. PSQ analysis performed on the patient's respiratory specimen showed resistance to oseltamivir due to the NA-H274Y substitution. Telephone inquiry with the mother did not reveal any contact of the index patient with other influenza cases and/or antiviral treatment at any time. Transmission of the virus might have occurred by household contact; the mother reported mild influenza symptoms such as headache, coughing and fever (38.3 °C) one day after the onset of symptoms in the index patient.

Oseltamivir-resistance and sensitivity to zanamivir was confirmed by phenotypic analysis for the virus isolates obtained from patient 1 and 2 (Table 2). Other resistance-associated mutations of viral NA were not detected. The immunocompetent and otherwise healthy patients suffered from typical influenza symptoms, suggesting no differences in the clinical symptoms caused by resistant and by sensitive viruses. Since the patients were not treated with oseltamivir, these two resistant pandemic A/H1N1 viruses seem to have emerged in the absence of selective drug pressure. A possibility for occurrence and spread of resistant viruses would be the evolution of resistance in an infected patient and transfer of the strain among personal contacts (Janies et al., 2010). Therefore, in these two cases person-to-person transmission from unidentified index cases cannot be fully excluded.

In six other cases, selection of multidrug-resistant pandemic viruses during oseltamivir treatment was detected. These viruses were collected from patients affected by different underlying conditions: cardiomyopathy (patient 3), a developmental disorder (patient 4) and four patients (patients 5–8) were severely immunocompromised (Table 3). Respiratory samples from two of these patients (patients 5 and 6) were obtained before starting the oseltamivir regimen; these early viruses did not carry the NA-H274Y substitution. After 21 days of oseltamivir treatment, a respiratory sample from patient 5 contained a mixture of approximately 80% sensitive and 20% resistant viruses by PSQ analysis. In order to confirm this finding, 18 molecular clones of the NA PCR products obtained by TA/TOPO cloning™ kit (Invitek, Berlin, Germany) were analysed. The PSQ assay revealed one resistant and 17 sensitive molecular clones and confirmed the emergence of resistant viruses as subpopulation of the viral quasispecies under oseltamivir treatment.

Table 3
Characteristics of eight patients infected with oseltamivir-resistant pandemic A/H1N1 2009 influenza viruses.

Patient	Age/sex	Sample type	Health status	Hospitalized	Clinical outcome
1	21/M	Virus isolate	Healthy, immunocompetent	No	Mild
2	9/M	Nasal swab	Healthy, immunocompetent	No	Medium, otitis media
3	31/M	Virus isolate	Cardiomyopathy	Yes	Severe, pneumonia
4	5/M	Nasopharyngeal swab	Developmental disorder	Yes	Fatal
5	2/M	Nasopharyngeal swab	Immunocompromised	Yes	Severe, prolonged virus shedding (>2 months)
6	10/F	Virus isolate	Immunocompromised	Yes	Severe
7	59/F	Throat swab	Immunocompromised by multiple myeloma	Yes	Severe pneumonia, prolonged virus shedding (>1 month)
8	4/M	Nasopharyngeal swab	Immunocompromised by biphasic leukemia	Yes	Severe, prolonged virus shedding (>1 month)

Pandemic A/H1N1 influenza infection was determined by qRT-PCR (Schulze et al., 2010) from respiratory specimens mainly provided by physicians working on influenza surveillance (Szecsenyi et al., 1995). Samples from immunocompromised patients with severe influenza infections were sent to the German National Reference Centre for Influenza at the Robert Koch-Institut, Berlin, Germany for conformation of influenza infection and/or antiviral resistance testing. M: male, F: female.

A mixture of resistant and sensitive viruses within the viral quasispecies was also detected in samples from patient 7, which were collected at different time points. The PSQ analysis of the earliest respiratory specimen indicated an initial degree of oseltamivir resistance of about 30% at day 15. At day 23, almost half of the viral quasispecies carried the H274Y substitution; sequencing of 19 molecular clones revealed seven resistant clones (40%). The coexistence of resistant and sensitive viruses was also reflected in the phenotypic assay that determined an IC_{50} of 200 nM for oseltamivir (Table 2). At day 31, only the resistant mutant was detected.

NAIs, especially the orally administered oseltamivir, are the first choice for treating and preventing influenza virus infections (WHO, 2009c). At present, there is no evidence of extended circulation of oseltamivir-resistant pandemic influenza viruses (WHO, 2010). However, the unexpected emergence and unprecedented spread of oseltamivir-resistant seasonal A/H1N1 viruses indicate the possibility of rapid spread of resistant viruses once they appear. The WHO reported oseltamivir resistance for 285 pandemic 2009 viruses; 25% of them occurred in severely immunocompromised patients, 34% of the remaining cases were associated with antiviral treatment and only 17 (6%) occurred in the absence of treatment (WHO, 2010). In our study, 1570 pandemic viruses circulating recently in Germany were analysed. Almost all of them (95%) were sampled from immunocompetent, otherwise healthy patients and especially before beginning the treatment with oseltamivir. We detected oseltamivir resistance in eight pandemic A/H1N1 specimens, 75% of them were associated with oseltamivir administration and co-morbidity. Our findings, that severely immunocompromised patients were at a higher risk for selecting oseltamivir-resistant viruses during treatment, confirmed previous results and emphasises the importance of monitoring resistance development during the course of treatment (WHO, 2009d; Harvala et al., 2010; Wang et al., 2010). However, our data indicated also oseltamivir-resistant pandemic A/H1N1 influenza infection in immunocompetent and otherwise healthy patients who were not treated with oseltamivir.

Conflict of interest

No conflict of interests is declared.

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