Comparison of the performance of the rapid antigen detection *actim* Influenza A&B test and RT-PCR in different respiratory specimens

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Nowadays, influenza antigen detection test kits are used most frequently to detect influenza A or B virus to establish the diagnosis of influenza rapidly and initiate appropriate therapy. This study was conducted to evaluate the performance of the *actim* Influenza A&B test (Medix Biochemica). Overall, 473 respiratory specimens were analysed in the *actim* Influenza A&B test and the results were compared with those from an RT-PCR assay; 461 of these samples originated from paediatric patients aged 7 weeks to 6.5 years either with influenza-related symptoms or from the intensive care unit, and 12 samples originated from adults with underlying lung or haematological diseases. Diagnosis of influenza A or B virus could be established using the *actim* Influenza A&B test (9/473 samples for influenza A virus and 6/473 for influenza B virus). RT-PCR revealed 23 patients with influenza virus (13/473 for influenza A virus and 10/473 for influenza B virus). The sensitivity and specificity of the *actim* Influenza A&B test were 65 and 100 % compared with the RT-PCR assay. However, 32 external quality assessment samples containing seven different strains of influenza A subtypes H1N1 and H3N2 and the avian H5N1 were detected correctly by the *actim* Influenza A&B test. No cross-reactivity to a range of bacterial, fungal and other viral pathogens was observed. In conclusion, the *actim* Influenza A&B test is reliable for positive results due to its high specificity. Nevertheless, negative results from this test need to be confirmed by a more sensitive assay because of the low sensitivity observed with diagnostic samples.

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INTRODUCTION

Influenza viruses cause significant morbidity and mortality in both children and adults (Heikkinen, 2006; van der Wouden *et al.*, 2006) during local outbreaks or epidemics. Outbreaks and epidemics may appear at any time but are usually concentrated in the winter months in countries with temperate climates (Brammer, 2002).

The rapid detection of influenza viruses is important to apply preventative strategies, initiate antiviral therapy, detect avian influenza viruses and exclude influenza-like bacterial and viral diseases (e.g. anthrax, smallpox). Moreover, it is important to determine not only whether influenza virus is present, but also which type of influenza virus is present (Noyola *et al.*, 2000a; Chan *et al.*, 2002; Effler *et al.*, 2002; Poehling *et al.*, 2002).

Although the gold standard reference method for the diagnosis of influenza is isolation of the virus, commercially available rapid antigen detection tests have the advantage of providing results much more quickly, i.e. within approximately 30 min. These tests, also referred to as near-patient or point-of-care tests, are mostly immunoassays that detect influenza virus antigen, although one test detects viral neuraminidase activity (Noyola *et al.*, 2000b; Uyeki, 2003).

They detect and distinguish between influenza A and B viruses, detect but do not distinguish between influenza A and B viruses or detect influenza A virus only. The tests vary in complexity, type of respiratory specimen acceptable for testing and the time needed to obtain results (Seno *et al.*, 1990; Rodriguez *et al.*, 2002; Uyeki, 2003; Hurt *et al.*, 2007).

The *actim* Influenza A&B test (Medix Biochemica) is a point-of-care test utilizing influenza-specific mAbs for rapid diagnosis and differentiation of influenza A and B viruses. The aim of this study was to conduct a prospective evaluation of this test for qualitative, simultaneous and differential detection of influenza A and B antigens in different respiratory specimens. For this purpose, the *actim* Influenza A&B test was compared with an established in-house RT-PCR assay (Schweiger *et al.*, 2000) for sensitivity, specificity and the time needed to obtain results.

METHODS

Viruses and other micro-organisms tested. The performance of the *actim* Influenza A&B test was assessed for human influenza A virus (H1N1, H3N2 and H5N1) and influenza B virus samples ($n=32$) obtained from the quality assurance institution INSTAND

(Düsseldorf, Germany) (Table 1). A panel of other micro-organisms, including 11 bacteria (*Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli*, *Haemophilus* sp., *Klebsiella pneumoniae*, *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pneumoniae*), four *Candida* spp. (*C. albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis*) and ten viruses (adenovirus, human bocavirus, human cytomegalovirus, enterovirus, Epstein–Barr virus, herpes simplex virus, human metapneumovirus, respiratory syncytial virus, rhinovirus and varicella-zoster virus) were used to test for cross-reactivity in the *actim* Influenza A&B test. Bacteria and yeasts were cultured and resuspended in Dulbecco's PBS at a concentration of 10^7 – 10^8 c.f.u. ml⁻¹ or, in the case of *M. pneumoniae*, at a concentration of 10^7 – 10^8 colour-changing units ml⁻¹. For testing cross-reactivity to viruses other than influenza, patient specimens positive for the respective viruses were used. The *actim* Influenza A&B test was carried out with the investigator blinded to the nature of the pathogen.

Patients and clinical specimens. Paediatric patients ($n=461$) who presented (if they presented with symptoms) with fever, cough, coryza and/or myalgia/headache were eligible. Paediatric patients with immunosuppression secondary to drugs and human immunodeficiency virus infection were excluded from enrolment in the study. Nasopharyngeal aspirates and swabs ($n=367$), bronchoalveolar lavages ($n=11$) and the remainder as tracheal secretions, submitted for virus diagnostics between November 2004 and April 2005, October 2005 and April 2006, September 2006 and January 2007, and October 2007 and April 2008, were used for this study. The age of the patients ranged from 7 weeks to 6.5 years with the majority (63%) being between 0.5 and 1.5 years. Secretions were aspirated into a mucus trap by inserting a catheter into the nasopharynx of each nostril in turn. Specimens were collected at the time of admission and held at 4–8 °C (for periods of 1–6 h, depending on time of admission) until they were transported to the laboratory. Specimens collected more than 24 h prior to arrival in the laboratory were excluded from the study. Transport to the laboratory was carried out at ambient temperature; the maximal duration of exposure to

ambient temperature was less than 3 h. Aliquots of the respiratory specimens were taken for RT-PCR assay of the respiratory viruses. A second aliquot was used for analysis in the *actim* Influenza A&B test and a third aliquot was frozen at –70 °C for resolution of discrepant results.

Additionally, 12 respiratory specimens (stored at –20 °C) collected from adults with underlying lung or haematological diseases who had tested positive for influenza virus were included in the panel of 473 samples.

Ethical approval was obtained from the institutional review boards for this study.

***actim* Influenza A&B test.** The *actim* Influenza A&B test was carried out according to the manufacturer's instructions. The test was performed with the investigator blinded to the results of the RT-PCR. For liquid specimens, 300 µl sample was dispensed into the test tube together with three drops of extraction buffer, and nasopharyngeal swabs were inserted into test tubes containing six drops of NaCl buffer and three drops of extraction buffer, mixed and incubated for 5 min at room temperature to allow virus particles in the specimens to be disrupted, exposing internal viral nucleoproteins. The appearance of a blue–black control line on the *actim* test strip confirmed that the test had been performed properly and that the results were therefore valid.

BinaxNOW Influenza A & B test. The BinaxNOW Influenza A & B test (Binax) is an immunochromatographic membrane assay that uses highly sensitive mAbs to detect influenza type A and B nucleoprotein antigens in nasopharyngeal specimens. The test was carried out according to the manufacturer's instructions.

RNA extraction and cDNA synthesis. Total viral RNA was extracted using a commercial kit (QIAamp Viral RNA kit; Qiagen) according to the manufacturer's instructions. Viral RNA was extracted from 140 µl of each sample. The RNA was eluted from columns with 50 µl RNase-free water (Qiagen) and used immediately in the following experiments or stored at –80 °C. cDNA synthesis

Table 1. RT-PCR and *actim* Influenza A&B test results for the influenza A and B virus quality assessment strains

NA, Not applicable.

Controls/samples	Influenza A virus		Influenza B virus		Virus designation
	RT-PCR	<i>actim</i>	RT-PCR	<i>actim</i>	
Control*	NA	+	NA	–	A/Taiwan/1/1986 (H1N1)
Control*	NA	–	NA	+	B/Qingdao/102/1991
20033, 20039, 20043, 20052	+	+	–	–	A/New Caledonia/20/1999 (H1N1)
20034, 20041, 20044	–	–	+	+	B/Hong Kong/330/2001
20036	+	+	–	–	A/Moscow/10/99 (H3N2)
20038	+	+	–	–	A/Wyoming/3/2003 (H3N2)
20040, 20042, 20053, 20061	–	–	+	+	B/Shanghai/361/2002
20051, 20054	+	+	–	–	A/California/7/04 (H3N2)
20055, 20057, 20060, 20062	+	+	–	–	A/Duck/Vietnam/TG24-01/2005 (H5N1)†
20066, 20070, 20073	+	+	–	–	A/Wisconsin/67/2005 (H3N2)
20064, 20072, 20074	–	–	+	+	B/Malaysia/2506/2004
20069, 20063, 20071, 20075	+	+	–	–	A/Whooper swan/Germany/R65-2/2006 (H5N1)†
20067, 20068, 20076	+	+	–	–	A/Fukushima/141/2006 (H1N1)

*Controls supplied with the *actim* Influenza A&B test kit.

†H5N1 avian influenza virus strain.

was carried out at 37 °C for 1 h using 10 µl eluted RNA, 100 U murine leukaemia virus reverse transcriptase (Gibco/Invitrogen), 10 mM dithiothreitol, 20 U RNasin (Promega) and 0.25 µM random hexamer primers (Gibco/Invitrogen).

FastCycler PCR. PCR was carried out in a 20 µl volume containing 2.5 µl cDNA, 2 × master mix (FastCycler 9800, 2 × GeneAmp Fast PCR Master Mix; Applied Biosystems) and 0.25 µM each primer. Primer sets specific for influenza A and B virus (custom synthesized by MWG-Biotech) were used to detect and differentiate the two influenza viruses as described by Schweiger *et al.* (2000). PCR conditions were initial denaturation at 95 °C for 10 s, 40 cycles of 95 °C for 2 s and annealing and extension at 67 °C for 15 s, and final extension at 72 °C for 7 min. Amplicons were detected by gel electrophoresis.

Statistical analysis. Specimens positive for influenza A or B virus in the RT-PCR were regarded as true positives. The sensitivity, specificity, and positive (PPV) and negative (NPV) predictive values of the *actim* Influenza A&B test results compared with those of the RT-PCR assay were calculated using two-by-two contingency tables.

RESULTS

Cross-reactivity of the *actim* Influenza A&B test

To determine the cross-reactivity of the antigen detection kit *actim* Influenza A&B, commensal and pathogenic micro-organisms (11 bacteria, 10 viruses and four *Candida* species) that may be present in the nasal cavity or nasopharynx were tested. All of the samples were negative by the *actim* Influenza A&B test.

Analytical sensitivity of the *actim* Influenza A&B test in quality assessment influenza samples

A total of 32 quality assessment influenza A and B samples of different designation (INSTAND), including avian influenza virus, were evaluated by the *actim* Influenza A&B test and RT-PCR. All of these samples tested positive by both methods (Table 1), revealing a sensitivity and specificity of 100 % for the *actim* Influenza A&B test.

Comparison of the *actim* Influenza A&B test and RT-PCR assay

Among the 473 specimens, nine (1.9%) were antigen-positive for influenza A virus in the *actim* Influenza A&B test and 13 (2.8%) were positive in the RT-PCR assay. Six specimens (1.3%) were positive for influenza B virus by the *actim* Influenza A&B test and ten (2.1%) in the RT-PCR assay (Table 2). All influenza samples that were detected by the *actim* Influenza A&B test were also positive in the RT-PCR assay.

The results for the first batch of PCR amplicons ($n=9$) were confirmed by sequencing, whilst the other results were confirmed by haemagglutinin and neuraminidase gene-specific PCR or by the use of other detection methods (virus culture, direct immunofluorescence, rapid antigen detection

Table 2. Comparison of the *actim* Influenza A&B test with RT-PCR in detection of influenza A and B viruses in respiratory specimens ($n=473$)

	RT-PCR	
	Positive	Negative
Influenza A		
<i>actim</i> -positive	9	0
<i>actim</i> -negative	4	460
Total	13	460
Influenza B		
<i>actim</i> -positive	6	0
<i>actim</i> -negative	4	463
Total	10	463

test or RT-PCR). Of the influenza A viruses detected, 11 were H3N2 and two were H1N1 (data not shown).

The overall sensitivity of the *actim* Influenza A&B test compared with the RT-PCR assay was 65.2 % (69.2 % for influenza A virus and 60 % for influenza B virus) with a specificity of 100 %. The *actim* Influenza A&B test revealed a PPV of 100 % (95 % CI 78.2–100 %) for both influenza virus types, whereas the NPV was approximately 98 % (95 % CI 96.5–99.2 %).

From the panel of 473 samples, 149 paediatric respiratory samples were also analysed using a BinaxNOW Influenza A & B kit. The sensitivity of the BinaxNOW Influenza A & B kit [4/7 RT-PCR-positive samples (57 %) for influenza A virus and 3/7 RT-PCR-positive samples (43 %) for influenza B virus] was lower than the sensitivity of the *actim* Influenza A&B test (71 % for influenza A virus and 57 % for influenza B virus when compared for the 149 samples). The BinaxNOW Influenza A & B test kit revealed a specificity of 100 % for both influenza A and B viruses compared with the RT-PCR assay. When compared with the *actim* Influenza A&B test as the reference, the BinaxNOW Influenza A & B test yielded a sensitivity of 78 %. RT-PCR-positive samples were confirmed as described above.

Clinical symptoms of influenza A and B virus-positive cases

The majority of the influenza virus-positive samples were detected between February and March 2005 and between January and April 2008. Among the patients with influenza, the youngest was 7 weeks old and the eldest 61 years old (with influenza A and B virus, respectively; Table 3). The clinical presentation of the patients with influenza virus detection as well as the type of specimen in which influenza virus was detected are shown in Table 3. No dual infections with influenza A and B viruses were detected.

Table 3. Characteristics of influenza A and B virus-positive patients and samples

Age	Specimen*	actim	RT-PCR	Intensive care	Clinical presentation†	Underlying disease
6 years	TS	A	A	No	Febrile URI	Acute lymphocytic leukaemia
1 month	NPA	Negative	A	Yes	Febrile URI	Pre-term, gastrointestinal dysfunction
9 month	NPA	A	A	No	Febrile URI	Pre-term, invagination
1 month	NPS	Negative	A	Yes	Bronchitis	Pre-term, heart failure
6.5 years	TS	B	B	No	Febrile URI	None
9 month	NPA	A	A	Yes	Bronchitis	Pre-term, bowel inflammation and bleeding
6.5 years	NPA	B	B	No	Febrile URI	Unknown
3.5 years	NPA	B	B	No	Febrile URI	None
2 years	TS	B	B	No	Bronchitis	None
58 years	BAL	Negative	A	Yes	Respiratory failure	Pulmonary fibrosis
51 years	BAL	Negative	A	No	Fever, chills, diarrhoea, syncope	Lung transplantation
51 years	BAL	Negative	B	No	Shortness of breath, cough	Chronic obstructive pulmonary disease
3 years	TS	Negative	B	Yes	Asymptomatic	None
4 years	NPS	Negative	B	No	Fever, cough	Acute lymphocytic leukaemia
38 years	BAL	A	A	No	Unknown	Lung transplantation
40 years	BAL	A	A	No	Fever, cough, chills	Lung transplantation
17 years	BAL	A	A	No	Deterioration of lung function	Lung transplantation
26 years	BAL	B	B	No	Pneumonia	Wegener's granulomatosis
35 years	BAL	A	A	Yes	Fever, sepsis	Cystic fibrosis
15 years	BAL	A	A	No	Deterioration of lung function	Lung transplantation
22 years	BAL	A	A	No	Unknown	Lung transplantation
28 years	BAL	B	B	No	Unknown	Lung transplantation
61 years	NPA	Negative	B	No	Bronchitis	Stem-cell transplantation

*BAL, Bronchoalveolar lavage; NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; TS, tracheal secretion.

†URI, Upper respiratory tract infection.

DISCUSSION

Although the gold standard for detecting influenza virus infection is virus isolation in cell culture, the result is usually obtained too late to initiate antiviral treatment in time and to take measures to prevent transmission to other patients. Therefore, tests that yield results in a time of minutes to hours can improve the clinical management of suspected influenza cases among outpatients, emergency room patients and inpatients. Currently available tests that produce results in this time frame include immunofluorescence (direct and indirect), RT-PCR, real-time PCR and rapid antigen detection tests. Generally, positive results by the rapid antigen detection methods correlate well with actual infection as determined by shell vial culture (Dunn *et al.*, 2003). The sensitivities of the different rapid assays for influenza virus available at present vary from 40 to 96 % among different studies, depending on the reference methods used (Noyola *et al.*, 2000b; Herrmann *et al.*, 2001; Chan *et al.*, 2002; Effler *et al.*, 2002; Poehling *et al.*, 2002; Dunn *et al.*, 2003; Hurt *et al.*, 2007).

For example, Herrmann *et al.* (2001) compared an optical immunoassay for rapid detection of influenza A and B

virus with RT-PCR and immunofluorescence on nasopharyngeal aspirates and swabs. The sensitivity and specificity of the optical immunoassay in nasopharyngeal aspirates were 40.4 and 94.3 %, respectively, compared with the RT-PCR. In general, a greater yield of positive samples can be obtained with PCR techniques than with rapid antigen detection tests. This has been observed particularly when the sample is taken 3–4 days after the onset of symptoms, when the viral load drops sharply, as does the infecting capacity (Cherian *et al.*, 1994).

The Directigen FluA+B antigen test (BD Diagnostic Systems) was evaluated by Chan *et al.* (2002) in a study design similar to our present work. Most of the patients studied were children. Compared with an RT-PCR assay, the sensitivity, specificity, and PPV and NPV of the Directigen FluA+B test for influenza virus A were 96, 99.6, 96 and 99.6 %, and for influenza virus B were 87.5, 96.8, 80 and 98 %, respectively. In a French study of the QuickVue Influenza antigen test (Quidel), these values were 87.5, 100, 100 and 89.5 %, respectively (Arsene *et al.*, 2004). These results seem to suggest a better performance of the Directigen FluA+B and QuickVue Influenza

antigen tests than of the *actim* Influenza A&B test in the present study. However, Ruest *et al.* (2003) compared a number of antigen tests with an RT-PCR assay with different results. A poor specificity (35–58 %) and a poor PPV (41–60 %) were reported for the compared antigen tests, with the Directigen FluA + B test having a higher sensitivity than the QuickVue Influenza antigen test but being associated with a larger number of invalid results. For comparison, the *actim* Influenza A&B test had a sensitivity, specificity, and PPV and NPV of 65, 100, 100 and 98 %, respectively, and no invalid results were observed in the present study. Cross-reactivity of the *actim* Influenza A&B test with other bacteria, fungi and viruses that may be encountered in the respiratory tract was not observed. Moreover, false-positive results were not obtained using the *actim* Influenza A&B test in the panel of 473 patient specimens tested (Table 2). Therefore, the specificity and robustness of the *actim* Influenza A&B test are probably superior to those of the above-described rapid antigen detection kits.

As the sensitivity of the *actim* Influenza A&B assay in diagnostic samples seemed to be lower than that of the above-mentioned antigen detection kits, we analysed a subgroup of 149 respiratory samples in a direct comparison of the BinaxNow Influenza A & B and *actim* Influenza A&B tests. This revealed that the BinaxNow Influenza A & B test did not yield better results than the *actim* Influenza A&B test. Moreover, the *actim* Influenza A&B assay achieved a sensitivity of 100 % in comparison with RT-PCR and the expected results in a panel of quality assessment samples (including influenza A H1N1, H3N2 and H5N1) obtained from INSTAND (Table 1).

In summary, the studies cited above, together with our results, suggest that the reported sensitivities of influenza rapid antigen tests may vary highly in relation to the panel of diagnostic specimens tested. If a panel contains diagnostic specimens with low virus concentrations (perhaps because these were taken more than 2 days after the onset of symptoms), the sensitivities of antigen assays drop in comparison with that of RT-PCR, which can still detect influenza RNA due to its low detection limit. Thus RT-PCR offers an alternative method of diagnosing influenza virus infections. It has high sensitivity and specificity (Herrmann *et al.*, 2001), but requires a high level of skill and a complex laboratory infrastructure, and takes several hours to perform, as was also shown in our study (150–170 min). Therefore, RT-PCR does not fill the niche of tests that are rapid and easy to perform with a low level of expertise.

The low influenza activity in the Magdeburg area during the respective seasons between 2004 and 2008 was disadvantageous for this study, resulting in a limited number of positive specimens being obtained. Thus studying a larger number of positive specimens would make evaluation and determination of sensitivity and specificity more reliable. However, during times of low

influenza incidence, the NPV of a rapid antigen detection test is higher and the PPV is lower than in times of high influenza activity (WHO, 2005). Therefore, the high PPV of the *actim* assay observed in this study indicates that positive results do not need to be confirmed by RT-PCR and should be reported to the treating physician immediately in times of both low and high influenza incidence. However, negative *actim* Influenza A&B results, as well as negative results of other rapid antigen assays, should be confirmed by RT-PCR. This is even more important during the influenza season, because of decreasing NPV with increasing influenza virus incidence.

In conclusion, the *actim* Influenza A&B test is a rapid *in vitro* assay that detects and differentiates influenza A and B virus antigens (nucleoprotein) extracted from respiratory specimens. Because the *actim* Influenza A&B test is easy to perform and has a specificity and PPV of 100 % for each virus, this test is appropriate for acute cases as a first-line emergency diagnostic method. A negative result warrants confirmation with a more sensitive method.

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