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Original Article

Surveillance for antiviral resistance

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In the 10 years since licensure of neuraminidase inhibitor drugs, their use has steadily increased, especially during the pandemic of 2009. Experience now indicates that factors which influence the emergence of high level resistance include the nature of drug binding to target, viral subtype, the use of post exposure prophylaxis and a lack of immunity in the host as seen in children and immunocompromised individuals. These factors point towards targetted surveillance programmes for the early identification of transmissible drug resistance.

Keywords Neuraminidase inhibitor, antiviral susceptibility, monitoring, transmission.

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Introduction

The licensure of a new class of anti-influenza drug, the neuraminidase inhibitors (NAIs), in 1999/2000 heralded a new era for the control of human influenza. Two new agents, zanamivir (topical, inhaled) and oseltamivir (parenteral, oral), acting against the same viral target, the neuraminidase (NA) protein, offered more options for the treatment and prophylaxis of seasonal epidemic and pandemic influenza.

With any new class of antimicrobial drug, it is necessary to screen for the emergence of resistance, as presciently foreseen by Alexander Fleming in 1945, well before the genetic flexibility of microorganisms was understood, ….'There is the danger that the ignorant man may easily under dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant'.[1] The emergence of HIV as a major human pathogen and the intensive use of lifesaving antiretroviral compounds have provided a better understanding of the genesis of antiviral resistance. Resistance to antiviral agents is to be expected, mutations conferring high level resistance may be drug specific and may be delayed or overcome by combination therapies.

Unfortunately, practical application of combination therapies against influenza is limited by a very narrow drug spectrum. The restricted repertoire of new antivirals and combination treatments in development indicates the need for new influenza antiviral targets. This review summarizes our understanding of resistance to NAI drugs following the first ten years of their use and the implications for surveillance programmes.

Influenza antivirals

The only antivirals previously available to treat influenza were the M2 ion channel blockers, amantadine and rimantadine. These compounds are, however, only active against influenza A. They have not been widely used to treat influenza since their discovery in the 1960s, partly due to frequent emergence of resistance *in vitro*, with no apparent reduction in virus fitness or transmissibility, and partly due to lack of efficacy against influenza B. Resistance mutations are well characterized, occurring at five key sites in the transmembrane region of virus M2 protein. Resistance segregates with subtype and has been maintained and transmitted in the absence of drug pressure. The vast majority of human H3N2 viruses now circulating are resistant[2], and the dominance of resistant virus has been maintained in the absence of selective pressure through drug use. Amantadine resistance is also a characteristic of currently circulating A(H1N1)pdm2009 viruses, acquired by genetic reassortment from a resistant swine virus.[3] It varies according to genetic lineage of H5N1 in the avian reservoir (A. Hay, personal communication); however, how resistance to amantadine emerged in viruses circulating in the animal reservoir remains unknown.

By contrast, NAIs are active against both influenza A and B, and resistance emerges *in vitro* with much lower frequency. Early experience of NAI-resistant viruses with point mutations in the virus NA, arising during preclinical evaluation or clinical trials, indicated such viruses were compromised in fitness, with reduced transmissibility.

Existing surveillance

Significant factors to consider in developing targeted surveillance for antiviral resistance to any new class of anti-influenza drug include drug use, the effect of virus type/subtype, association with genetic/antigenic characteristics of circulating viruses and patient risk groups. The existence of a global surveillance network for influenza, underpinning vaccine strain selection, is a tremendous asset when seeking to track the emergence of antiviral resistance.[4] The routine sampling of circulating influenza viruses and their detailed characterization gives a composite picture of the relentless evolution of influenza viruses and variation in their antigenic properties. This, together with clinical experience developed over ten years of NAI drug use, now highlights the surveillance strategies necessary to provide early warning of significant antiviral resistance.[5]

First decade of drug use

At the outset, following the introduction of the NAI class of drug in 1999/2000, it was necessary to link data available on the emergence of resistant viruses during randomized clinical trials (RCTs) to the comprehensive global surveillance programme focused on antigenic variation in circulating viruses. A number of different mutations associated with antiviral resistance were recognized, but the correlation between these and virus type/subtype was not well understood, nor was the potential for cross-resistance to different antivirals. Technical challenges included the fact that the highly developed global surveillance system already in existence for influenza was geared to analysis of the virus haemagglutinin (HA)[4] rather than the neuraminidase (NA), and there was no definition of antiviral resistance or an agreed methodology for its measurement. Further challenges included uncertainty as to the resources required for this activity at national public health level when drug use was extremely limited. Over the ten-year period, there has been a gradual increase in antiviral use, peaking during the pandemic period 2009–2010, and a very wide variation in use geographically. The high per capita use in Japan during influenza seasons contrasts with relatively little use in Europe, South America, South-East Asia and Oceania, directly reflecting national policies (Figure 1).

Clinical and laboratory surveillance

When establishing any surveillance system *de novo,* it is necessary to determine to what extent laboratory data can be linked to epidemiological and clinical data. This remains a considerable challenge even in the extremely well-developed global influenza surveillance system coordinated by WHO, where virus isolate characterization and severe disease surveillance monitoring activities remain largely separate.[4] Orchestration of a global surveillance programme to scan for drug resistance has included the necessity to standardize methodology and to elucidate any differences between the antivirals, which have implications for the emergence of resistance.[5]

Drug resistance may be defined (i) clinically, when a treated individual is refractory to drug treatment, or if there is person to person transmission of a virus which is not susceptible to drug treatment; (ii) phenotypically, by the measurement of virus isolate susceptibility to drug in a

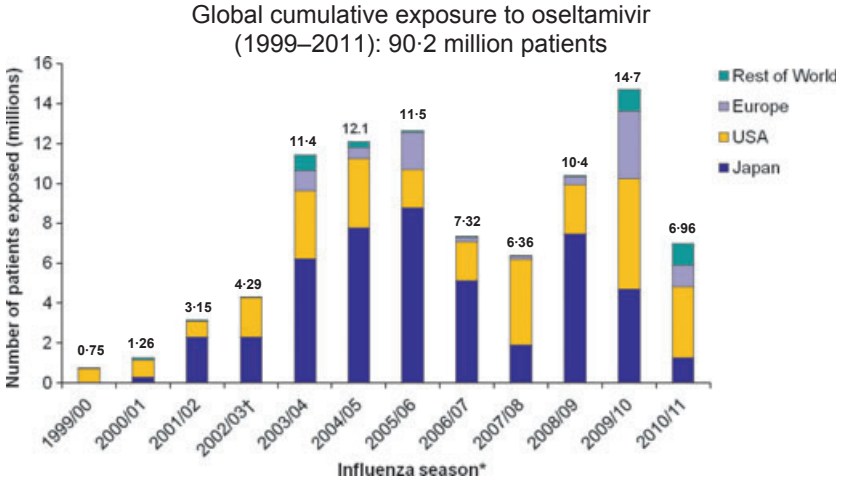

Figure 1. Global oseltamivir usage since 1999. *Defined as 12 months of data (October–September), except for 2003/04 (October–March); 2004/05 (April–March); 2005/06 (April–September); and 2010/11 (October–June). †USA and Rest of World data are combined for the 2002/03 season.Source: IMS prescription figures up to September 2010 and IMS sales figures for October 2010 onwards. Data for Europe in the 2009/10 season included UK government exposure data.

model system, with definition of resistance correlating with a measurable alteration in a virus property; or (iii) genetically, by a change in the virus genome correlating with a measurable phenotypic loss of susceptibility and/or clinical resistance.

Surveillance based on the detection of clinical resistance requires a link to clinical networks with defined clinical outcome monitoring, but is difficult to establish when there is limited drug use.

Laboratory surveillance, which focuses on phenotypic or genotypic monitoring of virus isolates, even if unlinked to clinical information, has the advantage of being practical and generates useful data about circulating viruses. Unfortunately, genetic and phenotypic resistance do not necessarily identify the same thing. A virus may appear to lose susceptibility *in vitro*, as a result of growth in a particular biological test system (phenotypic testing), yet retain the genetic characteristics of a fully sensitive virus, adding complexity and highlighting the necessity for standardized methodology for laboratory-based surveillance. Use of assays to assess inhibition of viral neuraminidase activity provides a measure of susceptibility to drug, usually expressed as the inhibitory concentration required to inhibit 50% of enzyme activity (IC_{50}). This approach is usually applied to cultured virus isolates, but the IC_{50} values obtained can vary significantly according to the format of the IC_{50} assay, culture substrate used to grow virus, and assay methodology if using a kinetic enzyme assay. Whilst the correlation between very high IC_{50} values (>1000 nmol) and lack of clinical efficacy is demonstrated, as with H1N1 H275Y variants, the relationship between IC_{50} and clinical efficacy is otherwise poorly understood, underlining the necessity for harmonizing methodologies.

On the introduction of NAIs, the priorities for laboratory surveillance were to

1. establish standardized methodology
2. search for the evidence of drug resistance occurring naturally prior to drug use
3. analyse resistance in contemporary circulating viruses.

Results of early surveillance

The application of standardized NA susceptibility assays[6,7] identified no pre-existing resistance to NAIs among globally representative isolates collected prior to their introduction. However, oseltamivir and zanamivir susceptibility of approximately 1000 clinical isolates collected between 1996 and 1999 showed a wide variation[7] (Figure 2). The NAs of influenza B viruses have approximately 10-fold lower susceptibility than those of influenza A viruses, yet the viruses remain clinically responsive to drug treatment *in vivo*. This emphasizes the continuing difficulty in establishing a practical definition of drug resistance which is applicable to all influenza A and B viruses.

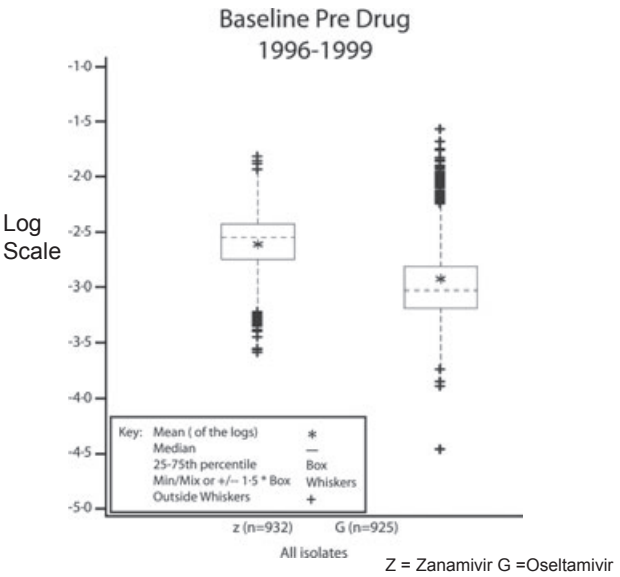

Figure 2. Antiviral susceptibility baseline, from 1996 to 1999 isolates. Plot showing distribution of IC_{50} values for zanamivir and oseltamivir susceptibility of human influenza isolates prior to licensure of drugs.

Over the first few years of surveillance after NAI introduction, the majority of isolates analysed had been collected for antigenic surveillance. This was a pragmatic solution to the development of a resistance screening programme, making use of the existing surveillance infrastructure. Establishing systematic screening for resistance involved the application of a two step approach, with initial phenotypic susceptibility (NA inhibition) screening of a wide range of isolates, followed by sequence analysis of NA genes of a much smaller set of isolates, which had an IC_{50} for susceptibility in the upper range (Figure 2). One of the difficulties with this approach, whilst efficient, is that the genotype–phenotype correlation may vary. For some NA mutations, a single amino acid substitution, for example, H275Y in N1, results in a several hundred fold decrease in susceptibility as measured *in vitro*, whereas mixtures of sen-

Table 1. Neuraminidase inhibitor resistance profiles (7,23)

NA mutation	NA type/ subtype	Susceptibility in the NAI assay (fold change in IC_{50})		
		Oseltamivir	Zanamivir	Peramivir
E119V	A/N2	R (>50)	S (1)	S (1)
R292K	A/N2	R (>1000)	S (4–25)	R (40–80)
H274Y	A/N1	R (>700)	S (1)	R (40–100)
R152K	B	R (>30–750)	R (10–100)	R (>400)

R, Resistant; S, Sensitive.

sitive and resistant viruses or different amino acid substitutions may not give clear shifts in susceptibility.[7]

Important information from the initial surveillance programme was the way in which different substitutions conferring resistance were associated with different virus type/subtype or drug[8] (Table 1). Understanding these observations was enhanced by structural studies[9], which indicated that amino acid substitutions occurring in the active site of the enzyme directly impact substrate binding or catalytic activity, whereas other mutations affect the framework of the NA and may affect protein stability. Crystal structure determination and phylogenetic analysis demonstrated that NAs could be clustered into two groups: Group 1 and 2, with important differences in substrate binding sites. Group 1 has an extended catalytic site, which may contribute to a propensity for the selection of some of the changes associated with drug resistance, suggesting that the group 1 NAs (including N1) might be more prone to tolerate resistance mutations, compared with group 2 NAs (including N2). Observations of H5N1 infections in humans[10,11] indicate the relative ease with which clinically significant resistance may emerge, and clinical studies in H1N1-infected children indicated that a significant number of children (15–20%) who were treated and otherwise healthy shed resistant virus during oseltamivir treatment, but without clinical consequence or apparent onwards transmission.[12,13]

Surveillance in Japan, 2004–2007

From licensure, health policy in Japan supported extensive NAI drug use, which eventually achieved the highest per capita use of oseltamivir in any country of the world. Surveillance in Japan therefore focussed on a location where selective drug pressure might have a greater influence on the emergence of drug resistance. The frequency of oseltamivir resistance detected in over two thousand virus isolates from Japan between 2004 and 2007 using the sequential phenotypic and genotypic screening approach (Figure 3) was <1%, despite the extensive drug use. Resistance due to some novel mutations was detected, emphasizing the necessity of a broad-based screening approach.[14]

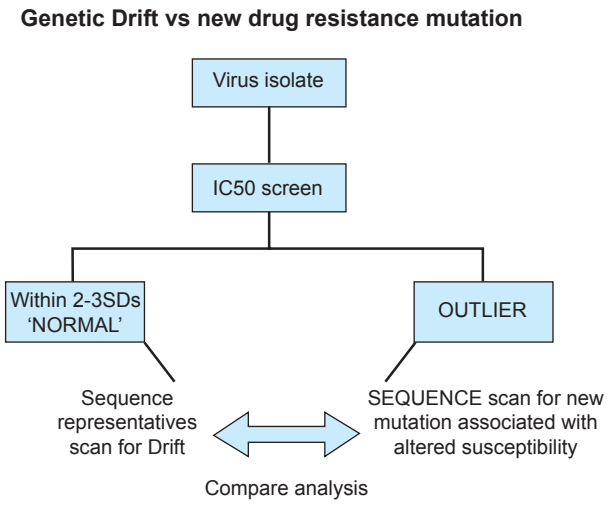

Figure 3. Schematic of laboratory testing algorithm.

Emergence of transmissible oseltamivir resistance in 2008

The establishment of systematic surveillance for drug resistance in Europe, correlating epidemiological and virological information via data linkage and IT systems in a specific EU funded health project (VIRGIL) covering 30 countries, proved unexpectedly useful in detecting and tracking the emergence and spread of oseltamivir-resistant H1N1 viruses, with a H275Y substitution, in the winter of 2007-2008.[15] Within 12 months, virtually all H1N1 viruses circulating globally were oseltamivir resistant.[16] As for amantadine resistance, the emergence of oseltamivir resistance occurred against a background of very little drug use, with resistant viruses outcompeting sensitive viruses.

Detailed phylogenetic analysis indicated that the oseltamivir-resistant NAs grouped in a single evolutionary clade.[17] Whilst structural characteristics may predispose the N1 NA to oseltamivir resistance, mutations arising through genetic drift may compensate for enzymatically unfavourable resistance substitutions by enhancing NA activity[17,18], or by a reduced requirement for NA as a result of altered binding affinity of HA. Virus fitness is dependent on well-matched biological activities of the two virus proteins. These observations re-emphasize the relationship between the enzyme activity of NA and receptor binding of the HA, and the desirability of linking surveillance of drug resistance with global virus surveillance programmes.

Pandemic influenza H1N1 2009

Informed by experience of the previous decade, the emergence and spread of antiviral resistance was recognized as a distinct possibility at the outset of the 2009 H1N1 pandemic. The overall global health response was coordinated through WHO, with guidelines for clinical use of antivirals. For the first time, consolidated laboratory resistance surveillance data, from all WHO regions, were available in one place on the WHO website[19] in real time, with a massive increase in the number of viruses being screened genetically for key resistance markers, such as H275Y. This emphasized the necessity for clinical laboratory capability to detect influenza A and B infections and distinguish between them and further to be able to provide subtype information

close to the patient. Clinical data associated with antiviral resistance detection indicated the importance of immunocompromised individuals with prolonged shedding and high virus loads in the generation of resistant viruses. When treated with multiple therapies, such patients may be a source of unusual and multiple drug resistant viruses.[20] The detection of resistant viruses post-treatment in healthy adults, particularly when using sensitive molecular detection techniques, is not necessarily the most important parameter to measure, as the key public health concern lies with transmissible resistance. This is more appropriately measured through surveillance in the community, or of pre-treatment samples taken from individuals with no known contact with drug-treated individuals.

Novel surveillance strategies

The unpredictable evolution of the influenza viruses and the need to focus on transmissibility of drug resistance requires tracking of different clinical patient categories where information about how drug is used is very important. Understanding the relative contributions of different patient groups to the emergence of antiviral resistance is an important source of information to guide prescribing strategies to minimize the emergence of resistance (Figure 4). The group with no known association with drug use is the best sentinel indicator for the emergence of transmissible drug resistance, represented by those in the community with no healthcare contacts. Developments in information technology infrastructure and sophistication of surveillance reporting suggest that this hitherto unattainable goal may be within reach. It is necessary to develop surveillance strategies to track resistance emergence in the community that are efficient but also relate to drug use. During the 2009 pandemic, England used a pandemic flu service, a dedicated telephone line with the principle of 'treat all'. An individual could call a dedicated number and go through a clinical triage system to get antiviral drug.[21] This was linked to a surveillance strategy, where about 500 individuals per day across England were randomly selected to receive self-sampling kits and asked to return the self sampled swabs by post to the national centre. Swabs were analysed for the detection of influenza and if positive were also analysed genetically for resistance (H275Y screen). Whereas self-swabbing did introduce some delay in receiving samples, results compared favourably with receipt of swabs from sentinel GPs. This mechanism allowed analysis of possible emergence of resistance in the community by age and time post-treatment,[22] providing a simple, scalable means of intensifying surveillance for drug resistance in the community, linked directly to drug use. This method could also generate a supply of virus isolates for more detailed characterization.

Conclusions

The development of regional networks for the surveillance of antiviral resistance is important in establishing the link between drug use and resistance detection, with more community or risk-based sampling to provide an early window into transmission of unusual variants. The last decade has demonstrated the importance of linking observational surveillance data with genetic and structural characteristics of viruses and animal model studies of virus transmissibility, to provide a good basic understanding of the biology of the virus–host interaction and deduce principles to be applied to new antivirals. As the use of NAI drugs reach maturity, with the recent licensure of laninamivir and peramivir in Japan[23], it is necessary to intensify surveillance for drug resistance, in the light of knowledge that transmissible resistance can occur and resistant viruses can outcompete sensitive strains.

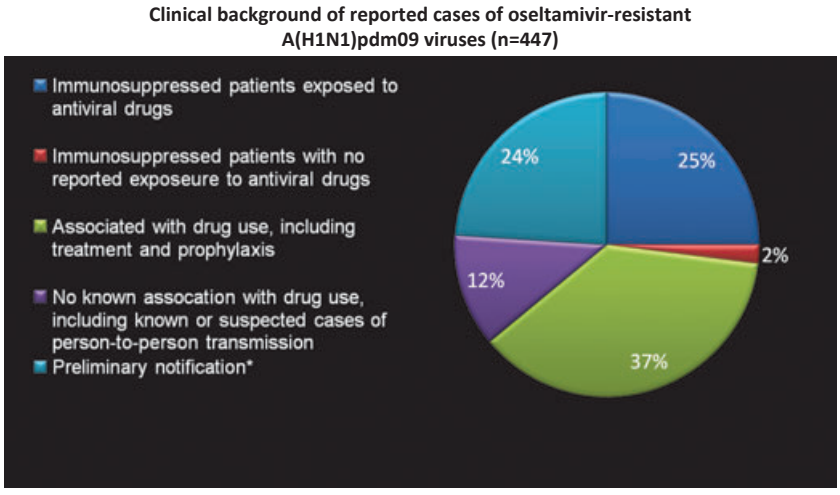

Figure 4. Clinical background of reported cases of oseltamivir-resistant A(H1N1)pdm09 viruses (n = 447).

Priorities to minimize emergence of antiviral resistance now include more widespread use of clinical guidelines for physicians to promote prescribing stewardship aimed at reducing prophylactic use of drugs. All this is in the sure knowledge that 'Evolution will outsmart intelligent (drug) design every time'.[24]

Key learning points from 10 years of NAI drug resistance surveillance

1. Resistance to NAI drugs is primarily associated with substitutions in virus NA gene.
2. Drug resistance mutations may affect substrate binding, catalysis or framework of virus NA protein.
3. Relationship between resistance phenotype and genotype is not always predictable.
4. For N1-containing viruses, the major mutation conferring oseltamivir resistance is likely to be H275Y.
5. Mutations conferring NAI resistance differ between virus subtypes.
6. Some *in vitro* systems for the measurement of drug susceptibility may generate anomalous results.
7. Drug resistance mutations have a variable pattern of cross-resistance.
8. Emergence of drug resistance is not necessarily linked to drug use.
9. Compensatory mutations occurring as a result of genetic drift may overcome fitness deficits due to drug resistance.

Priorities

1. Develop guidelines for physicians for treatment and prophylaxis and prescribing stewardship.
2. Establish community-/risk-based sampling.
3. Develop regional networks for surveillance.
4. Evaluate transmission potential of different mutations.
5. Link to structural and biological model work.
6. Develop new drug pipelines.

Conflict of interest

Maria Zambon is a Committee member of the UK Scientific Advisory Group in Emergencies (SAGE) and IHR Emergency Advisor to WHO.

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