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ARTICLE

# Evaluation of the ESPLINE® Influenza A & B-N assay for the detection of influenza A and B in nasopharyngeal aspirates

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**Abstract** Several direct antigen tests for the detection of influenza often lack sensitivity compared to immunofluorescence (IF) on the specimens and viral culture (VC). We evaluated the performance of a rapid test, the ESPLINE® Influenza A & B-N assay. A total of 302 respiratory specimens were collected at the University Hospital of Antwerp. A first group of 60 samples taken during the H1N1 outbreak (2009–2010) and a second group of 242 samples stored during the seasonal influenza epidemics (2000–2009) were analyzed with the ESPLINE® test. A subset of samples were also evaluated with the BinaxNOW Influenza and the Clearview Exact Influenza. The results were compared to IF on the specimens, VC with IF, and the combination of both, which was considered as the gold standard. The ESPLINE® test's overall sensitivity and specificity were 91% and 97%, during the H1N1 season 80% and 93%, and for the detection of seasonal influenza 93% and 97%, respectively. In comparison to the BinaxNOW Influenza and the Clearview Exact Influenza, all tests demonstrated a similar specificity of 92.0–100% but a significantly different sensitivity of 44.4–86.0%, with the ESPLINE® test being significantly more sensitive. Due to its very good performance and simplicity, the ESPLINE® test facilitates urgent testing. The test seems less sensitive to detect H1N1 compared to seasonal influenza, although the difference is borderline not significant ($p$=0.067).

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## Introduction

Influenza viruses (Orthomyxoviridae) cause seasonal epidemics or even pandemics, which are associated with a high morbidity and mortality, including flu symptoms, such as high fever, headache, cough, myalgia, persistent fatigue, and exhaustion. The clinical diagnosis is often difficult because of other circulating respiratory viruses in periods of epidemic prevalence. Currently, it has become a challenge to develop strategies to limit the spread of the virus to high-risk individuals by combining rapid diagnosis with appropriate antiviral therapy and infection control measures, both improving the patients' clinical outcome and reducing hospital costs [4, 9, 10]. Furthermore, the laboratory diagnosis permits the antigenic surveillance of circulating influenza strains, which is necessary for present vaccine efficacy evaluations and the creation of future effective vaccine formulations.

For the detection of respiratory viruses by conventional culture techniques, although often still considered as the gold standard, the results are frequently available too late to have an impact on patient management [1]. Polymerase chain reaction (PCR)-based tests have been proven to be more sensitive than culture-based techniques and are gradually replacing culture as the gold standard, but these tests are still very expensive and not available in every routine clinical laboratory [5]. The use of direct specimen testing is recommended as an adjunct to culture isolation for the identification of the influenza virus, so that the unnecessary use of antimicrobial agents is minimized. There are several rapid tests available to detect influenza

A and B in just 15 to 30 min which have an excellent specificity but often lack sensitivity. This can be explained by the differences in specimen type, influenza type or subtype circulating at that moment, patient age, and the gold standard used [4].

The goal of this study was to evaluate the performance of a new, not yet CE labeled, rapid immunochromatographic test, the ESPLINE® Influenza A & B-N (Fujirebio, Tokyo, Japan). We also compared the performance of the ESPLINE® Influenza A & B-N test to the BinaxNOW Influenza and the Clearview Exact Influenza (Inverness Medical) in order to find a good alternative for viral culture and immunofluorescence (IF) to detect influenza outside normal working hours.

## Materials and methods

### Clinical specimens

A total of 302 nasopharyngeal aspirates (NPA) were included from patients with flu-like symptoms, either positive or negative for influenza, at the University Hospital of Antwerp. A first group of 60 samples taken during the H1N1 outbreak between October 2009 and January 2010 were consecutively stored at −80°C and retrospectively tested in February 2010 with the rapid antigen test ESPLINE® Influenza A & B-N (Fujirebio, Tokyo, Japan). A second group of 242 samples stored during the seasonal influenza epidemics between 2000 and 2009 at −80°C were also retrospectively analyzed. When the samples were too viscous, they were diluted with a minimum of Dulbecco's phosphate buffered saline (PBS; Gibco®, Invitrogen).

Ninety specimens of these NPA were used to compare the three different antigen tests: 45 samples taken from the first group and 45 samples from the second group were analyzed in parallel using the three influenza assays. The results were compared to the gold standard.

### New rapid influenza test

Each ESPLINE® Influenza A & B-N kit (Fujirebio, Tokyo, Japan) consisted of ten enzyme immunoassay-based immunochromatographic tests. The collected specimens had to be mixed with a swab in the extraction solution of a squeeze tube and an applicator tip was inserted. Two drops of sample solution were dropped at the sample window and were mixed with the developing solution. At that moment, influenza A antigen and/or influenza B antigen, if present, reacted with alkaline phosphatase (ALP)-labeled monoclonal influenza A and B antibodies to form complexes. These complexes and/or free ALP-labeled antibodies flew through the device by the developing solution and were bound to the corresponding antibodies at the judgement and reference part to form three-part sandwich complexes. The testing batch did not include positive or negative controls.

### Reference methods

The NPA were transported within 2 h to the virology laboratory, where they were processed immediately for routine viral diagnosis using an indirect IF assay with monoclonal antibodies on the specimen and/or viral culture (VC), depending on the application form. If available, an aliquot of the sample was frozen at −80°C.

### Immunofluorescence on the specimen (IF)

To carry out IF, a part of the sample was suspended in PBS and centrifuged at 800 rpm for 5 min. After vortexing, the resulting cell pellet was spotted onto a multispot slide. These spots were dried, fixed with cold acetone, covered by a drop of influenza A and B-specific monoclonal antibodies (Argene), incubated in a $CO_2$ incubator (37°C) for 15 min, washed with PBS, and dried. Then, conjugate was added and, after an additional incubation and washing step, the slides were screened with a fluorescence microscope after mounting and covering the spots with a coverslip.

### Viral cell culture (VC)

Viral cell culture for influenza detection was performed by using IF on inoculated, centrifuged shell vials with Madin–Darby canine kidney (MDCK) cells. First of all, a part of the sample was diluted in a shell vial with glass beads using Minimal Essential Medium (MEM), followed by vortexing and centrifuging 30 min at 3,000 rpm. Then, five drops of the resulting supernatant were added to an MDCK shell vial, which was centrifuged and incubated at 32–34°C. After 2 days of incubation, the supernatant was removed from the vials, the cells were washed with PBS and fixed with methanol, followed by a PBS rinse. Infection was confirmed by staining using the same monoclonal antibodies and conjugate as described above. After incubation, 1 ml of PBS was added and the inoculated slide was removed from the vial onto a substrate glass in order to screen for influenza infection with a fluorescence microscope.

### Gold standard

The combination of IF on the specimen and/or VC was considered as the gold standard, which means that if one or both methods were positive, the result was reported to be positive.

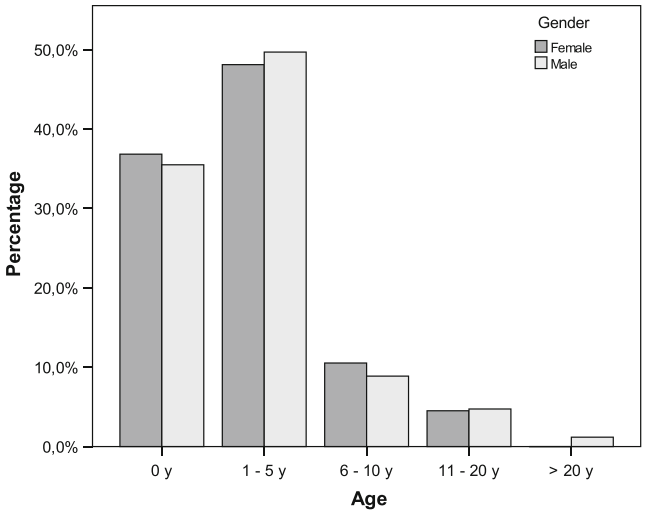


**Fig. 1** Overall age and gender distribution

### Other rapid influenza antigen tests

Two comparator tests were used to evaluate the performance of the ESPLINE® test against other commercially available rapid tests: the BinaxNOW Influenza and the Clearview Exact Influenza. These are also two immunochromatographic assays with a turnaround time of 15 min, but have another method of sample application.

### Statistical analysis

The ESPLINE® test was evaluated by calculating the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using the SPSS analytical software. The results of the ESPLINE® test were compared with the gold standard as described above and evaluated by performing a Pearson Chi-square test. A $p$-value$<0.05$ was considered as statistically significant.

## Results

This retrospective, blind study was conducted on 302 specimens from 133 female and 169 male patients with a mean age of 2.8 years. Figure 1 shows the age and gender distribution for the overall population. Out of these 302 collected samples, 285 results were available for IF on the specimen and 298 for VC. This difference in number can be explained by the lack of volume in some samples after performing the ESPLINE® test, so the second reference method that was missing (IF or VC) could not be accomplished. Concerning the result for this study, a

**Table 1** Performance of the ESPLINE® Influenza A & B-N test for the two groups of samples compared to the two reference methods and the gold standard

| Winter period | | No. | | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|---|
| | | Negative | Positive | | | | |
| 2000–2009 | | | | | | | |
| VC | Negative | 118 | 57 | 93.7 | 67.4 | 50.9 | 96.7 |
| | Positive | 4 | 59 | | | | |
| IF | Negative | 118 | 5 | 95.7 | 95.9 | 95.7 | 95.9 |
| | Positive | 5 | 112 | | | | |
| VC and/or IF | Negative | 112 | 3 | 92.7 | 97.4 | 97.4 | 92.6 |
| | Positive | 9 | 138 | | | | |
| 2010 | | | | | | | |
| VC | Negative | 39 | 3 | 81.3 | 88.6 | 72.2 | 92.9 |
| | Positive | 3 | 13 | | | | |
| IF | Negative | 28 | 4 | 84.6 | 87.5 | 73.3 | 93.3 |
| | Positive | 2 | 11 | | | | |
| VC and/or IF | Negative | 27 | 2 | 80.0 | 93.1 | 88.9 | 87.1 |
| | Positive | 4 | 16 | | | | |
| 2000–2010 (total) | | | | | | | |
| VC | Negative | 157 | 62 | 91.1 | 71.7 | 53.7 | 95.7 |
| | Positive | 7 | 72 | | | | |
| IF | Negative | 146 | 9 | 94.6 | 94.2 | 93.2 | 95.4 |
| | Positive | 7 | 123 | | | | |
| VC and/or IF | Negative | 139 | 5 | 90.9 | 96.5 | 96.3 | 91.4 |
| | Positive | 13 | 130 | | | | |

**Table 2** Difference in sensitivity and specificity between the two groups of specimens

| VC and IF | 2009–2010 | 2000–2009 | *p*-value |
|---|---|---|---|
| Sensitivity | 16/20 (80.0%) | 114/123 (92.7%) | 0.067 |
| Specificity | 27/29 (93.1%) | 112/115 (97.4%) | 0.260 |

**Table 4** Difference in the overall sensitivity (*p*-value) between the three tests

| Difference in sensitivity (*p*-value) | BinaxNOW | Clearview Exact |
|---|---|---|
| ESPLINE | 0.041 | <0.001 |
| BinaxNOW | – | 0.040 |

specimen was found to be positive if the singular method was positive; if negative, no conclusion could be made regarding the gold standard. The specimens of the 2009–2010 group were defined as samples of the H1N1 period due to the lack of seasonal H3N2 activity in this time window (http://www.iph.fgov.be).

Twenty samples tested with the ESPLINE® test were influenza B-positive (20/135 positive ESPLINE® tests). Eighteen of these were positive with the gold standard. As we could not tell the difference between influenza A and B with VC or IF, there was no possibility to check the performance results for influenza A and B separately. However, this was not the main purpose of our study of "finding a good alternative for viral culture and IF to detect influenza outside normal working hours."

A total of 135/302 (45%) tests were influenza-positive compared to the gold standard, resulting in an overall sensitivity and specificity of 91% and 97%, respectively. During the H1N1 season, the sensitivity and specificity were 80% and 93%, respectively; if compared to culture or IF alone, the results were 81% and 89% and 85% and 88% respectively. For the detection of seasonal influenza, the sensitivity and specificity were 93% and 97%, respectively; compared to culture or IF alone, the results were 94% and 67% and 96% and 96%, respectively. The results described above are shown in Table 1. The difference in sensitivity between the two groups, compared to the gold standard, is borderline not significant ($p=0.067$) (Table 2).

### Evaluation in comparison to the BinaxNOW Influenza and the Clearview Exact Influenza assays

The three antigen assays have a similar specificity ($p=0.39$) but a significantly different sensitivity. A total of 43/90 (48%) samples were found to be positive with the gold standard. The ESPLINE® test showed an overall sensitivity and specificity of 86% and 95.7%, the BinaxNOW Influenza 67.4% and 95.7%, and the Clearview Exact Influenza 44.4% and 100%, respectively. The performance results for the two groups of specimens are presented in Tables 3 and 4.

## Discussion

Our results show that the ESPLINE® test seems to be less sensitive to detect H1N1 compared to seasonal influenza. The obtained sensitivity for the NPA in the seasonal influenza epidemic group of 92.7% is comparable with the results of previous studies, such as Mitamura et al. with 93% compared to cell culture and Hara et al. with 94.5%, even though Hurt et al. published a sensitivity of 67% for influenza A detection with VC/IF as the reference method [3, 4, 8]. Yang et al., Cheng et al., and Lévêque et al. used the same direct antigen test in comparison to molecular methods and showed, respectively, a sensitivity of 64% (64/100) and

**Table 3** Performance of the three direct antigen tests for the two groups of samples

| Winter period | No. | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|
| ESPLINE | | | | | |
| 2010 | 45 | 80.0 | 92.0 | 88.9 | 85.2 |
| 2007–2009 | 45 | 91.3 | 100.0 | 100.0 | 91.7 |
| 2007–2010 (total) | 90 | 86.0 | 95.7 | 94.9 | 88.2 |
| BinaxNOW | | | | | |
| 2010 | 45 | 60.0 | 96.0 | 92.3 | 75.0 |
| 2007–2009 | 45 | 73.9 | 95.5 | 94.4 | 77.8 |
| 2007–2010 (total) | 90 | 67.4 | 95.7 | 93.5 | 76.3 |
| Clearview EXACT | | | | | |
| 2010 | 45 | 30.0 | 100.0 | 100.0 | 64.1 |
| 2007–2009 | 34 | 62.5 | 100.0 | 100.0 | 75.0 |
| 2007–2010 (total) | 79 | 44.4 | 100.0 | 100.0 | 68.3 |

55.9% (90/161) for seasonal influenza and H1N1 and 62% (37/60) and 64% (16/25) for the detection of the H1N1 virus [2, 7, 11]. Possible reasons for these lower results in comparison to our sensitivity of 80% for the 2009–2010 group can be the difference in study population, mainly pediatric NPA, the use of molecular techniques instead of IF on the specimens and VC as the reference method, and the clinical specimens used: throat swabs (Yang et al.), 25 nasal swabs (Lévêque et al.), and a combination of respiratory specimens (Cheng et al.) [2, 7, 11].

Thus, there are still some questions about its sensitivity in the H1N1 pandemic, which can be explained by the yearly altering nucleoproteins of the influenza virus besides already known factors, such as specimen collection, transportation to the laboratory, storage of the samples, and patient characteristics. This is consistent with the findings of Kok et al., where a significant reduction in rapid antigen testing sensitivity was observed when H1N1-positive samples were tested compared to nucleic acid testing [6]. Similarly, Yang et al. confirmed the same observation by demonstrating that the circulating influenza virus subtype determines the viral load, which affects the clinical sensitivity of several rapid antigen assays, rather than a diminished capacity of the rapid antigen test itself to detect these two subtypes of influenza A viruses [11].

It is important to emphasize that the evaluation of the ESPLINE® test was conducted on pediatric NPA, which usually contain a higher viral load than adult specimens, so the test may give other results than if it were to be performed on adults only. On the other hand, Yang et al. stated there was no significant correlation between the patient age and gender and the viral loads of the H1N1- nor the H3N2-infected populations [11].

In our evaluation of the ESPLINE®, the BinaxNOW Influenza, and the Clearview Exact Influenza assays, we found that these are all user-friendly and rapid antigen assays, although the ESPLINE® test had a sharper and easier to read test result. Overall, the ESPLINE® Influenza A & B-N test provided the best performance results to detect influenza A & B in respiratory specimens. Such an evaluation is of great value to the clinical laboratory and physicians in choosing between different commercially available influenza antigen tests. Other studies, such as that by Hurt et al., reported a sensitivity of between 67% and 71% compared to cell culture for five rapid tests (Binax-NOW Influenza A&B, Directigen EZ FluA+B, Denka Seiken Quick Ex-Flu, the ESPLINE® Influenza A&B-N, and Quidel QuickVue Influenza A+B Test). Booth et al. compared the Immuno*Card*STAT!, the NOW Flu A, and NOW Flu B, with sensitivities for all tests of 80% and 47% for influenza A and B, respectively, compared to IF and/or culture. As articulated by Smit et al., the BinaxNOW Influenza A & B, NOW Flu A, NOW Flu B, the Becton–Dickinson Directigen Flu A+B assays were compared with VC, resulting in no significant differences in the performance of all rapid antigen tests, with sensitivities of 53% to 59% for detecting influenza A compared with VC and IF (80%). Weinberg and Walker [10] showed that the BD Directigen Flu A+B (Directigen), Directigen EZ Flu A+B (EZ), and NOW Flu A and NOW Flu B (Binax) tests had comparable combined influenza virus A and B specificities, varying from 94% to 98%. In contrast, the sensitivity of EZ was significantly lower (39%) than that of NOW (76%) and marginally lower than that of Directigen (56%) [1, 4, 9]. With these lower sensitivity results for the point-of-care testing (POCT) of influenza in our minds, we can conclude that, in this evaluation, the ESPLINE® test has significantly higher results in comparison to other commercially available antigen tests. For this reason, the ESPLINE® assay certainly takes an important place in testing for influenza infection outside normal laboratory working hours and over weekends.

Together, these findings support the assertion that direct antigen assays need to be investigated regularly because of the continuing changes in the nucleoproteins of the influenza virus. Our results allow us to conclude that the ESPLINE® Influenza A & B-N assay is a user-friendly and rapid direct antigen assay with a very good performance for pediatric NPA. Nevertheless, the test is less sensitive to detect the 2009 H1N1 influenza virus and, thus, needs to be confirmed in case of a negative result.

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