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Evaluation of a new lateral flow test for detection of *Streptococcus pneumoniae* and *Legionella pneumophila* urinary antigen

Charlotte S. Jørgensen [a,*], Søren A. Uldum [b], Jesper F. Sørensen [c], Ian C. Skovsted [c], Sanne Otte [c], Pernille L. Elverdal [c]

[a] Department of Microbiological Diagnostics & Virology, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen, Denmark
[b] Department of Microbiology & Infection Control, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen, Denmark
[c] Department of Production and Development, SSI Diagnostica, Herredsvejen 2, 3400 Hillerød, Denmark

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ABSTRACT

Pneumonia is a major cause of morbidity and mortality worldwide. Early diagnosis of the etiologic agent is important in order to choose the correct antibiotic treatment. In this study we evaluated the first commercial combined test for the agents of pneumococcal pneumonia and Legionnaires' disease based on urinary antigen detection, the ImmuView® *Streptococcus pneumoniae* and *Legionella pneumophila* Urinary Antigen Test.
In this evaluation, the new test had a significantly higher sensitivity than the BinaxNOW® lateral flow tests and the Binax® EIA test. This identifies the ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test as a fast and sensitive point of care test for identification of the infectious agent in a major group of patients with pneumonia.

1. Introduction

The most common cause of community-acquired pneumonia is *Streptococcus* (*S.*) *pneumoniae*, but also *Legionella* (*L.*) *pneumophila* plays a major role (O'Brien et al., 2009; Mongardon et al., 2012; von Baum et al., 2008) especially among hospitalised cases.

The clinical symptoms of severe pneumococcal pneumonia and Legionnaires' disease (pneumonia caused by *Legionella*) are similar, and it is not possible clinically to distinguish between the two diseases. However, in Denmark the treatment of the two infections is different; *S. pneumoniae* is usually treated with penicillin, whereas *Legionella* requires macrolides or quinolones. A fast and correct treatment of the patients correlates with a successful outcome.

Laboratory diagnosis can be performed by culture, PCR, antibody tests on blood, or urine antigen tests for both agents. Urinary antigen tests are especially widely used for Legionnaires' disease and accounts for more than 80% of the diagnosis in Europe (Beauté et al., 2013; Ginevra et al., 2005). Some countries have introduced national guidelines or recommendations regarding diagnosis and treatment of community-acquired pneumonia (Mandell et al., 2007; Woodhead et al., 2011, and guidelines for UK: http://www.nice.org.uk/guidance/cg191). These guidelines recommend urinary antigen testing for both pneumococcus and *Legionella* besides X-ray and culture. Since culture is quite time consuming and especially for *Legionella* is slow and has a relatively low sensitivity, the urinary antigen tests, which are fast and easy to perform, are often the preferred choice.

In this study, we evaluated the first commercially available combined test for pneumococcus and *Legionella* urinary antigen, the ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test. According to the kit insert, the test detects *L. pneumophila* serogroup 1 and all 92 *S. pneumoniae* types.

2. Materials & methods

2.1. Urine samples for Legionella urinary antigen tests (sensitivity)

Urine samples from 55 culture confirmed *L. pneumophila* serogroup 1 cases (36 males, 19 females, median age 63 year [range: 41–82]) and 44 probable serogroup 1 cases (diagnosed by Binax® EIA, which preferentially detects serogroup 1 (Alere, Scarborough, Marine, USA); 29 males, 15 females, median age 64 year [range: 28–92]) were selected from the routine laboratory at Statens Serum Institut (SSI). All cases were laboratory confirmed according to the ECDC case definition (http://www.ecdc.europa.eu/en/publications/publications/1202-ted-eldsnet-operating-procedures.pdf and http://www.ecdc.europa.eu/en/activities/surveillance/eldsnet/pages/eu%20case%20definition.aspx). Culture was performed on lower respiratory tract specimens by standard methods. The samples were cultured undiluted and diluted 1:10 on MWY-O and BCYE agar plates (both from SSI Diagnostica, Statens Serum Institut, Denmark). Colonies identified as *L. pneumophila* were

* Corresponding author.
E-mail addresses: csv@ssi.dk (C.S. Jørgensen), su@ssi.dk (S.A. Uldum), jfd@ssi.dk (J.F. Sørensen), ics@ssi.dk (I.C. Skovsted), sot@ssi.dk (S. Otte), pel@ssi.dk (P.L. Elverdal).

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investigated by the Dresden panel + MAb 3 of the international panel of monoclonal antibodies to determine the serogroup and subgroup (if applicable) of the isolates (Helbig et al., 2002). Among the 55 culture positive serogroup 1 cases 13 were MAb 3/1 positive (Pontiac group) and 42 were MAb 3/1 negative (non-Pontiac group).

In addition to the *L. pneumophila* serogroup 1 cases, we investigated 50 samples from culture confirmed patients, who had infections caused by other *L. pneumophila* serogroups than serogroup 1; twenty-eight samples from patients with *L. pneumophila* serogroup 3 infection, two from serogroup 4, five from serogroup 5, 14 from serogroup 6, and one from a patient with serogroup 15 infection.

The urines were tested without heat treatment. All urines were stored in the freezer at −20 °C until investigated.

2.2. Urine samples for pneumococcus urinary antigen tests (sensitivity)

Ninety-nine urine samples (58 males, 38 females, median age 65 year [range: 6–93]; 3 samples were from anonymous patients) were selected among positive *S. pneumoniae* samples stored at SSI (previously boiled for 10 min, analysed and found positive in the in-house latex agglutination test described below, and stored in the freezer at −20 °C). Since boiling is a part of the validated procedure, all positive samples have been boiled before analysis. Both lateral tests are intended for investigation of un-boiled samples, so these samples were tested with this deviation from the instruction of the manufacturers.

Moreover, 71 urine samples from patients (anonymous adults above 18 years of age) with positive blood cultures for *S. pneumoniae* were selected for investigation (stored, without boiling, in the freezer at −20 °C until investigated).

For the samples from the routine laboratory at SSI, exemption for review by the ethical committee system and for obtaining informed consent was obtained from the Committee on Biomedical Research Ethics for the Capital Region (protocol number 2001-54-0200) in accordance with Danish law on quality development projects. Culture positive *S. pneumoniae* urines were covered by the ethical committee protocol number H-3-2013-027.

2.3. Binax® Legionella urinary antigen EIA

The enzyme immunoassay from Alere (Scarborough, Marine, USA), which qualitatively detects the presence of *L. pneumophila* serogroup 1 antigen in urine, was performed according to the instruction of the manufacturer. Briefly, urine samples were added together with anti-*Legionella* HRP conjugate to the microtitre wells, coated with polyclonal antibodies raised against *L. pneumophila* serogroup 1 antigen. After 2 h incubation, the plate was decanted and washed. A colour developer was added. Following another incubation of 15 min the reaction was stopped and the resultant absorbance read on a microplate reader at 450 nm.

2.4. ImmuView® S. pneumoniae and L. pneumophila Urinary Antigen Test

The lateral flow assay for the qualitative detection of *S. pneumoniae* and *Legionella pneumophila* serogroup 1 antigen in urine from SSI Diagnostica (Hillerød, Denmark) was performed according to the instruction of the manufacturer. Briefly, three drops of patient urine and two drops of running buffer were gently mixed in a test tube. The test strip was inserted. Test results were read after 15 min incubation, interpreted by the presence or absence of visually detectable pink (*S. pneumoniae*) or blue (*L. pneumophila*) coloured lines.

2.5. BinaxNOW® Legionella urinary antigen card and BinaxNOW® S. pneumoniae antigen card

The lateral flow assays from Alere (Scarborough, Marine, USA) for the qualitative detection of *L. pneumophila* serogroup 1 antigen or for detection of *S. pneumoniae* antigen in urine was performed according to the instruction of the manufacturer. Briefly, a swab was dipped into the urine and then inserted into the test card/strip. Reagent buffer was added. Test results were read after 15 min incubation, interpreted by the presence or absence of a visually detectable pink-to-purple coloured line.

2.6. In-house serotype-specific latex agglutination test S. pneumoniae

The latex agglutination test was performed as described in Strålin et al., 2004. Briefly, urine was boiled for 10 min followed by mixture with the latex-suspension coated with type-specific pneumococcal antisera for identification of the 23-valent pneumococcal vaccine serotypes on a slide. If positive, aggregates occur within approximately 8 s.

2.7. Statistics

Statistical analysis was performed using GraphPad Prism 6. An unpaired Student's t-test was used for comparison of sensitivity performance for the selected urinary antigen assays. Comparisons were considered significantly different for $p < 0.05$.

3. Results

Urine samples from 99 *L. pneumophila* serogroup 1 cases (55 confirmed by culture and serotyping and 44 probable serogroup 1 cases identified by Binax® Legionella Urinary Antigen EIA), were investigated using the two different lateral flow tests, ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test and the BinaxNOW® Legionella Urinary Antigen Card. All together 74 samples were positive in the Binax® EIA assay. A total of 88 of the samples were positive in the ImmuView® assay, and 71 samples were positive in the BinaxNOW® kit, giving rise to the following sensitivities: the ImmuView® 88.9%, the Binax® EIA 74.7%, and the BinaxNOW® 71.7%. It should be mentioned that out of the 71 positive samples in the BinaxNOW® assay, 18 samples showed a very faint “band”, which might easily be overlooked (if these were scored negative the sensitivity would decrease to 53.5%).

For the 55 culture confirmed cases, the following sensitivities were observed: the ImmuView® 87.3% (100% for the Pontiac group, 83.3% for the non-Pontiac), the BinaxNOW® 78.2%, (92.3% for the Pontiac group, 73.8% for the non-Pontiac), and the Binax® EIA 54.5% (100% for the Pontiac group, 42.9% for the non-Pontiac). Surprisingly the Binax® EIA showed a very low sensitivity for the non-Pontiac cases compared to the ImmuView® and the BinaxNOW® kits.

Ninety-nine urine samples (boiled) selected from positive *S. pneumoniae* samples in the routine laboratory at SSI, and 71 urine samples (un-boiled) from patients with a positive blood culture for *S. pneumoniae* were tested in the ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test and the BinaxNOW® *S. pneumoniae* Antigen Card lateral flow test. For both of the lateral flow tests, the urine samples should be un-boiled, but since we routinely boil the urine samples for our in-house pneumococcus urine antigen test, we did not have antigen-positive un-boiled urine samples available. The 71 urine samples from patients with a positive blood culture had an unknown antigen status before this investigation.

Of the 99 boiled samples, 82 were positive in the ImmuView® assay, and 61 were positive in the BinaxNOW® assay, giving sensitivities of 82.8% and 61.6% respectively. Of the 71 samples tested directly, 60 were positive in the ImmuView® test, and 55 were positive in the BinaxNOW® assay, giving sensitivities of 84.5% and 77.5% respectively. The difference in sensitivity observed for the two different kits used for the boiled samples was statistically significantly different, with the ImmuView® kit showing the best sensitivity. For the un-boiled samples the observed difference was not significant. The difference in sensitivity between the two kits in the two groups might indicate that boiling

decreases the sensitivity of the BinaxNOW® assay but not of the ImmuView® assay, since BinaxNOW® performs relatively worse on the boiled samples compared to the ImmuView® assay. Another explanation can be that the 71 culture positive patients might have a higher overall antigen level, thus the lower sensitivity observed for the boiled samples is solely a consequence of lower sensitivity of the BinaxNOW® assay. Ten of the positive *L. pneumophila* samples were tested in the ImmuView® assay both before and after boiling with similar results in each case, indicating that boiling did not interfere with the *Legionella* results of the ImmuView® assay. Unfortunately, no material was left for the same investigation of the *S. pneumoniae* samples.

If the results for the specific types of *S. pneumoniae* identified in the in-house latex agglutination assay are compared with the results obtained using the ImmuView® and the BinaxNOW® assays, the two lateral flow tests had a decreased sensitivity for type 1 (especially the BinaxNOW® which only identified 6 out of 15 samples, while ImmuView® identified 14 out of 15), type 8 (BinaxNOW® identified 4 and ImmuView® identified 6 out of 15 samples), and type 33 (BinaxNOW® identified 1 and ImmuView® identified 2 out of 5 samples) (results not shown).

A comparison of the ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test results with the combined results of the two BinaxNOW® kits using the 99 *L. pneumophila* cases and the 71 culture confirmed *S. pneumonia* cases results in an overall sensitivity of 87.1% for the ImmuView® and 74.1% for the combined BinaxNOW® kits. Results are shown in Fig. 1.

The specificity of the ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test was investigated by testing 76 urines from patients with culture confirmed urinary tract infections. These samples were all negative for *L. pneumophila*, but one sample was positive for *S. pneumoniae*. This gave a 100% specificity for *L. pneumophila*, and 99% specificity for *S. pneumoniae*. All 76 samples were negative using the two BinaxNOW® kits. The positive sample was from a patient with urinary tract infection caused by *Klebsiella pneumoniae*. All together, three *K. pneumoniae* positive patients were tested, of which only one sample was positive. A co-infection with both *K. pneumoniae* and *S. pneumoniae* cannot be ruled out.

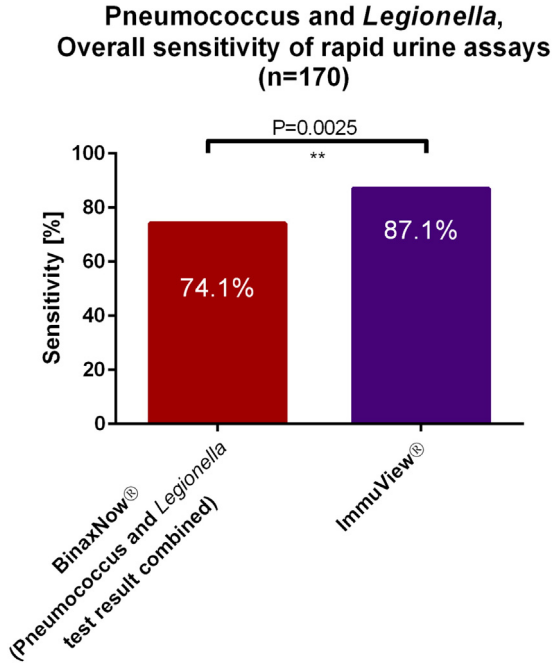

Fig. 1. Overall sensitivity of ImmuView® and the two BinaxNOW® kits (combining the results for both the *S. pneumoniae* and *L. pneumophila* cases) (n = 170).

Urines from two of the culture-positive *L. pneumophila* serogroup 1 Oxford/OLDA patients were negative for *L. pneumophila* in both the BinaxNOW® and the ImmuView® kits, but positive in the ImmuView® test for *S. pneumoniae*. Moreover, four of the urine samples from blood culture *S. pneumoniae* positive patients were found positive for both *S. pneumoniae* and *L. pneumophila* in the ImmuView® test. Unfortunately, no more sample material was left, so further analysis was not possible. We cannot exclude a possible double infection with both bacteria, which has been observed earlier (Dickgießer et al., 1985; Tan et al., 2002). However, it might also be a false positive reaction in the ImmuView® test.

In addition to the *L. pneumophila* serogroup 1 cases (confirmed and probable serogroup 1), we investigated 50 urine samples from culture confirmed cases of *L. pneumophila* non-serogroup 1. Of these, the ImmuView® kit was able to detect 10 out of 28 of the serogroup 3 cases, of which six were found positive by the Binax® EIA kit, and one was found positive by both the BinaxNOW®.

ImmuView® was also able to detect two out of the 14 serogroup 6 cases and one serogroup 15, which was not found by the BinaxNOW® or the Binax® EIA kit. All other non-serogroup 1 samples were negative in both the ImmuView® and the two Binax® kits.

4. Discussion

Three different lateral flow tests for the diagnosis of *L. pneumophila* and *S. pneumoniae* were compared: the ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test which detects both agents at one time, the BinaxNOW® Legionella kit and the BinaxNOW® *S. pneumoniae* kit. All three tests are fast methods for point-of-care diagnosis of the infectious agent in patients presenting with certain types of bacterial pneumonia.

Comparing the ImmuView® with the BinaxNOW® lateral flow test or the Binax® EIA showed that the sensitivity for detection of *L. pneumophila* serogroup 1 of the ImmuView® was significantly better than the other tests (88.9% compared to 71.7% and 74.7% respectively). Also for other serogroups than serogroup 1, the ImmuView® performed better — even though it only detected a small number of these patients. Both the ImmuView® and the Binax® EIA and BinaxNOW® assays are developed for detection of serogroup 1 antigen. But also other serogroups are involved in human infections. The clinical manifestations and the treatment of these infections are similar for all serogroups, so it is an advantage if the assays identify at least a proportion of these non-serogroup 1 infections.

For unknown reasons, we observed a low sensitivity (42.9%) of the Binax® EIA for cases with MAb 3/1 negative strains, the same strains that reacted very faintly in the BinaxNOW® lateral flow test. This is not in accordance with previous observations (Svarrer et al., 2012).

Comparing the ImmuView® assay with the BinaxNOW® for the detection of *S. pneumoniae*, showed that the ImmuView® had a significantly higher sensitivity when using boiled samples than the BinaxNOW® (82.8% compared to 61.6%), whereas the difference for the un-boiled samples was not significant (84.5% compared to 77.5%). Whether this is due to decreased sensitivity for boiled samples, or an overall lower sensitivity of the BinaxNOW® assay is not known, but this needs further investigation before a conclusion can be made. Both the ImmuView® and the BinaxNOW® assay is validated for un-boiled urine, but it may still be of interest how the tests perform on boiled samples, which may often be the case for studies on historical samples. Moreover, it cannot be ruled out that rare non-specific reaction can be minimised by boiling the samples (Birtles et al., 1990).

The investigation of 76 urines from patients with culture confirmed urinary tract infections indicates a high specificity of the lateral flow tests. The ImmuView® assay had 100% specificity for *L. pneumophila* and 99% for *S. pneumoniae*, which is slightly less than the BinaxNOW® kits which showed 100% specificity for both bacteria. This difference is not statistically significant. The result indicates that non-specific

binding is not an issue, even on un-boiled samples, but further investigation is needed.

The main method for the diagnosis of Legionnaires' disease in Europe is urinary antigen tests (Joseph et al., 2010). This may contribute to an underestimation of the actual number of cases, since the urinary antigen tests primarily identify serogroup 1 cases. In Denmark, only approximately 60% of the culture-confirmed cases are caused by serogroup 1 infection, and more than 20% are due to serogroup 3, followed by few cases of other serogroups (Kjelsø and Uldum, 2014). We tested 50 samples from cases with other serogroups than serogroup 1, but were only able to identify 13 of them using the ImmuView® kit, and just one using the BinaxNOW® and 6 using the Binax® EIA kit. Even though the ImmuView® assay did find a few of these samples and performed better than the BinaxNOW® and Binax® EIA, this emphasizes the importance of using other methods than the urinary antigen tests, leaving PCR combined with culture as the gold standard in order to be able to identify cases caused by other serogroups.

We are aware that the panel of Legionnaires' disease cases does not reflect what is seen in a normal diagnostic setting, since the proportion of MAb 3/1 negative cases are vastly overrepresented even for Denmark. The low sensitivity for Binax EIA for this group is unexpected and probably not a general problem, but this must be investigated further.

5. Conclusion

This investigation identified the ImmuView® *S. pneumoniae* and *L. pneumophila* Urinary Antigen Test as the most sensitive point-of-care test for patients presenting with pneumonia. Moreover, the ImmuView® also has the benefit of providing both results in one assay, making it easier to perform and cheaper to identify the correct infectious agent. This combined testing also fits very well with the national recommendation in different countries for simultaneous testing for these two agents.

Conflict of interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest. Jesper F. Sørensen, Ian C. Skovsted, Sanne Otte, and Pernille L. Elverdal are employed in the manufacturing company of the ImmuView test. But all analysis has been performed in the Department of Microbiological Diagnostics & Virology, Statens Serum Institut, without any involvement from Statens Serum Institut Diagnostica. There has been no financial support for this work that could have influenced its outcome.

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