

# Detection of influenza B viruses with reduced sensitivity to neuraminidase inhibitor in Morocco during 2014/15 season

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## اكتشاف فيروسات إنفلونزا B ذات حساسية منخفضة لمثبطات النورامينيداز في المغرب خلال موسم 2014-2015

فاطمة الفلكي، حسن احزماس، عبد الرحمان بيموحن، زكية الرركاكي، سميرة بنكروم، يوسف بكري، آمال بركات

الخلاصة: تهدف هذه الدراسة إلى رصد خصائص الحساسية المتعلقة بالنمط الظاهري والنمط الجيني لفيروسات الإنفلونزا في المغرب خلال موسم 2014-2015 للدوائين المثبطين للنورامينيداز، اوسيلتاميفير وزاناميفير. جُمعت العينات (مسحات حلقيّة أو أنفية) من مرضى خارجيين (بأعراض ما يشبه الإنفلونزا) ومرضى داخليين (بأعراض مرض تنفسي حاد وخيم)، وفُحصت عينات فيروسات الإنفلونزا A و B باستخدام مقاييسات "تفاعل البلمرة المتسلسل - الزمن الحقيقي" في الزمن الحقيقي البسيط. ولقّحت العينات الإيجابية على خلايا مادين-داربي كاندني، وقيمت خصائص حساسية النمط الظاهري للفيروسات المستفردة لمثبطات النورامينيداز باستخدام مقاييسات تثبيط النورامينيداز المتألق. من أصل 440 عينة كانت 135 (31%) عينة إيجابية لفيروس الإنفلونزا B الشبيه بياجاتا، وكانت 38 (8%) عينة A(H1N1)pdm09، و 25 (6%) عينة كانت A(H3N2). لقد وجد أن ستين فيروسة من فيروسات الإنفلونزا B التي استُفردت على MDCK وفُحصت حساسيتها لمثبطات النورامينيداز لم تظهر أي مقاومة تذكر لمثبطات النورامينيداز المتوفرة. واثنان من فيروسات الإنفلونزا B / - B /Morocco /176H/2015 و B /Morocco /CP10 /2015، وهما سلالتان استثنائيتان اشتملت عليهما فيروسات الإنفلونزا B التي استُفردت في MDCK - أظهرت انخفاضاً في الحساسية للأوسيلتاميفير. إن وجود اثنين من فيروسات الإنفلونزا B الجوالّة أثناء موسم 2014-2015 في المغرب حساسيتها منخفضة للأوسيلتاميفير يُظهر أن التّصّد المستمر للحساسية لمثبطات النورامينيداز أمر ضروري.

ABSTRACT We monitored phenotypic and genotypic susceptibility of influenza viruses circulating in Morocco during 2014-2015 to oseltamivir and zanamivir. Throat and nasal swab specimens were collected from outpatients (with influenza-like illness) and inpatients (with severe acute respiratory illness) and tested for influenza viruses using real-time reverse transcription polymerase chain reaction. Positive samples were inoculated in MDCK cells and virus phenotypic susceptibility to neuraminidase inhibitors (NAIs) was assessed using fluorescent NA inhibition. Of 440 specimens, 135 were positive for influenza B Yamagata-like virus, 38 were A(H1N1)pdm09 and 25 were A(H3N2). Sixty influenza B viruses isolated from MDCK cells showed no significant resistance to NAIs. However, two of these strains, B/Morocco/176H/2015 and B/Morocco/CP10/2015, showed reduced susceptibility to oseltamivir. The two influenza B viruses with reduced susceptibility to oseltamivir show that ongoing NAI susceptibility surveillance is essential.

## Maroc : dépistage de virus de la grippe B présentant une sensibilité réduite à l'inhibiteur de la neuraminidase durant la saison 2014-2015

RÉSUMÉ Nous avons surveillé le profil de sensibilité phénotypique et génotypique des virus de la grippe à l'oseltamivir et au zanamivir durant la saison 2014-2015 au Maroc. Des échantillons ont été prélevés (par frottis de gorge ou écouvillonnage du nez) chez des patients externes (présentant un syndrome de type grippal) et chez des patients hospitalisés (présentant une infection respiratoire aiguë sévère). Ils ont été soumis au test de détection des virus de la grippe A et B via la méthode d'analyse RT-PCR simplex en temps réel. Les échantillons positifs ont été inoculés à des cellules rénales canines Madin-Darby (MDCK) et le profil de sensibilité phénotypique des virus isolés aux INA a été évalué au moyen du test d'inhibition de la neuraminidase (NA) par fluorescence. Sur 440 échantillons, 135 (31 %) étaient positifs au virus de la grippe B de type Yamagata, 38 (8 %) à celui de la grippe A(H1N1)pdm09 et 25 (6 %) à celui de la grippe A(H3N2). Soixante virus de la grippe B isolés sur les cultures de cellules MDCK et soumis au test de sensibilité aux INA n'ont démontré aucune résistance significative aux INA. Toutefois, deux de ces souches, la B/Maroc/176H/2015 et la B/Maroc/CP10/2015, ont démontré une sensibilité réduite à l'oseltamivir. Les deux virus de la grippe B ayant une sensibilité réduite à l'oseltamivir montrent l'importance d'une surveillance permanente de la sensibilité à l'inhibiteur de la neuraminidase.

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## Introduction

Human influenza is responsible for an estimated 250 000–500 000 deaths annually worldwide (1). Although vaccination remains the primary means for the prevention of influenza, antiviral drugs are a key component in the prophylaxis and treatment of the population at high risk of influenza-associated complications. Antiviral drugs might be the only medical intervention available during the early phases of a pandemic. Two classes of specific anti-influenza drugs have been developed to date: the M2 ion channel blockers (adamantanes) and the neuraminidase inhibitors (NAIs) (oseltamivir and zanamivir) (2).

Susceptibility of influenza B viruses to NAIs has been less studied than susceptibility of influenza A viruses. Influenza B viruses, classified into 2 genetic lineages Victoria and Yamagata, are circulating globally with unpredictable temporal and spatial distributions (3). Recently, low-level resistance to NAIs has been documented but the known molecular markers, transmissibility and fitness of NAI-resistant variants are not well established (4). A worldwide antiviral surveillance study of influenza B viruses from 2004 to 2008 identified 1 extreme outlier and 3 mild outliers among 1070 viruses tested (0.4%) (5). A later study among 346 influenza B viruses isolated worldwide during 2011 identified 2 (0.6%) with reduced susceptibility to NAIs (6). These resistant viruses can emerge under drug selection pressure or occur naturally without drug interventions (7), and are associated with amino acid substitutions at the conserved NA residues or in surrounding locations (8).

In this study, we evaluated the sensitivity to oseltamivir and zanamivir of influenza B viruses isolated from specimens collected from the National Influenza Surveillance System in Morocco during the 2014–2015 season.

## Methods

### Sample collection

Throat and nasal swab specimens were collected during the 2014–2015 season from outpatients presenting with influenza-like illness (ILI) and inpatients presenting with severe acute respiratory illness (SARI). ILI was defined as ARI with onset during the past 10 days, temperature  $\geq 38^\circ\text{C}$  and cough (9). SARI was defined as ARI with history of fever or temperature  $\geq 38^\circ\text{C}$  and cough, with onset of symptoms in the past 10 days and requiring hospitalization (10). After collection, the samples were immediately stored at  $4^\circ\text{C}$  until delivery to the National Influenza Centre located at the Institut National d'Hygiène in  $< 48\text{--}72$  hours (9,10).

### Influenza B virus screening

Viral RNA was extracted directly from the collected specimens by automatic extraction (iPrep Purelink Virus Kit; Thermo Fisher Scientific, Carlsbad, CA 92008 USA). A 25- $\mu\text{L}$  reaction volume contained 12.5  $\mu\text{L}$  2 $\times$  polymerase chain reaction (PCR) Master Mix, 5.5  $\mu\text{L}$  RNase-free water, 0.5  $\mu\text{L}$  40  $\mu\text{M}$  reverse primer, 0.5  $\mu\text{L}$  40  $\mu\text{M}$  forward primer, 0.5  $\mu\text{L}$  probe 10  $\mu\text{M}$  (influenza A or B), 0.5  $\mu\text{L}$  enzyme mix (SuperScript III RT/Platinum Taq Mix; Thermo Fisher Scientific, Carlsbad, CA 92008 USA) and 5  $\mu\text{L}$  viral RNA extract. Amplification was carried out in a 7500 Fast 96-Well Thermocycler (Applied Biosystems, Foster City, CA, USA) with a single reverse transcription step ( $50^\circ\text{C}$  for 30 minutes), Taq inhibitor activation ( $95^\circ\text{C}$  for 2 minutes), PCR amplification over 45 cycles ( $95^\circ\text{C}$ , 30 seconds/ $55^\circ\text{C}$ , 30 seconds), according to the US Centers for Disease Control (CDC) protocol (11). Influenza B primers and probes were provided by CDC as part of a material transfer agreement that ensures privacy and non-publication.

### Subtyping of influenza B virus lineages

The 25- $\mu\text{L}$  reaction volume contained 12.5  $\mu\text{L}$  2 $\times$  PCR Master Mix, 5.5  $\mu\text{L}$  RNase-free water, 0.5  $\mu\text{L}$  25  $\mu\text{M}$  reverse primer, 0.5  $\mu\text{L}$  25  $\mu\text{M}$  forward primer, 0.5  $\mu\text{L}$  probe 25  $\mu\text{M}$  (Yamagata or Victoria; Thermo Fisher Scientific, Carlsbad, CA 92008 USA), 0.5  $\mu\text{L}$  enzyme mix (SuperScript III RT/Platinum Taq Mix), and 5  $\mu\text{L}$  viral RNA extract. Amplification was carried out in a 7500 Fast Thermocycler (Applied Biosystems) with a single reverse transcription step at  $50^\circ\text{C}$  for 30 minutes, Taq inhibitor activation at  $95^\circ\text{C}$  for 15 minutes, and PCR amplification over 45 cycles ( $95^\circ\text{C}$ , 10 seconds/ $54^\circ\text{C}$ , 30 seconds/ $72^\circ\text{C}$ , 10 seconds) (11).

### Virus isolation

PCR-positive samples (100  $\mu\text{L}$ ) were inoculated into 2 wells of a 24-well plate with 70–90% confluent monolayers of MDCK cells. After 30 minutes adsorption at room temperature, 2 mL/well minimal essential medium supplemented with 2 mg/mL tosyl phenylalanyl chloromethyl ketone trypsin was added. Cells were incubated in sealed bags at  $35^\circ\text{C}$ . After 2 days, viral multiplication was detected by haemagglutination assay using guinea pig red blood cells. Virus identification was carried out by haemagglutination inhibition test using WHO Haemagglutination inhibition reagent kits (12).

### NAIs

Oseltamivir carboxylate, the active compound of the ethyl ester prodrug oseltamivir phosphate, was kindly provided by Hoffmann–La Roche (Basel, Switzerland), and zanamivir by GlaxoSmithKline (Uxbridge, UK).

### Reference viruses

A matching pair (sensitive and resistant to oseltamivir and/or zanamivir) of reference viruses for each subtype to be tested was included in each assay run. The panel containing drug-sensitive/

resistant control virus pairs was delivered by the WHO Collaborating Centre, Melbourne (13).

### Fluorescent NA inhibition assay

The phenotypic evaluation of influenza B virus susceptibility to NAIs was performed using the NA-Fluor Influenza Neuraminidase Assay Kit (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) in 96-well, opaque black, flat-bottom microplates, according to the CDC protocol (available from the CDC upon request: [flu-antiviral@cdc.gov](mailto:flu-antiviral@cdc.gov)). This assay utilized 2-(4-methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid (MUNANA) (Applied Biosystems, Foster City, CA, USA) as substrate. Viruses were diluted at concentrations corresponding to the target fluorescence signal generated by 1000 pmol/well of 4-methylumbelliferone standard. Subsequently, 25  $\mu$ L of each diluted virus was mixed with 25  $\mu$ L of a range of concentrations of each NAI inhibitor (0.0015–4000 nM) and incubated at 37°C for 45 minutes. Then, 50  $\mu$ L of 200 mM MUNANA was added to the virus and inhibitor mixture and incubated at 37°C for 1 hour. The reaction was stopped with 100  $\mu$ L NA-Fluor Stop Solution. Fluorescence was detected on the Victor X3 (WSLH. Series Multilabel Plate Readers PerkinElmer, Inc. Waltham, MA 02451 USA) equipped with filters for excitation ( $\lambda = 365$  nm) and emission ( $\lambda = 450$  nm) (14).

### Data analysis

Raw fluorescence NA inhibition assay data expressed as RFU were plotted against drug concentration (nM) to calculate the median 50% inhibitory concentration (IC<sub>50</sub>) using JASPER version 1.2 curve-fitting software (CDC, Atlanta, GA, USA) (15).

### Interpretation of IC<sub>50</sub> values

Fold changes in IC<sub>50</sub> were determined by comparing the IC<sub>50</sub> of test viruses

with the mean IC<sub>50</sub> (outliers excluded) according to drug and influenza type/subtype (16). IC<sub>50</sub> was interpreted using the WHO Antiviral Susceptibility Expert Working Group criteria for influenza B viruses: < 5-fold change in IC<sub>50</sub> represents normal inhibition; 5–50-fold change represents reduced inhibition; and > 50-fold change is highly reduced inhibition (17).

### Sequencing of the NA gene

Real-time PCR was carried out for individual gene fragments with the primers panel from the CDC with PCR conditions as described in the MyTaq One-step RT-PCR Kit (Bioline, London, UK). The PCR products were purified using the GE Healthcare/USB ExoSAP-IT PCR Product Clean-Up (Affymetrix, Cleveland, OH, USA). Purified PCR products were sequenced with the use of the ABI Big Dye Terminator, version 3.1 (Applied Biosystems) and M13 primers (CDC protocol unpublished). The latest PCR products were purified using Big Dye X Terminator purification (Applied Biosystems)

and then analysed on an ABI 3130XL sequencer.

### Sequencing analysis

The sequences of nine NA gene were assembled, edited and aligned using the Sequencher programme (version 4.10) (Gene Codes Corporation, Ann Arbor, MI, USA). The construction of phylogenetic trees was performed using MEGA version 4.0 with the neighbour-joining method and bootstrap analysis of 1000 replicates.

## Results

### Influenza virus surveillance

From September 2014 to August 2015, 440 specimens were collected from 273 ILI and 167 SARI patients (Table 1). Two hundred and one (46%) specimens tested positive for influenza virus: 135 (31%) were type B, 38 (8%) were A(H1N1)pdm09 and 25 (6%) were A(H3N2). Subtyping of influenza B virus circulating in the 2014/15 season

**Table 1 Demographic characteristics and influenza prevalence among patients with ILI and SARI, Morocco, 2014–2015**

Characteristic	ILI (n = 273) No. (%)	SARI (n = 167) No. (%)	Total (n = 440) No. (%)
<b>Age</b>			
0–23 months	5 (1.8)	71 (42.5)	76 (17.3)
2–5 years	19 (7.0)	13 (7.8)	32 (7.3)
6–15 years	50 (18.3)	14 (8.4)	64 (14.6)
16–49 years	112 (41.0)	28 (16.8)	140 (31.8)
50–64 years	59 (21.6)	17 (10.2)	76 (17.3)
≥ 65 years	16 (5.9)	15 (9.0)	31 (7.1)
Unknown	12 (4.4)	9 (5.4)	21 (4.8)
<b>Sex</b>			
Female	155 (56.7)	81 (48.5)	236 (53.6)
<b>Influenza subtype</b>			
A (H1N1)pdm09	24 (8.8)	14 (8.4)	38 (8.6)
A (H3N2)	22 (8.1)	3 (1.8)	25 (5.7)
A unsubtypeable	NA	3 (1.8)	3 (0.7)
B	113 (41.4)	22 (13.2)	135 (30.7)
Negative	114 (41.8)	125 (74.9)	239 (54.3)

ILI = influenza-like illness; SARI = severe acute respiratory illness.



**Table 2** NAI susceptibility of influenza B/Yamagata isolates by fluorescent NAI assay

NAIs	Origin of specimen	No. analysed	IC50 (nM)		NAI susceptibility (fold change)
			Median (range)	Mean ± SD	
<b>Oseltamivir</b>	ILI	28	33.94 (11.60–46.44)	31.98 ± 9.57	S (1)
	SARI	30	31.84 (12.04–59.01)	31.52 ± 11.17	S (1)
		2	225.40 (200.60–250.20)	225.44±35.07	RS (6-8)
<b>Zanamivir</b>	ILI	28	3.45 (0.67–7.51)	3.37 ± 1.79	S (1)
	SARI	32	3.60 (0.56–8.61)	3.74 ± 2.11	S (1)

Median IC50 values for the drug among susceptible viruses (outliers excluded in calculation).

IC50 values calculated using JASPER curve-fitting software.

Mean and SD of IC50 values for the drug among susceptible viruses (outliers excluded in calculation).

Fold changes in IC50 determined by comparing IC50 of test viruses to the mean IC50 (outliers excluded) by respective drug and influenza type/subtype.

ILI = influenza-like illness; NAI = neuraminidase inhibitor; RS = reduced susceptibility; S = sensitive; SARI = severe acute respiratory illness; SD = standard deviation.

showed that all belonged to B/Yamagata/16/88 lineage.

### Phenotypic analysis

Sixty of 135 influenza B viruses had a titre of  $\geq 1$  in 4 in the haemagglutination test and were subject to the NA inhibition assay. The assay showed that all influenza B viruses were sensitive to NAIs, except B/Morocco/176H/2015 and B/Morocco/CP10/2015, with IC50 values of 200.60 and 250.20 nM, respectively (Table 2). Therefore, these 2 viruses were considered as outliers and demonstrated a 6–8-fold reduced susceptibility to oseltamivir compared to the mean IC50 (31.52 nM) of the same virus types. The mean IC50 for all influenza B viruses tested, not including the outliers, for zanamivir (3.62 nM) was low compared with the mean IC50 for oseltamivir (31.74 nM), reflecting increased sensitivity ( $\times 9$ ) of influenza B virus to zanamivir compared with oseltamivir.

### Phylogenetic and molecular analysis

The NA of 9 of 60 influenza B virus strains tested in the fluorescent NA inhibition assay was sequenced: B/Morocco/CP10/2015, B/Meknes/35/2014, B/Sale//70/2014, B/Agadir/68/2014,

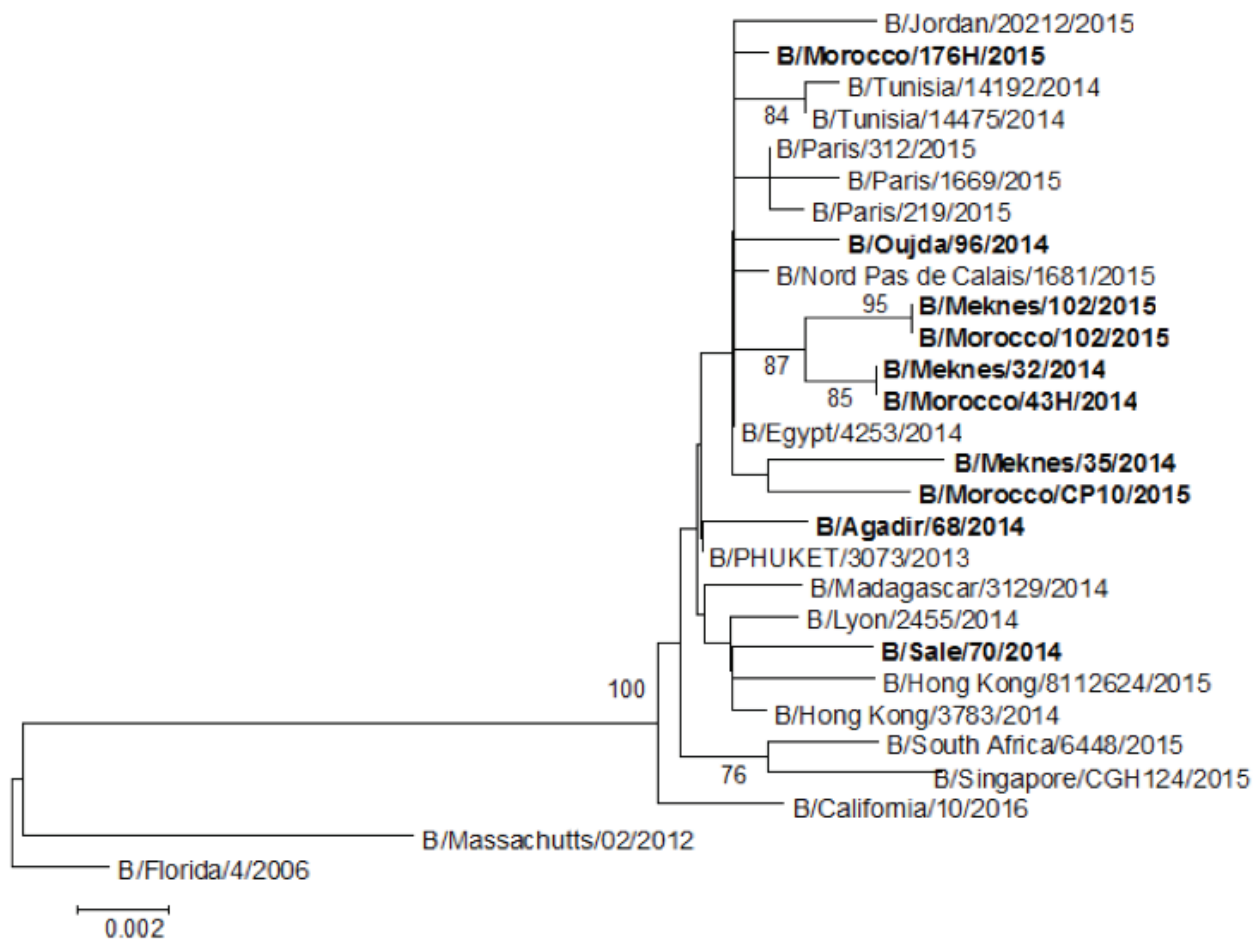
B/Morocco/176H/2015, B/Oujda/96/2014, B/Morocco/102/2015, B/Morocco/43H/2014 and B/Meknes/32/2014. The respective GISAID accession numbers were: EPI\_ISL\_216533, EPI\_ISL\_175139, EPI\_ISL\_17514, EPI\_ISL\_175086, EPI\_ISL\_215754, EPI\_ISL\_171966, EPI\_ISL\_215717, EPI\_ISL\_215716 and EPI\_ISL\_171964). The phylogenetic and molecular analysis of the 9 NA sequences (Figure 1) showed that they were related genetically to B/Phuket/3073/2013; the vaccine prototype strain for the 2015–2016 season (18). The NA of the 2 isolates with reduced sensebity to oseltamivir harboured the specific mutations of B/Phuket/3073/2013 (Figure 2). The B/Morocco/CP10/2015 strain collected from a deceased patient confirmed as influenza positive harboured the specific K371N amino-acid substitution mutation, which is not known to confer drug resistance on influenza B viruses.

### Discussion

We analysed the antiviral susceptibility of the predominant influenza B virus circulating in Morocco during the 2014/15 season using the phenotypic

NA inhibition assay (NA Fluor Influenza Neuraminidase Assay kit) as well as sequencing of the NA gene. We found that all the influenza B viruses exhibited normal sensitivity for zanamivir. This is in accordance with studies of influenza drug susceptibility worldwide, in which only a few strains with reduced sensitivity to zanamivir have been reported (19,20). This could be explained by the characteristic structure and conformation of this drug. The higher structural homology with the NA natural substrate, sialic acid, and lower use of zanamivir (compared with oseltamivir) are the most probable factors to account for the infrequent isolation of zanamivir-resistant variants worldwide (8).

Sixty influenza B viruses isolated in MDCK cells and tested by fluorescent NA inhibition test were sensitive to oseltamivir ( $< 5$ -fold change in IC50), except for 2 isolates that demonstrated a 6–8-fold reduced susceptibility compared with the mean IC50 (outliers excluded) according to drug and influenza virus type/subtype. Both isolates were collected from SARI patients without historical drug exposure. A few previous studies have revealed influenza B virus with reduced susceptibility to NAIs, particularly oseltamivir (2,21,22).



**Figure 1** Phylogenetic comparison based on nucleotide sequences of B/Yamagata lineage of NA gene with that of vaccine viruses and strains from other countries (the tree was rooted with B/Florida/02/2012 Na gene and was created using the neighbour-joining and bootstrap analysis of 1000 replicates method).

As indicated by substantially higher oseltamivir IC50 values, influenza B viruses appear to be more sensitive to zanamivir than oseltamivir. Most systematic antiviral surveillance evaluating NAI susceptibility of influenza B viruses has showed that oseltamivir is less effective at treating influenza B than influenza A infections in children (23,24,25). Sequencing analysis of the NA gene of 9 influenza B viruses compared with the reference B/Massachusetts/02/2012 strain showed that they were related genetically to the B/Phuket/3073/2013 vaccine virus for the 2015–2016 influenza season.

B/Morocco/CP10/2015 and B/Morocco/176H/2015 showing reduced sensitivity to oseltamivir did not harbour any substitution mutations

known to confer reduced sensitivity to influenza B strains, such as G109E, G402S, D198N and I221T detected in isolates from patients treated with NAIs (2, 8). However, B/Morocco/CP10/2015 harboured amino acid substitution K371N (B numbering) located among the highly conserved catalytic NA residues. This residue seems highly conserved from other alignment, so this looks like an unusual change and may mean that it is responsible for the change. Residue substitution R374I (B numbering) is only 3 amino acids long and does have a proven effect on NAI susceptibility. To prove the probable role of the K371N mutation in NAI resistance, reverse genetics analysis is needed.

In Morocco, prior to the pandemic of 2009, influenza drugs, especially NAIs, were rarely used for influenza prophylaxis or treatment. This may be a reason for drug-sensitive strains. It also shows that there has been no circulation of “fit” resistant viruses in Morocco that are highly transmissible such as the H275Y A(H1N1) seasonal variant in 2007/08.

## Conclusion

We identified influenza B viruses with reduced susceptibility to oseltamivir, thus highlighting the importance of influenza antiviral susceptibility surveillance in Morocco, even though the use of NAIs is low.

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**Competing interests:** None declared.

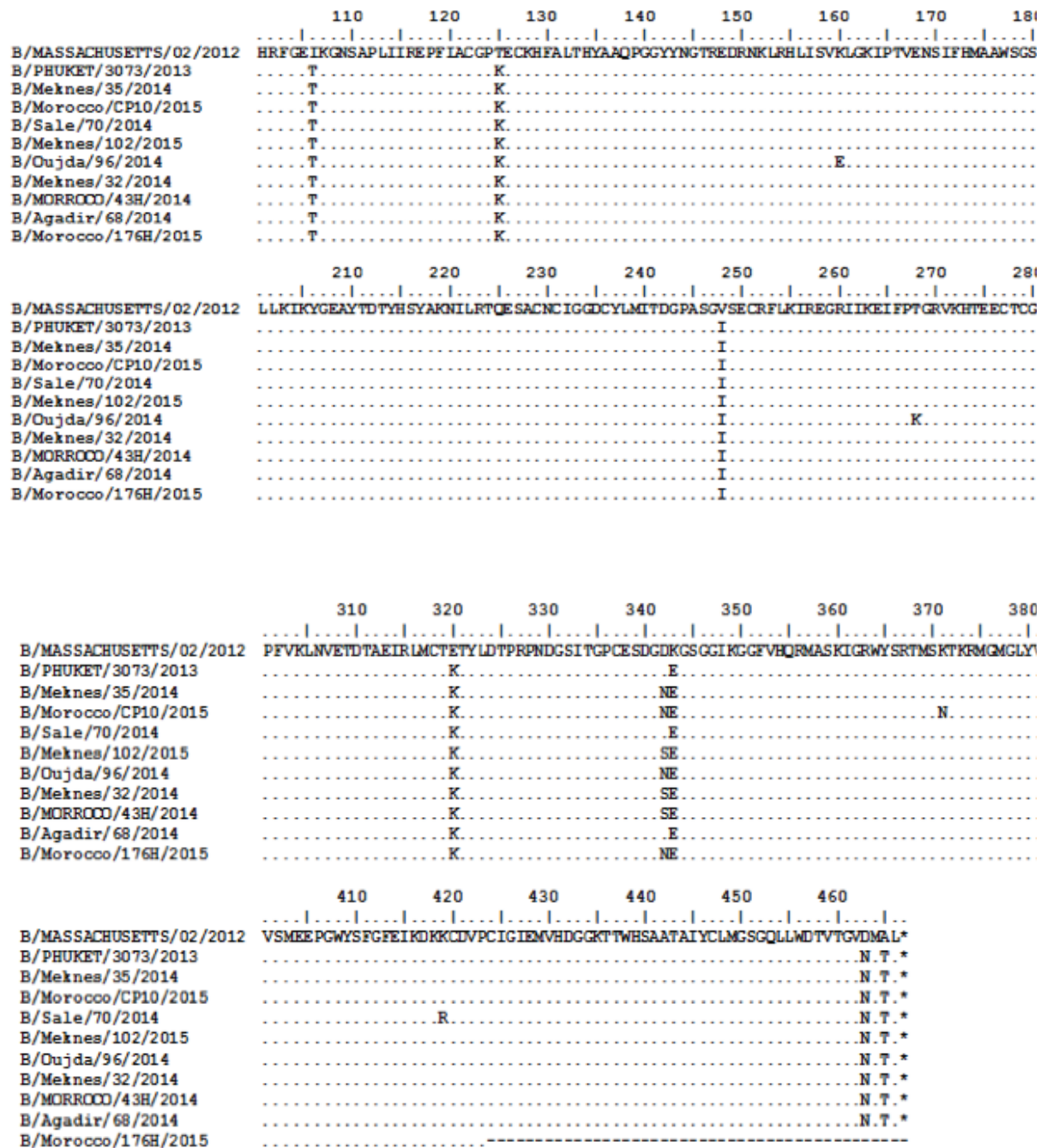


Figure 2 Comparison of amino acid sequences of NA gene of Yamagata lineage Moroccan influenza B virus strains with reference strain B/Massachusetts/02/2012, showing the specific substitutions.

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