neuraminidase inhibitors of influenza A(H3) circulating in Portugal during 2016/2017 season

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Background:

The influenza antiviral surveillance is one of the key areas for influenza control. The neuraminidase inhibitors (NAI) play an important role in flu treatment especially for high risk patients. This study aims to determine the NAI susceptibility profile of influenza A(H3) detected during 2016/2017 season in Portugal and to evaluate the emergence of new variants by deep sequencing analysis related to NAI resistance.

Methods:

Nasopharyngeal swabs were collected from patients with influenza like illness (ILI) selected in primary care settings in the scope of the National Influenza Surveillance Program during 2016/2017 season. For 134 A(H3) viruses, isolated in MDCK-Siat1 cells, the phenotypic antiviral drug susceptibility assay to NAI (Oseltamivir and zanamivir) was performed. Viral RNA was extracted directly from biological samples and after multiplex PCR amplification, the whole genome was sequenced for 144 influenza A(H3) viruses by deep sequencing on a MiSeq platform. The neuraminidase gene sequences were assembled using a in-house multi-software pipeline with a mean depth of coverage of 1144x. Multiple gene alignments and mutational analysis was performed on MEGA software 6.0. All neuraminidase sequences were submitted to Flusurver (http://flusurver.bii.a-star.edu.sg/) in order to detect any mutation associated with susceptibility to neuraminidase inhibitors. Were analysed intra-host single nucleotide variants (iSNV) with frequencies between 1% and 50% (minority iSNVs).

Results:

Phenotypic profile

All 134 A(H3) strains had IC_{50} compatible with susceptibility to both NAI. The IC_{50} mean values were 0,30 (IQR 0,22-0,36) for oseltamivir and 0,49 (IQR 0,40-0,59) for zanamivir. The IC_{50} were unchanged for viruses that belonged to both co-circulating genetic groups: 3C.2a (A/Hong Kong/4801/2014-like) and 3C.2a1 (A/Bolzano/7/2016-like), although amino acid replacements were observed in NA.

Table I - Neuraminidase inhibitor susceptibility of influenza A(H3) during 2016/2017 season. IC_{50} values (nM) by fluorescent neuraminidase inhibition assay (MUNANA).

2016/2017	IC_{50}							
Influenza A(H3) Genetic group	Oseltamivir				Zanamivir			
	Min.	Max.	Median	IQR	Min.	Max.	Median	IQR
All	0,12	0,49	0,3	0,22-0,36	0,24	0,89	0,49	0,40-0,59
3C.2a	0,22	0,43	0,3	0,25-0,34	0,38	0,62	0,50	0,43-0,53
3C.2a1	0,12	0,48	0,3	0,23-0,36	0,24	0,76	0,48	0,41-0,59

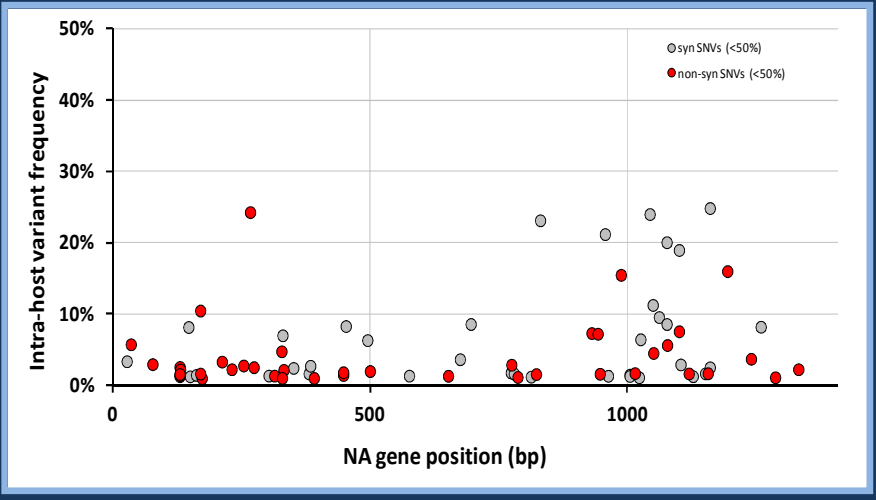

Figure 1 - Frequencies of each minority iSNV (1%-50%) found in the neuraminidase gene. In grey and red – synonymous and non-synonymous (amino acid-altering) iSNV's, respectively.

Genotypic profile

- All A(H3) expressed the wild-type aa residue compatible with NAI susceptibility.

- Analysing neuraminidase sequences we found 68 nucleotide sites displaying minority iSNVs among 54 (37.5%) viruses (Figure 1). From these 68, 7 sites revealed intra-host variation in more than 1 sample (with the iSNV in the nucleotide position 130 being found in 4 distinct patients).
Of note, none of the detected iSNVs is associated with any of the known resistant markers for NAI.

- This scenario at gene level corresponds to the existence of 33 minority iSNVs at amino acid level, detected in a total of 32 viruses (22.2%). Regarding amino acid sites displaying intra-host variation in more than one patient, the following 4 amino acid mixtures were found: S44P/F, I57T, A110D/T and R150C/S. Remarkably, 2 out of 4 viruses revealing the S44P (nucleotide change T130C) intra-host variation clustered in the same phylogenetic branch at genome level, and the patients involved are from the same city and have been sampled in the same week. No potential transmission link could be established for the 2 other patients as the viruses involved fall in another genetic subgroup.
For all remaining sites revealing redundancy in intra-host variation and potential viral transmission patterns, no transmission links could be unveiled due to the lack of phylogenetic and epidemiological congruence.

- Still, the observed intra-host variation specifically targeting the same amino acid position in more than one patient may suggest that those specific protein sites are under selective pressure, although we cannot exclude the random scenario.

Conclusions:

All of the tested A(H3)viruses were susceptible to oseltamivir and zanamivir. Isolates possessing iSNV`s expressed the wild-type aa residue and had $IC_{50}$ compatible with NAI susceptible. NAI`s should be considered a good therapeutic options for influenza A(H3) infection, prioritized to high risk groups. Whole genome deep sequencing, directly from the biological samples, proved to be a good laboratory method to monitor the emergence of iSNV that could lead to a resistant genotype. This approach could be used in management of influenza severe infections under NAI treatment.
Selective pressure during the course of infection could be an explanation for the observed redundancy of amino acid positions displaying intra-host variation in more than one virus. In addition, for one case this analysis could disclose a potential transmission event.