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## Detection of Adamantane-Resistant Influenza on a Microarray

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### Abstract

**Background**—Influenza A has the ability to rapidly mutate and become resistant to the commonly prescribed influenza therapeutics, thereby complicating treatment decisions.

**Objective**—To design a cost-effective low-density microarray for use in detection of influenza resistance to the adamantanes.

**Study Design**—We have taken advantage of functional genomics and microarray technology to design a DNA microarray that can detect the two most common mutations in the M2 protein associated with adamantane resistance, V27A and S31N.

**Results**—In a blind study of 22 influenza isolates, the Antiviral Resistance-Chip (AVR-Chip) had a success rate of 95% for detecting these mutations. Microarray data from a larger set of samples were further analyzed using an artificial neural network and resulted in a correct identification rate of 94% for influenza virus samples that had V27A and S31N mutations.

**Conclusions**—The AVR-Chip provided a method for rapidly screening influenza viruses for adamantane sensitivity, and the general approach could be easily extended to detect resistance to other chemotherapeutics.

### Introduction

Influenza virus is a significant human pathogen that results in a large number of deaths each year (Layne et al., 2001). Upon identification of the influenza virus, antivirals are often prescribed to limit the severity and duration of infection. However, the frequency of antiviral drug resistance has increased dramatically over the last 10 years, and prompted the need to identify drug susceptibility.

Amantadine, and its methylated derivative rimantadine, inhibit ion flow through the ion-channel (designated M2) of influenza, thereby preventing a productive infection. (Weinstock and Zuccotti, 2006). These drugs have been used extensively worldwide, but, unfortunately, adamantane resistance readily develops both *in vivo* and *in vitro* (Belshe et al., 1989).

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Resistance generally results from single point mutations in M2, although double mutations have been observed (Belshe et al., 1988, Cheung et al., 2006). Mutations result in single amino acid changes at residues 26, 27, 30, 31 or 34 of the M2 protein and do not appear to result in significant virus growth impairment (Bright et al., 2005, Bright et al., 2006, Hay et al., 1986, Saito et al., 2003, Suzuki et al., 2003, Sweet et al., 1991, Ziegler et al., 1999).

The ability to reproducibly and rapidly detect resistance to antivirals has been a major challenge (Hayden et al., 1980). ELISA of infected MDCK cells has historically been the standard for resistance determination of uncharacterized viruses (Belshe et al., 1988). More recently, identification of the most common mutable positions has allowed detection by genomic sequencing or by novel techniques that quickly assay a few nucleotide positions of the genome. A restriction fragment length polymorphism methodology was developed to determine resistance mutations in patient samples (Klimov et al., 1995, Saito et al., 2002). Pyrosequencing can also be used to detect mutations (Ahmadian et al., 2000), however, detecting resistance to multiple drugs or to detect mutations that do not cluster in one region of the genome would require multiple assays.

An oligonucleotide microarray is a highly attractive approach to genotyping due to the multiplexing capability and has been applied to other antiviral resistance problems (Pannucci et al., 2004). For example, microarrays to detect point mutations that give rise to drug resistant *M. tuberculosis* and *S. aureus* have been demonstrated (Couzinet et al., 2005, Troesch et al., 1999). Booth et al. developed a low-density microarray for detecting drug-resistant *N. gonorrhoeae* (Booth et al., 2003).

Here we report the development and characterization of a low-density DNA microarray to distinguish between adamantane resistant and sensitive influenza A viruses. The two most common mutations, V27A and S31N, were specifically targeted using a small set of capture and label sequences. A blind study demonstrated that the microarray could rapidly and accurately detect resistance and identify the mutation responsible.

## Methods

A generalized schematic representing the two-step hybridization used is shown in Figure 1. Briefly, an amino modified “capture” sequence is immobilized on the microarray surface and binds the target RNA during hybridization. The RNA is labeled by hybridization to a secondary sequence with an attached fluorophore. The microarray is then washed, scanned and visualized.

### Sequence Selection

Sequences were selected using a modification of the protocol described in (Mehlmann et al., 2006, Smagala et al., 2005). Two novel programs were written for use in the sequence selection process. The *rm\_dup* program was written in Python and eliminated identical sequences from an aligned FASTA file. The program, *rm\_dup*, removed duplicates by comparing both accession numbers and genome sequence data. Removal of duplicate genome data, while occasionally eliminating valid sequences, prevented artificial weighting of the database to clusters of genomes that were collected in one region over a short period of time. The *gen\_mismatch\_report* program compared chosen capture and label sequences with the genomes present in each subtree of the database. The average number of mismatches between each capture/label sequence was output and this value used to determine the best sequences that covered each database.

## Modifications to the Sequence Selection Methodology

Due to the high variability of portions of the M2 gene, the capture and label sequences were chosen separately and allowed to be between 6–25 nt apart. Capture probes targeted the highly variable region covering the mutable positions and label sequences targeted the more conserved neighboring regions. An additional benefit of this approach was that only a few label sequences were necessary to cover the entire influenza database.

## Microarray Sequence Selection

We used 1018 M gene segment sequences from the Los Alamos National Labs Influenza Sequence Database<sup>1</sup> (Macken et al., 2001) and 1194 sequences of ‘influenza segment 7’ (M gene) from NCBI’s nucleotide database<sup>2</sup> on 2/1/06. Sequence files with either identical accession numbers *or* identical nucleotide sequences were removed using *rm\_dup*. The final database of 1086 influenza sequences was aligned using Clustal W (Thompson et al., 1994).

From this database, DNADIST was used to create a phylogenetic tree using only sequence data from nucleotide positions 754–849. Analysis, phylogenetic grouping and conserved region selection were performed as previously described (Mehlmann et al., 2006). A modified version of *find\_oligos* was used to select single sequences (either capture or label) independently, without the restriction that they be 0 or 1 nt apart. Oligos were scored and picked using *score\_oligos* and *pick\_oligos*. All picked oligos were examined using *gen\_mismatch\_report*, to determine which sequences best covered the 1086 sequences in the database. Label sequences were chosen from the more conserved nucleotide regions 754–789 and 816–849. Capture sequences were selected that covered the five known mutable positions (Table 1) within the M2 gene and in which the mutable position was at least 6–8 nucleotides from either end of the oligo. A total of 111 sequences ranging from 16–25 nucleotides were “picked” by the automated *pick\_oligo* program. From these sequences, 8 label sequences (4 from nt 754–789 and from nt 816–849) were selected, based on their coverage of the 1086 influenza sequences, and ordered with a conjugated Quasar-570 fluorophore (Biosearch Technologies, Novato, CA). A total of 9 capture sequences (5 targeting position 805 and 4 targeting position 793) were initially selected. For each capture sequence selected, two probes were synthesized (Operon, Huntsville, AL). The first was a perfect match with a sensitive virus (sensitive specific) and the second was a perfect match for a resistant virus (resistant specific). Selected sequences are available for non-profit organizations by request to CU Technology Transfer Office<sup>3</sup>.

## Microarray Preparation

Capture and label sequences were spotted as described previously (Townsend et al., 2006). Capture sequences designed to probe for mutations at either nucleotide 805 or 793 were spotted in triplicate. Immobilized positive control (PC) sequences that bound directly to label sequences were used both to test chip spot quality and hybridization performance and provide orientation when viewing the array.

## Samples and Sample Processing

The samples for the blind study contained purified vRNA from 7 H1N1 and 15 H3N2 influenza isolates obtained during the 2005–2006 season. Samples used for the NN study included H1N1 and H3N2 viruses isolated during 2003–2006.

<sup>1</sup><http://www.flu.lanl.gov/>

<sup>2</sup><http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=NucleotideGenBank>

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Nucleic acid purification, amplification and microarray hybridization and analysis were performed as described previously (Townsend et al., 2006). Briefly, purified viral RNA was RT-PCR amplified with the M specific primers SZAM+ and T7-SZAM- (which incorporated a T7 promoter site in addition to the standard primer) (Zou, 1997). PCR products were used for run-off transcription of RNA. Reverse transcribed RNA was briefly fragmented to eliminate secondary structure and hybridized to the array for 2 hours at room temperature. Slides were scanned using a Bio-Rad Laboratores (Hercules, CA) VersArray scanner. Contrast optimized images were used for the blind study. The VersArray analyzer software was used for image quantitation.

### Artificial Neural Network Sample Identification

EasyNN Plus Software<sup>4</sup> (Version 7.0j) was used for analysis by an artificial neural net (ANN). The ANN program is a supervised pattern recognition algorithm that was used to identify input data from “unknowns” based on its training with “known” samples. The ANN was developed and used as described in (Dawson et al., 2006).

## Results

### Detection of Antiviral Resistance on a Microarray

Two capture probes were designed for each sequence chosen; one a perfect match for a sensitive virus and one a perfect match for a resistant virus. As outlined in Figure 1, it was anticipated that RNA from a sensitive virus would hybridize more efficiently to the “sensitive” capture probe and less efficiently to the “resistant” capture probe due to the mismatch with the resistant probe. Thus, by examining the signal from sensitive and resistant probes, a determination could be made as to the antiviral susceptibility of the virus.

Figure 2 shows the microarray layout (a, b) and contrast optimized images (c–f) used to query positions 793 and 805. Hybridization of H3N2 sensitive viruses produced a pattern where greater fluorescence intensity was present on most of the sensitive specific probes. By comparison, hybridization of H3N2 resistant viruses generally resulted in higher intensity hits on the resistant specific probes. Although all probes were not equally sensitive to the presence of a mismatch, the built in redundancy provided several options to detect resistance mutations. A simple visual examination could determine virus drug sensitivity. It is instructive to note that the probes were designed to provide identification for a range of influenza viruses. Thus, while some sequences appear non-discriminatory, they may work more appropriately for a different virus.

### Blind Study Results

Using a set of 22 H3N2 and H1N1 influenza virus isolates, we performed a blind study to evaluate the ability of the AVR-Chip to identify drug-resistant and–sensitive viruses. Visual interpretation of the microarray images was based on evaluation of fluorescence intensity differences between the sensitive and resistant-specific capture/label set. These results are summarized in Table 2. Overall, 95% of the samples were correctly typed. The single incorrectly identified sample contained a V28I mutation in addition to the S31N mutation, which yielded a fluorescence image pattern sufficiently different than the expected patterns that the results for the virus were categorized as “inconclusive.” In contrast, samples 13–15 also contained additional mutations but were correctly identified.

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<sup>4</sup>[www.easynn.com](http://www.easynn.com)

## ANN Identification of Antiviral Drug Sensitivity

To test the feasibility of automated analysis, data from all samples were quantified and used for drug sensitivity determination with an Artificial Neural Network (ANN). Negative control samples were included in both training and as unknowns. The output options provided for the ANN were Negative, Sensitive, and Resistant. The 94 images in this data set were divided into 10 groups. Each group was individually queried while the ANN was trained with the samples from the other 9 groups. As shown in Table 3, 94% of the samples were correctly identified, 4% were false negatives, and 2% were false positives. The AVR-Chip's results correspond to a sensitivity [true positives / (true positives + false negatives)] of 95% and specificity [true negatives / (true negatives + false positives)] of 89%. These values compare well with previous diagnostic tests used to determine influenza drug sensitivity (St George et al., 2002). The full result set from the ANN output is available as a supplement (Table S1).

Of the samples that were incorrectly identified, two were false positives from negative control samples. Careful analysis of these two samples showed no significant fluorescence signal on any of the influenza specific sequences or other spurious signals and they would easily be identified as negative by visual analysis.

## Discussion

The extensive impact of influenza, along with the cyclic global pandemics, has prompted efforts to improve the speed and comprehensiveness of influenza diagnostics. We previously demonstrated a microarray-based approach for rapidly typing and subtyping influenza (Mehlmann et al., 2006, Townsend et al., 2006). Here we have extended this work to detect antiviral resistance.

Being able to rapidly assess whether or not a patient is infected by a drug-resistant strain of influenza could enhance both the treatment of the patient and help limit the spread of resistance. Currently only a small number of patient samples are tested for resistance due to the time and expense involved, and these limited data are then used for widespread treatment recommendations. Increased surveillance would allow more timely changes in treatment recommendations, thereby helping limit the propagation of drug resistant virus.

The V27A and S31N mutations account for up to 97% of adamantane resistance (Bright et al., 2005, Bright et al., 2006, Hay et al., 1986, Saito et al., 2003, Suzuki et al., 2003, Sweet et al., 1991, Ziegler et al., 1999). By choosing appropriate capture and label sequences along with a simple array design, we found that we could visually identify viruses as sensitive or resistant with high accuracy. Additionally, we have shown that the data can be accurately analyzed using an ANN. Furthermore, we anticipate that as the size of the training set is increased, the accuracy of the ANN will improve.

One implicit assumption for these studies was that mismatches at nucleotides other than the queried position should reduce binding to *both* the sensitive and resistant specific probes. Since such mutations would result in an additional mismatch for binding to both the sensitive and resistant probe, the signal from both probes should be reduced similarly. Since three of the four samples containing additional mutations were correctly identified, this assumption appears generally correct.

To our knowledge, this is only the second application of a low density array for detection of antiviral resistance for influenza viruses (Booth et al., 2003). As compared with higher-density arrays, benefits of low-density arrays include ease of data management and lower cost while having the multiplexing ability of microarrays. The total cost for this assay, including slides and all reagents used during printing, amplification and hybridization, is less than \$20.

Although only 18 probes were utilized for this analysis, the current array production format would readily allow incorporation of additional sequences with minimal extra cost. These properties make low-density arrays attractive for routine diagnostic applications. In addition, although the current protocol utilized virus isolates, we have demonstrated that an analogous analysis of influenza type and subtype present in patient samples only requires ~7 hr (Dawson et al., 2006). Thus, microarray-based differentiation of drug-sensitive and drug-resistant virus from patient samples should also be ~7 hr.

A limitation to all sequence based technologies is that there must be *a priori* knowledge of the mutable position responsible for resistance. Adamantane resistance has been well characterized and provided a starting point for the development of the AVR-Chip. Although the current proof-of-principle array tests for resistance at the two current most important positions, future resistant viruses with mutations at other M2 positions could become prominent. Importantly, the established sequence selection methodology, as well as the ease with which the array could be modified, would allow easy update for detection of these new mutations. Furthermore, the technology developed herein could be extended to detect resistance mutations in other virus subtypes such as the H5N1 avian influenza viruses, and potentially to detect resistance to other antiviral drugs. For example, resistance to neuraminidase inhibitors has been observed (15,32). Although there is not yet a clear and predictable relationship between all mutations and functional neuraminidase resistance, commonly observed mutations that are known to be correlated with resistance could be queried in a microarray format to provide some degree of screening.

## Conclusions

We developed a simple and inexpensive method to determine adamantane drug resistance in influenza viruses. The success rate for identifying resistance due to the mutations V27A and S31N exceeded 94%. This approach is amenable to combination with previously described microarrays (Dawson et al., 2006, Townsend et al., 2006) where virus type and subtype are determined, and could also be adapted to future arrays designed to provide further genetic analysis of influenza. Indeed, it should be possible to extend this approach to detect resistance to the other primary class of influenza drugs, the neuraminidase inhibitors, thereby providing a complete profile of what drugs will work with a given influenza infection.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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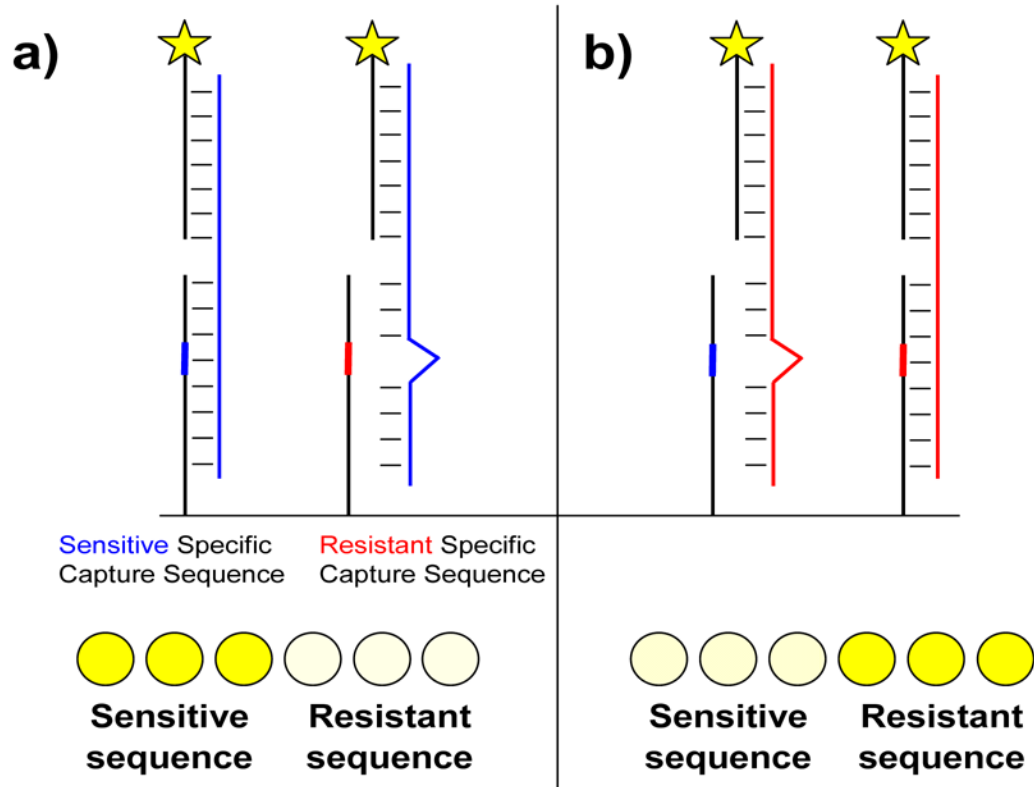
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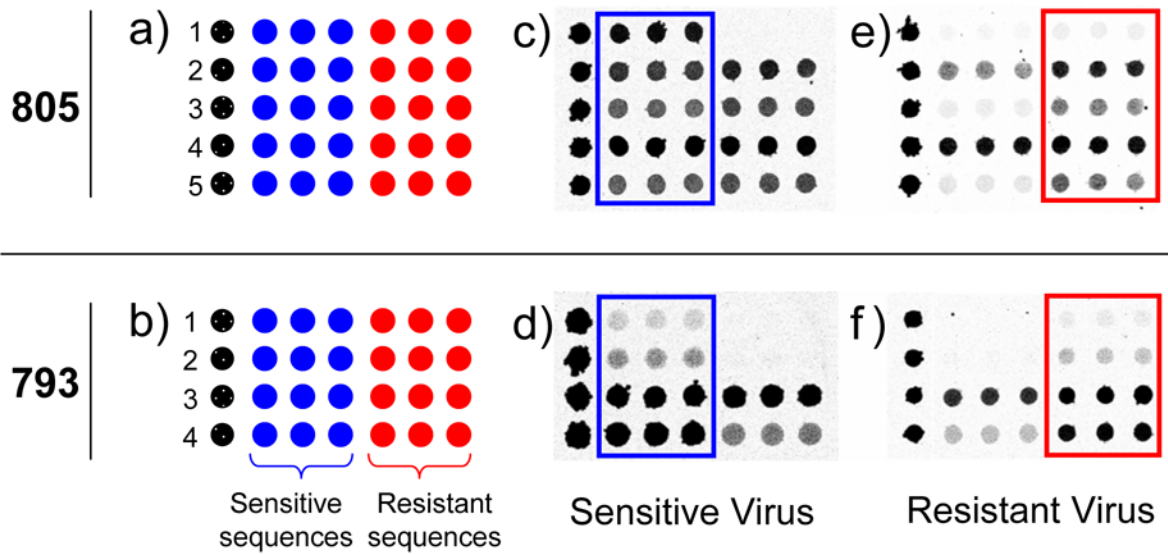
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**Figure 1.**

Schematic for two-step hybridization and detection of antiviral resistance on a microarray. **a)** Viral RNA from a sensitive virus (blue) hybridizes perfectly to the sensitive specific capture sequence but not to the resistant specific sequence. Captured RNA is detected by hybridization to a fluorophore-conjugated secondary sequence. The row of spots at the bottom represents a typical pattern observed when RNA from a sensitive virus hybridizes to triplicate spots of each capture sequence. Both capture sequences are identical except for the single position designed to probe resistance. **b)** The reverse scenario where RNA from a resistant virus (red) hybridizes to the same sequences.



**Figure 2.**

Microarray layout (a and b) and hybridization images (c-f) for probes designed to query positions 805 (S31N mutation) and 793 (V27A mutation). Each numbered row represents a specific probe sequence designed for either the 805 or 793 mutation. In (c-f) darker grey represents higher fluorescence intensity. The drug sensitive specific sequences are boxed in blue and the drug resistant sequences are boxed in red for ease of viewing. Images c and d were from drug sensitive A/H3N2 viruses. Images e and f were from drug resistant A/H3N2 viruses.

**Table 1**

Mutations associated with adamantane resistance.

Amino acid position	Mutation	Nucleotide position <sup>a</sup>	Nucleotide change <sup>a</sup>
26	Leu → Phe	789	C → U
	"	791	A → U,C
	"	791	G → U,C
27	Val → Ala	793	U → C
30	Ala → Thr	801	G → A
31	Ser → Asn	805	G → A
34	Gly → Glu	814	G → A

<sup>a</sup>Mutations are provided in terms of the change found in the coding strand (+ sense RNA or cDNA) of the virus.

**Table 2**  
Blind study results for microarray detection of drug resistance

Sample	Subtype	Visual Assignment	Position	Correct?	Additional Mutations
1	H1N1	Sensitive	-	✓	
2	H3N2	Sensitive	-	✓	
3	H3N2	Resistant	V27A	✓	
4	H3N2	Resistant	S31N	✓	
5	H3N2	Sensitive	-	✓	
6	H1N1	Resistant	S31N	✓	
7	H1N1	Sensitive	-	✓	
8	H1N1	Resistant	S31N	✓	
9	H3N2	Resistant	V27A	✓	
10	H3N2	Resistant	S31N	✓	
11	H3N2	Resistant	S31N	✓	
12	H3N2	Resistant	S31N	✓	
13	H3N2	Resistant	S31N	✓	
14	H3N2	Resistant	S31N	✓	
15	H3N2	Resistant	S31N	✓	A30T
16	H1N1	Sensitive	-	✓	V28A
17	H3N2	Resistant	S31N	✓	P25L
18	H3N2	Sensitive	-	✓	
19	H3N2	Sensitive	-	✓	
20	H3N2	Sensitive	-	✓	
21	H1N1	Resistant	S31N	✓	V28I
22	H1N1	Resistant	S31N	✓	

**Table 3**

Summary of ANN - aided identification of drug resistance

Sample Type	Number of Sample Images in Data Set	Number Correctly Identified	Number of False Negatives	Number of False Positives
Influenza Negative	16	14		2
S-H1N1	16	15	1	
R-H1N1 (V27A)	2	2		
R-H1N1 (S31N)	5	4	1	
S-H3N2	28	28		
R-H3N2 (V27A)	2	1	1	
R-H3N2 (S31N)	25	24	1	
All Samples	94	88	4	2