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Synergistic effect of the homologous PB1-NA gene constellation in Influenza A virus reassortants: Evaluation and characterization of reassortant influenza variant viruses crossresistant to influenza Neuraminidase Inhibitors.

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Previous work: Team research areas include: genetic and evolutionary analysis of influenza virus, role of gene reassortment in the genetic evolution, virulence determinants and antiviral drug resistance. From 2012 to 2014 I took part in the project: "Evaluation and characterization of the emergence of resistance to influenza antiviral drugs in the context of acute respiratory infection", regarding the genotypic and phenotypic characterization of influenza virus A(H1N1)pdm09 and A(H7N9). The work plan has focused on the phylogenetic and mutational analysis of influenza viral strains circulating in Portugal from 2009-2013. Based on the international collaboration of the team, it was possible to develop complementary experimental work in the Respiratory Virus Unit at Public Health England, U.K. During this period I have developed experimental work for the optimization of a non-infectious transfection system to express non-human influenza virus proteins and further characterize the profile of a N9 neuraminidase (NA) from a zoonotic influenza A(H7N9) virus. Recently, I have been involved in the project: "Neuraminidase Inhibitors: new antivirals and new brands" at INSARJ. This study aimed to compare available cost-efficient alternative reagents for NA inhibition assay (NIA) and to assess the phenotypic susceptibility profiles of influenza virus from different (sub)types to both the classic (oseltamivir and zanamivir); and the new (laninamivir and peramivir) NA inhibitors (NAIs). Since 2015 I have been working on the project: "Functional compatibility of the replication complex as a determinant of virulence in influenza virus" [1][2]. In this context, we found functional compatibility when PB1 is homologous to antigenic proteins NA and hemagglutinin (HA), which is suggested by an increasing in viral fitness. The presence of PB1 homologous to HA and NA in the A(H1N1)pdm09 vaccine seed prototype prototypes in the backbone of A/Puerto Rico/08/34 [PR8:(A(H1N1)pdm09-HA,NA,PB1] resulted in statistically significant virus growth improvement, when compared to the 6:2 classical seed prototypes [PR8:(A(H1N1)pdm09-HA,NA] [2]
Also, we have identified mutations -R386K, I517V and L298I- in the PB1 protein of A(H1N1)pdm09, that may have contributed to an enhanced compatibility between PB1 and HA. L298I has already been associated with

increased pathogenicity in mice [3]. Our current line of research explores the phenotypic outcome of creating seed viruses bearing PB1 homologous to HA and NA, in the kinetics of viral growth and antigen yield. Additionally, we continuously analyze and characterize the phenotypic and genotypic NAIs susceptibility profiles of influenza viruses circulating in Portugal, using the in-house MUNANA-based IC50 (concentration of NAI required to inhibit 50% of the virus NA activity) fluorescence assay, provided by PHE [4]. Influenza antivirals are an important line of defense in case of a newly emerging influenza virus strain, and particularly in immunocompromised and elderly patients [5]. In this context, the emergence and spread of influenza A viruses, with diminished susceptibility to NAI, increase the need to understand the impact of specific mutations on evolution and viral fitness of drug-resistant virus.

INTRODUCTION: The neuraminidase (NA) inhibitors (NAIs) oseltamivir phosphate (Tamiflu®) and zanamivir(Relenza®) are currently the only effective antiviral drugs available in Europe for the management of influenza - they have been approved for use in many countries since 1999/2000 [6]. Each one presents known limitations in scope, effectiveness and emergence of resistance strains [7]. The emergence in 2007/2008 of an oseltamivir-resistant seasonal A(H1N1) variant (carrying the H275Y NA mutation) and the detection of sporadic cases of this variant among A(H1N1)pdm09 viruses in Australia (2011) and Sapporo, Japan and USA (2013-2014) have demonstrated the potential for drug-resistant influenza viruses to arise and spread globally within the community in the absence of drug-selective pressure [8][9]. Additional NA mutations detected in A(H1N1)pdm09 viruses may have potentially compensated for the expected fitness deficits and reveal why H275Y mutants were able to emerge and become widespread [7]. I223R and S247N NA mutations of H275Y mutant A(H1N1)pdm09 viruses have been reported to have a synergistic effect with the H275Y substitution on the reduction of NA inhibitor susceptibility, prompting the concern that these variants may have acquired cross-resistance to other NAIs [9][10]. Recent studies reporting variants harboring mutations conferring cross-resistance to approved NAIs [10] expose the potential for emergence and spread of resistant viruses and reinforce the demand for a close evaluation of virus susceptibility to NAIs. Albeit various mutations in the NA conferring reduced susceptibility to NAIs have been identified, the amino acid substitutions that confer cross-resistance with undiminished viral fitness have not been comprehensively characterized and thus remain poorly understood [10]

OBJECTIVE: This project aims to elucidate the functional impact of specific mutation induced changes in the NA and PB1 genes on the viral fitness and cross-resistance profile of A(H1N1)pdm09 viruses.

METHODS: A genomic library of the parental viruses A/Puerto Rico/8/1934(H1N1) (PR8) and A/Portugal/82/2009 (pdm09) has been previously constructed in a bidirectional plasmid vector [2] and is available for study. The prototype reassortant PR8:(A(H1N1)pdm09-HA,NA,PB1 has been generated in vitro by plasmid-based reverse genetics and are also available for this study. Site-directed mutagenesis using

appropriate primers and a commercial mutagenesis kit will be used to induce mutations in NA and PB1 genes and the mutated viral reassortants will be generated by reverse genetics. The plasmid containing the NA gene will be used for the introduction of single (H275Y) and double (H275Y/S247N) mutations; the plasmid containing the PB1 gene will be used for the introduction of L298I mutation. The resulting plasmids: p-NAH275Y, p-PB1L298I, p-NAH275Y/S247N and pNAH275Y/S247N:PB1L298I will be evaluated for their specific impact on viral replicative kinetics and on the NA enzymatic properties of the generated PR8:(A(H1N1)pdm09-HA,NA,PB1 reassortants. Growth kinetics will be tested from 12 to 60h post-infection by Hemagglutination titer (HA) and Tissue Culture Infectious Dose (TCID50). NA activity and NA inhibition assay will be determined by an in-house fluorescence MUNANA-based assay [4] in independent assays at different time points post-infection. EXPECTED RESULTS: By constructing reverse genetics reassortants bearing different genetic features (individual or a group of specific mutations) involving the NA and PB1 genes, we expect to appraise their impact on viral phenotype, regarding the viral replication kinetics and susceptibility profile to NAIs. Additionally, the results obtained in this study may contribute to a better clarification on the phenotype-genotype relationship. Elucidating evolutionary trends in the genes encoding influenza virus internal proteins and profiling of influenza A viruses to antiviral drugs are essential to assess the risk of influenza virulence and antiviral effectiveness, contributing significantly to future public health strategies.

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