Rapid diagnostics: the detection of neuraminidase activity as a technology for high-specificity targets

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The accurate detection of influenza by clinical symptoms is challenging since multiple pathogenic viruses and bacteria mimic similar symptoms in a patient. With new and more effective influenza therapeutics available, there is a growing need for highly accurate and rapid diagnosis of influenza, particularly when the window of opportunity for proper treatment is measured in hours. A parallel technology, which is also used in the treatment of influenza, was developed for the rapid diagnosis of influenza by exploiting the enzymatic activity of influenza neuraminidase. This technology, which is called Pathozyme, offers the high specificity inherent from the conservation of the neuraminidase active site. The ZstatFlu test uses a small molecule derivative of sialic acid chemically coupled to a reporter group together with simple point-of-care reagents for directly detecting influenza from a patient specimen with high specificity. A second-generation platform technology using this neuraminidase detection system coupled with a more sensitive chemiluminescent reporter has been developed and formatted for reading on high-speed instant film. This modification resulted in a platform technology many-fold more sensitive than the former while maintaining its inherent high specificity. Preliminary data from a prototype tested during the mild 2000–2001 influenza season demonstrated that an optimized chemiluminescent test system could approach the accuracy of 14 day viral culture in a convenient 10–20 min test. This platform technology is currently being explored for the rapid detection of other pathogenic organisms where sensitivity, specificity and speed are essential in a point-of-care setting.

Keywords: influenza; neuraminidase; high-specificity target; rapid diagnostics; chemiluminescence; disease management

1. THE SIGNIFICANCE OF COMPREHENSIVE DISEASE MANAGEMENT

The best method of ‘treating’ disease is through a comprehensive disease management programme that includes disease surveillance, effective vaccination, accurate diagnosis and timely treatment. When properly administered, each of these components provides an essential piece of a programme for reducing the overall morbidity and mortality of any disease, including influenza. The absence of any one of these components reduces the effectiveness of disease control. Because influenza is such a widespread disease, effective disease management benefits can be significant when health care costs are estimated at US\$12–15 billion each year. Vaccination and viral surveillance for influenza have been around for some time, whereas rapid diagnostics that can detect both influenza type A and B viruses and therapeutics that treat both virus types are new to influenza disease management.

2. INACCURACY OF INFLUENZA DIAGNOSIS BY CLINICAL SYMPTOMS

Upper respiratory disease infections account for more than 100 million visits to a doctor, emergency room or

clinic in the USA each year. It is estimated that more than half of these are due to viral infection. The infection rate of influenza in the USA is estimated to be between 30 and 60 million cases each year (Murphy & Webster 1990). Influenza infection is accompanied by a myriad of symptoms, some of which are believed to be characteristic of this illness. The symptoms of 546 patients presenting to physicians during the 1997–1998 influenza season who were clinically diagnosed as potentially having influenza were compared to viral culture confirmation in a study by Shimazaki *et al.* (2001). The symptoms of each patient were recorded and the results demonstrated that, in all age groups, diagnosis by clinical symptoms was correct in 36.6% of cases when compared to viral culture confirmation, while 63.4% of these upper respiratory cases were due to another aetiology. Upon examining single symptoms and combinations of symptoms and relating these to a positive outcome of viral culture for influenza, the strongest relationship was observed with trios of symptoms, but was never greater than 61% accurate for the overall population and this only accounted for less than 6% of the total true influenza-positive patients.

This does not mean that influenza diagnosis by clinical symptoms is unwarranted. However, it does show that there is limited accuracy of a diagnosis for influenza based upon clinical symptomology alone and demonstrates that there are other factors that clinicians need to

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Table 1. Oral bacterial isolates tested against influenza neuraminidase substrates.

(The fluorescence observed upon incubation of oral bacterial isolates with selected influenza neuraminidase substrates.)

bacterial isolate	dilution	4MU-Neu5 Ac	4MU-4-OMe Neu5 Ac	4MU-4,7-diOMe Neu 5Ac
blank	none	reference	reference	reference
<i>Streptococcus mitis</i> isolate 1	1:2	408.2	1.0	-1.6
<i>Corynebacterium</i> sp. isolate 2	1:2	512.6	-4.0	0.0
<i>Streptococcus mitis</i> isolate 3	none	344.4	1.9	1.6
not available isolate 4	none	394.7	-0.8	-1.2
<i>Streptococcus mitis</i> isolate 5	none	606.8	2.9	-0.6
<i>Streptococcus mitis</i> isolate 6	1:4	437.0	-3.7	0.1
<i>Staphylococcus hominis</i> type 1 isolate 7	1:4	544.2	-5.2	0.5
<i>Streptococcus mitis</i> isolate 8	1:4	519.3	-5.3	-2.7
<i>Streptococcus mitis</i> isolate 9	1:2	469.9	-2.6	-0.9
<i>Streptococcus mitis</i> isolate 10	1:2	453.1	-2.9	-0.1
<i>Streptococcus mitis</i> and <i>Neisseria subflava</i> or <i>mucosa</i> isolate 11	none	143.3	-1.6	0.8

take into account when forming a clinical diagnosis for influenza. This is particularly important during the early and later periods of the influenza season, when many other infectious organisms have a higher prevalence than influenza and mimic symptoms similar to influenza. These other infectious organisms can include rhinovirus, respiratory syncytial virus, adenovirus, parainfluenza virus and even bacterial or mycoplasma infections.

3. THE NEED FOR RAPID AND ACCURATE DIAGNOSTICS

With the introduction of more effective therapy, namely neuraminidase inhibitors, for treating both influenza type A and B viruses which has fewer side-effects than previous antiviral therapy with tricyclic amines, the need for rapid and accurate diagnostics is more acute. Since the new anti-neuraminidase therapies must be administered within a window of no later than 36–48 h after the onset of symptoms to be effective, diagnosis must be definitive and accurate in order to determine proper therapy and rule out potential unnecessary use of antibiotics. The consequences of treatment based upon inaccurate diagnosis for influenza are significant and have resulted in a health care advisory statement from the Food and Drug Administration (FDA) regarding the usage of the new anti-neuraminidase antivirals. For instance, a presumptive diagnosis of influenza when it is indeed a bacterial infection can result in severe complications and, as occurred in a limited number of cases, even death. The consequences of presumptively diagnosing a bacterial infection when it is actually influenza may result in the incorrect prescription of unnecessary antibiotics. Incorrect diagnosis of upper respiratory infections has led to the overuse of antibiotics and is a growing problem with the emergence of drug-resistant bacteria. New and more rapid point-of-care tests for influenza have now become available. A critical criterion of any diagnostic test is specificity. A positive test result must be accurate since it would initiate the prescribing of antiviral medication, whereas a false positive would initiate antiviral treatment when ultimately antibiotics may be the correct treatment.

4. INFLUENZA DETECTION BY VIRAL CULTURE CONFIRMATION IS CURRENTLY THE GOLD STANDARD

The gold standard for influenza detection is currently viral culture with confirmation by monoclonal antibodies. Unfortunately this method of detection can take from 2 days to as long as 21 days for definitive culture results. Some viral culture laboratories are moving to testing by direct fluorescence antibody detection in order to reduce the turnaround time to less than 1 day. However, because of the trained technical expertise and facilities necessary for performing this method, this type of testing is still impossible for routine influenza diagnosis. Viral cultures have value in that they provide surveillance monitoring of the circulating influenza type and strain (viral surveillance), supply information necessary for determining the composition of the following season's influenza vaccine and provide stored isolates for future testing and study. However, these types of test are not an option for timely prescribing of the new influenza drugs. When one includes the high costs of viral culture, the labour-intensive requirements and the fact that these services are provided by few laboratories with a long turnaround-time, viral culture testing for influenza has had limited usage for incorporation into treatment for influenza.

5. MOST RAPID DIAGNOSTICS ARE HISTORICALLY BASED UPON ANTIGEN-ANTIBODY TECHNOLOGY

Historically, most rapid diagnostics have been based upon the specificity provided by antibody-antigen recognition technology. This has been the basis for numerous types of assays, such as enzyme-linked immunosorbent assays and rapid point-of-care diagnostic testing. They have served well for the detection of a target antigen that is stable and conserved. However, influenza is not only capable of point mutations, but also evades the host immune system by reassortment of its genome segments. This reassortment is the primary method by which new influenza pandemics arise and is the reason why a new influenza vaccine must be formulated each

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Figure 1. Amino acid conservation in the active site of influenza type A virus (compiled from Colman *et al.* 1983).

year in order to provide immunization against a potentially new strain. New strains that have been quite troublesome from a health care perspective, such as Hong Kong influenza (H5N1), have emerged and concerns have been aroused about the potential emergence of the next pandemic strain, such as that seen during 1918–1919 where 20 million people died due to the virulent ‘swine influenza’ circulating at that time. However, there are portions of the virus that have been shown to be highly conserved, even in spite of the antigenic shift and drift mutations that occur annually with influenza virus. These conserved regions are the targets for the pathoyme technology.

6. INFLUENZA NEURAMINIDASE TECHNOLOGY AND SPECIFICITY IMPLICATIONS

Influenza virus encodes for a neuraminidase enzyme that is essential for the supportive functions that contribute to viral infectivity and, ultimately, pathogenesis. The fact that this enzyme is also membrane bound and expressed on the surface of the virion makes it an interesting target for both therapeutic and diagnostic applications. The pathoyme technology is the exploitation of these subtle differences between seemingly similar enzymes for use in the rapid detection of pathogenic organisms.

It is known that specific amino acids in the binding site of influenza neuraminidase have been highly conserved over time (figure 1) and that this binding site plays a key role in the recognition and processing of substrates. An excerpt of the sequence of influenza neuraminidase from Coleman *et al.* (1983) showing conservation of specific residues in the active site of influenza virus sequenced over 27 years ago is given in figure 1. Most significant is the conservation of the amino acids that make up the binding site of this enzyme (highlighted in figure 1).

7. PATHOZYME SUBSTRATE MODIFICATION AND SPECIFICITY

Sialic acid or *N*-acetyl-neuraminic acid (figure 2) is the natural substrate for influenza virus neuraminidase. It is the terminal sugar of glycosylated proteins, provides the receptor for the haemagglutinin of influenza virus and is the molecule that is cleaved by influenza neuraminidase. This molecule served as the basis for the chemical modifications that ultimately led to the current substrate used in the ZstatFlu diagnostic test. During the development of the ZstatFlu test, it was found that multiple species of

Figure 2. Sialic acid structures.

organisms other than influenza also contain and encode for neuraminidase enzymes. These enzymes are found in bacteria, mammalian cells and even other respiratory viruses. A key aspect of a good diagnostic test is the ability to distinguish between potentially cross-reactive organisms and still provide the necessary recognition and detection capabilities without false-positive results from competing organisms. A major accomplishment of this technology was the selective differentiation of neuraminidase activity through the modification of this natural substrate (Liav *et al.* 1999). Numerous molecules were

Figure 3. ZstatFlu test chemistry.

synthesized and tested through a series of single and dual modifications at each of the hydroxyl residues in assays of neuraminidase activity of viral, bacterial and mammalian origin. This method determined that selected modifications in position 4 eliminated bacterial and mammalian sialidase recognition and that the implementation of a position 4,7-modified molecule eliminated the remaining non-influenza viral recognition (Liav *et al.* 1999). The position 7 modification appears to have provided the needed interspecies selection of viral neuraminidase activities for influenza types over that of parainfluenza and mumps viruses, which also have neuraminidase activity. This selective substrate molecule is the key component of the ZstatFlu test and serves as the recognition portion of the substrate.

8. BACTERIAL, MAMMALIAN AND OTHER NON-INFLUENZA VIRAL NEURAMINIDASES

The ZstatFlu synthetic substrate was developed with a position 4 and 7 modification that provides for exquisite specificity for influenza types and has been demonstrated to detect all strains and variants tested. In addition, this substrate was recognized by avian influenza virus and the recently isolated Hong Kong influenza isolates. ZstatFlu chemical processing occurs when the influenza neuraminidase enzyme recognizes the specific chromogenic substrate and cleaves the glycosidic bond between the reporter and recognition molecules. Dimerization takes place once the cleavage occurs, forming an insoluble blue indigo dye which is captured in a funnel filter device and read as a positive (blue) or negative (white) test for the presence of influenza (figure 3).

Analytical laboratory and clinical testing with the modified sialic acid substrates were conducted on actual specimens in order to examine their specificity for influenza neuraminidases. Groups of viral, bacterial and mammalian neuraminidase-producing organisms or purified neuraminidases were tested under standardized assay conditions for the detection of neuraminidase activity. The viral neuraminidase-producing organisms included the parainfluenza type 1–4 viruses, which contain a haemagglutinin–neuraminidase surface protein, and mumps virus, which also encodes for a neuraminidase. The bacterial organisms have been described in the literature, these being *Clostridium perfringens*, *Arthrobacter* and *Vibrio cholerae*. In addition, we isolated numerous oral bacteria that contained neuraminidase activity at levels much higher than that found in any influenza infection. These included *Streptococcus sanguis*, *Corynebacterium* and *Neisseria* (tables 1 and 2). The mammalian sialidase tested was purified from Chinese hamster ovary cells (figure 4). No significant activity was detected relative to that detected with influenza type A and B viruses in all of the testing conducted with viral, bacterial and mammalian sialidases.

9. ZSTATFLU TESTING PROCEDURE

The ZstatFlu test procedure has four simple steps (which are outlined in figure 5) and uses a patient-friendly and doctor-preferred throat swab as the specimen for influenza testing. This test is currently waived from the Clinical Laboratories Improvement Amendment (CLIA) in the USA, that means that it has been determined by the FDA to be simple and reproducible enough to exempt it from the requirements of CLIA 1988. Other

Table 2. Colour intensities in chromogenic neuraminidase substrate testing with human respiratory viruses.
(Selected examples from US Patent 5 719 020 issued on 17 February 1998.)

virus	titre	4CN-4OMe-Neu5 Ac	BI-4OMe-Neu5 Ac	BI-4,7diOMe-Neu5 Ac
human influenza type A virus	harvest 1:50	2 +	1 + / 2 +	2 +
human influenza type B virus	harvest 1:10	3 +	3 +	3 +
human parainfluenza type 1 virus	undiluted isolate no. 2	> 3 +	2 +	negative
human parainfluenza type 2 virus	undiluted day 2 harvest	> 3 +	1 + / 2 +	negative
human parainfluenza type 3 virus	undiluted isolate no. 1	2 +	indeterminate	negative
mumps virus	undiluted day 7 harvest	> 3 +	3 +	negative
respiratory syncytial virus	undiluted harvest	negative	negative	negative
adenovirus	undiluted harvest	negative	negative	negative

Figure 4. Activity of Chinese hamster ovary cell neuraminidase with position 4-modified and unmodified fluorescent substrates.

features of this test include specimen transport that has no refrigeration requirements and remains stable for 24 h, a test shelf life of 3 years and minimal user hands-on time (less than 2 min).

10. CHEMILUMINESCENCE FORMAT AND CLINICAL TESTING RESULTS

The same principle as that of pathozyme was applied to a chemiluminescent reporter group with an inherently higher sensitivity (figure 6). Previous use of chemiluminescence assays has been limited to research laboratories and has principally replaced radioactive isotopes for instrument-based assays. These uses of chemiluminescence have also moved to solution-based assays where expensive tabletop luminometers have provided more sensitive alternatives to fluorescence and ultraviolet-based instrument assays. ZymeTx has developed the ZstatFlu-II assay in collaboration with Polaroid Corporation.

Clinical testing with a pre-production prototype was conducted with samples of 300 nasal wash aspirates that were prospectively gathered from a hospital setting for determination of the feasibility of using this technology in human samples. The testing was performed during the 2000–2001 influenza season and the results were compared to the current gold standard viral culture confirmation and against another competitor's antigen-based test (Hamilton *et al.* 2001). The discordant samples are currently being analysed by the most sensitive method of a polymerase chain reaction (PCR). These results were analysed and compared to consensus results and are described in table 3. The specificity of this pre-production, instant film-based chemiluminescence prototype test is 96.4% and the sensitivity is 89.5%. Further optimizations are being developed in order to improve its sensitivity and ease of use. The test is performed with a minimum of hands-on time (*ca.* 2 min) and a total test time of *ca.* 20 min. The results are

Figure 5. ZstatFlu test procedure pictures.

available on instant film that can be placed in a patient's chart or saved on record.

11. THE NEXT-GENERATION RAPID DIAGNOSTIC PLATFORM—FIRST APPLICATION TO INFLUENZA

Based upon promising clinical prototype testing of this chemiluminescence technology, ZymeTx is refining the further development of this product into a commercializable assay for use as a point-of-care test for influenza prior to treatment. With modifications, this format can also be used in regional laboratories and hospital or acute care settings where high throughput is necessary for batching larger numbers of samples. Additional applications for other viral disease targets are already being examined as the development of the influenza application matures. Because of the high sensitivity of this platform format additional applications for testing for other viral diseases of humans and pathogenic organisms in veterinary medicine and those for bioterrorism agents such as anthrax and smallpox are being explored.

Table 3. Clinical trial results of a chemiluminescent-based test for the rapid detection of influenza.

(These are ZstatFlu chemiluminescence results compared to a reference viral culture and consensus positives. False positives and false negatives are being evaluated by PCR. The sensitivity was 89.5% and the specificity was 96.4%. $n = 300$.)

	positive reference	negative reference
positive	68	8
negative	8	216

12. WHAT RAPID AND ACCURATE DIAGNOSIS WILL DO FOR HEALTH CARE

With the recent introduction of new and more effective antiviral medications for the treatment and prophylaxis of influenza, rapid and accurate diagnosis of influenza is becoming more critical to proper therapy. Rapid point of care diagnostic tests aid in the correct diagnosis of

Figure 6. ZstatFlu colorimetric and chemiluminescent formats.

influenza and provide the means needed for distinguishing between bacterial and viral aetiology that will result in the proper administration of new anti-influenza medications. The more widespread use of rapid diagnostic tests for influenza should result in a reduction in unnecessary antibiotic prescriptions since a definitive diagnosis of influenza will alleviate the uncertainty of bacterial *versus* viral infections. This benefit alone will assist in reducing the proliferation of rapidly emerging antibiotic-resistant bacteria.

It is important that rapid diagnostics deliver the sensitivity and specificity for detecting low levels of virus in patients presenting with symptoms and provide the specificity needed for detecting rapidly mutating strains of influenza such as in a pandemic situation. The patho-enzyme technology has produced the first rapid test for influenza type A and B viruses and is the only one that is based on the activity of influenza neuraminidase for its detection. Historically, the active site of influenza neuraminidase has been well conserved, making it an ideal target for both diagnostic and therapeutic applications. Because of the parallel technology used for exploiting the neuraminidase in both the diagnostic and therapeutic arenas, it is possible that the ZstatFlu diagnostic may be a good predictor of the new antiviral medication's efficacy in patients that test positive for influenza by ZstatFlu.

ZymeTx is also developing an ultrasensitive second-generation platform technology based upon patho-enzyme by adapting this system for a chemiluminescent substrate reporter molecule in order to produce an image on high-speed Polaroid instant film (figure 6) with the convenience needed in a point-of-care setting. The result is a platform system that is much more sensitive than any

other point-of-care test and that can be adapted for multiple other disease detection targets of viral and bacterial origin.

Because influenza poses both an annual medical and economic cost to patients and health care systems, a complete and comprehensive disease management programme can reduce the toll it has on society. Proper disease management can reduce the incidence of the disease through vaccination, while disease surveillance can alert the local community and regions at large to proper preventative measures. Accurate and rapid diagnostics can reduce unnecessary testing and point to proper therapy, while effective therapeutics can reduce morbidity and mortality.

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