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Evaluation of the antiviral drug susceptibility of influenza viruses in Italy from 2004/05 to 2009/10 epidemics and from the recent 2009 pandemic

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ABSTRACT

Antiviral monitoring of influenza viruses circulating in Italy has been carried out since 2007 by the National Influenza Centre (NIC), using both phenotypic and sequence-based assays. Here, we report results of the susceptibility evaluation to neuraminidase (NA) inhibitors (NAIs, zanamivir and oseltamivir) and adamantanes of nearly 300 influenza type A and B seasonal viruses isolated in Italy during six recent seasons, together with over 30 pandemic (H1N1) 2009 virus strains. The present work is the first such study conducted in Italy, aimed to develop national data on antiviral drug profile and to establish a nationwide surveillance programme on antiviral susceptibility. Sequencing of the NA gene was undertaken either to confirm the phenotypic findings or to identify any NA change, in potentially resistant viruses (outliers), which might be associated with reduced susceptibility to NAIs. The 50% inhibitory concentration values (IC_{50}s) showed slightly different sensitivities of the seasonal Italian isolates to the two NAI drugs, depending on the specific NA subtype. We found mean zanamivir IC_{50}s of 0.74, 1.33 and 7 nM, and oseltamivir IC_{50}s of 0.67, 2.34 and 30.1 nM for the N2, N1 and B NAs, respectively. The pandemic (H1N1) 2009 viruses showed IC_{50}values overall comparable to the seasonal N1 viruses from previous years, showing mean zanamivir IC_{50}s of 1.02 nM and mean oseltamivir IC_{50}s of 2.82 nM. Oseltamivir resistance was found in a total of 19 seasonal N1viruses of 2007/2008 and 2008/2009, and in three pandemic (H1N1) 2009 strains. A gradual increase of resistance to adamantanes was observed among the N2 viruses isolated in recent seasons; no resistant viruses were found among the seasonal N1 strains, whereas all the pandemic (H1N1) 2009 isolates analysed were resistant to the M2 blockers.

1. Introduction

Although vaccination represents the most effective tool against influenza, antiviral drugs are useful during epidemics and pandemics and can provide a valuable alternative prior to vaccine availability or in those for whom vaccination is unsuitable. Two classes of antiviral agents are available for the prophylaxis and treatment of influenza virus infection in humans: the adamantanes (amantadine and rimantadine) (Mould et al., 2000; Mast et al., 1991; Hayden et al., 1989) and the most common neuraminidase (NA) inhibitors (NAIs: oseltamivir and zanamivir) (McKimm-Breschkin et al., 2003).

The M2 ion channel blockers (adamantanes), unlike NAIs, are able to limit replication of the influenza A viruses only (McKimm-Breschkin et al., 2003) and they are associated with high level of resistance among A(H3N2) subtype, some seasonal A(H1N1) viruses (CDC, 2008) and among all the recently emerged pandemic (H1N1) 2009 viruses (Garten et al., 2009). The NAIs were introduced into clinical practice in 1999 and, although rarely used to treat seasonal influenza, they had been stockpiled by most European countries, including Italy, as part of pandemic preparedness plans (Meijer et al., 2007). During the recent emergence of the pandemic (H1N1) 2009 virus, the sudden increase in usage of these drugs for chemoprophylaxis and treatment of human cases (Dawood et al., 2009) reinforced the need for extensive antiviral susceptibility vigilance among circulating influenza isolates. Moreover, the unexpected emergence and spread of H1N1 oseltamivir-resistant viruses (ORV) among seasonal strains isolated worldwide in 2007/2008 (Lackenby et al., 2008; Hauge et al., 2009), in the absence of drug pressure, demonstrated that the replication and virulence of these mutant viruses may be surprisingly comparable to that of wild type strains (Baz et al., 2010).

The major aim of this study was to investigate the antiviral susceptibility profile of the Italian influenza viruses and to increase

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our knowledge of the origin and spread over time of antiviral resistance in Italy. The National Influenza Centre (NIC), located at the National Institute of Health (Istituto Superiore di Sanita'-ISS) in Rome, has been monitoring antiviral susceptibility since 2007, using methodologies established in collaboration with other European public health laboratories, within the framework of the European Surveillance Network for Vigilance against Viral Resistance (VIRGIL). We examined antiviral susceptibility, to both NAIs and adamantanes, of type A and B seasonal influenza viruses, collected over an extended period of time (from 2004 to 2010) throughout the country, together with pandemic (H1N1) virus isolates collected in 2009. Drug-susceptibility testing was performed for both oseltamivir and zanamivir, using a NA activity inhibition assay in conjunction with NA sequence analyses. A number of Italian influenza A viruses from multiple influenza seasons were also investigated for adamantane resistance, by sequencing the M gene.

2. Materials and methods

2.1. Viral strains tested

Monitoring of antiviral drug susceptibility in the Italian influenza strains is performed in the context of the national sentinel surveillance activities conducted by the NIC/ISS, according to a specific drug testing strategy (Fig. 1). A total of 294 influenza field isolates, of which 110 were A/H1N1, 107 A/H3N2 and 77 B viruses, were isolated from throat swabs collected from patients with influenza-like illness over an extended period of time (from 2004 to 2010). Thirty-one pandemic (H1N1) 2009 strains were obtained from clinical samples (nasal, pharyngeal, or nasopharyngeal swabs and/or tracheal aspirates) of hospitalized patients, collected between May and November 2009.

Information on antiviral use has not been obtained in association with these clinical samples. With regard to pandemic (H1N1) 2009 viruses, 23 (74%) clinical samples came from patients who developed a mild disease and 8 (25%) from subjects with severe illness. Information about the therapeutic regime of the NAIs treated subjects were obtained only in a few cases. Virus isolation was performed in MDCK cells, after no more than two passages.

2.2. Neuraminidase inhibitors

Oseltamivir carboxylate (GS4071) and zanamivir compounds were provided by Roche and by GlaxoSmithKline, respectively.

2.3. Fluorometric NA inhibition assay

The fluorescence-based enzyme inhibition assay, used in the present study to define viral resistance to the NAI drugs and for the calculation of the inhibitory drug concentration (IC_{50}), had been previously developed and standardised at HPA (Health Protection Agency, London, UK) (Lackenby et al., 2008). Viruses were screened for susceptibility to NAIs, using the methyl umbelliferone *N*-acetyl neuraminic acid (MUNANA) as substrate. Each isolate was initially titrated in black 96-well flat bottom plates, in order to standardise virus input and to ensure equivalent NA activities were compared against inhibitors. After titrating NA activities, the inhibition assay was performed pre-incubating 10 μl of drug and 10 μl

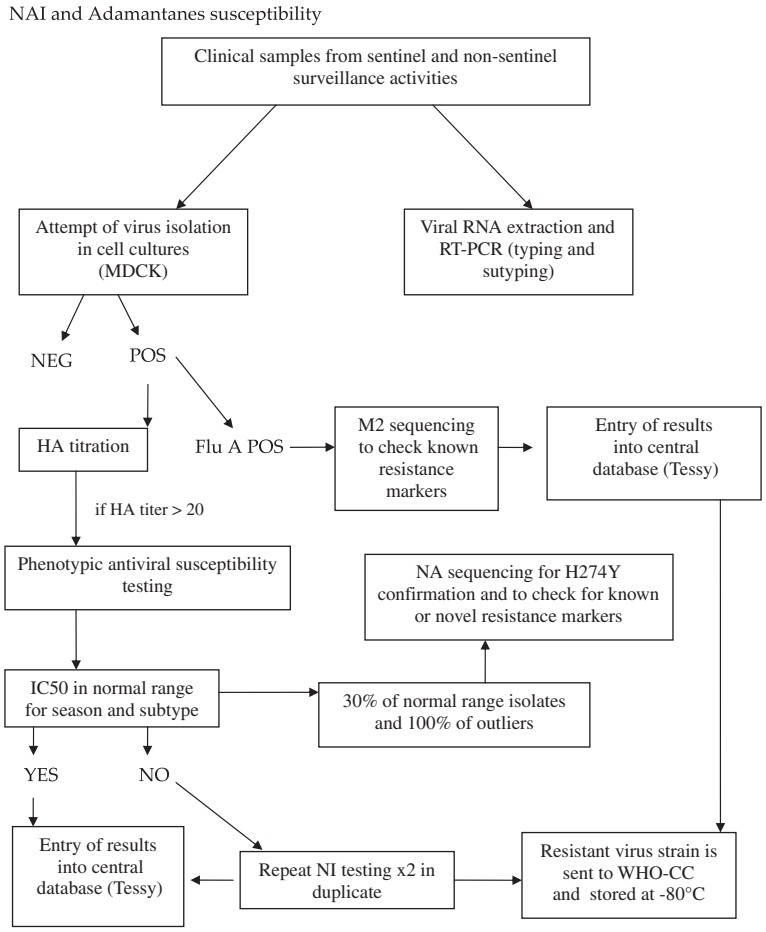

Fig. 1. Drug testing strategy.