A multicenter evaluation of the Biotest legionella urinary antigen EIA

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Objectives: To undertake a multicenter study to evaluate the Biotest legionella urinary antigen enzyme immunoassay (EIA) performance against those EIAs already in use in 14 European laboratories.

Methods: Each laboratory examined urine specimens from appropriate patients using both their current assay and the Biotest EIA. Each examined: a standard panel of 12 coded urine samples (distributed by Biotest); a panel of 10 coded urine samples provided as part of a European external quality assurance (EQA) scheme; urine samples from patients with proven legionnaires' disease (LD); urine samples from patients with pneumonia of microbiologically proven cause other than LD; and urine samples submitted for routine examination. Thus, the performance of the Biotest assay (in comparison with current EIAs), its specificity and utility, and the inter-laboratory agreement were assessed.

Results: Inter-laboratory agreement was excellent, with all participants obtaining the expected results for 20 of 22 coded urine specimens. Specificity, determined using 123 specimens from patients with infections of known etiology, was 100%. The Biotest EIA gave positive results in 86% of specimens which had been positive in the laboratories' current EIAs, and in 94.6% of those specimens which were positive for *Legionella pneumophila* serogroup 1.

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Conclusions: The Biotest EIA is simple to use and specific and the results obtained in different laboratories show excellent agreement. The assay compares well with existing EIAs, at least for *L. pneumophila* serogroup 1.

Key Words: Urinary antigen EIA, legionella, diagnosis

INTRODUCTION

Estimated rates of Legionnaire's disease (LD) in European countries averaged 4.45 cases per million of population in 1996, with a total of 1563 cases reported to the European Working Group on Legionella Infections (EWGLI) [1]. However, studies in Europe and elsewhere indicate that this probably represents 10% or less of actual cases [2,3]. This poor detection rate is due, at least in part, to the cumbersome diagnostic techniques in routine use. Culture of legionellae from respiratory specimens is relatively slow (3–10 days) and possibly insensitive, and requires a high degree of expertise. Direct fluorescent antibody staining of respiratory specimens is rapid and there are excellent reagents commercially available, but the sensitivity of this technique is poor (25–70%) [4]. The most widely used diagnostic method remains, therefore, estimation of serum antibody levels [4]. Unfortunately, this approach does not often allow a diagnosis to be established during acute illness, and so has little value in patient management or in the early detection of outbreaks.

In contrast, it is now well established that the use of enzyme immunoassays (EIAs) for the detection of *Legionella pneumophila* antigen in urine allows diagnoses of LD to be established early in the course of infection. Until recently, however, the use of such assays was restricted to those few reference laboratories able to develop and maintain in-house EIAs [5–7].

The first widely available commercial assay for the detection of legionella urinary antigen was a radioimmunoassay (Du Pont Co., Wilmington, USA) but, although apparently sensitive and specific [8], this was not widely used, probably because of both cost and safety considerations. More recently, the first commercial EIA kit (Binax Legionella Urinary Antigen EIA, Binax, Portland, USA) was produced. This kit, which is reported to be sensitive and specific, is only intended for the detection of *L. pneumophila* serogroup 1 infections and is also considered to be expensive to use [9]. Recently, a second commercial EIA has become available (Biotest Legionella Urin Antigen EIA, Biotest AG, Dreieich, Germany). In addition to *L. pneumophila* serogroup 1 urinary antigen, this kit is intended to detect antigen from other *L. pneumophila* serogroups as well as other *Legionella* species.

The study presented here had three aims: first, to evaluate the performance of this new assay against that of other EIAs currently in use, with particular reference to *L. pneumophila* serogroup 1, the commonest cause of legionellosis; second, to determine whether *L. pneumophila* nonserogroup 1 cases could be detected with confidence; and third, to investigate the comparability of results obtained in 14 European laboratories, each of which is a local or national legionella reference laboratory which contributes to the EWGLI surveillance scheme.

MATERIALS AND METHODS

EIA systems

Ten laboratories used the Binax EIA kit (Binax, Portland, USA), according to the manufacturer's instructions, as their 'current assay'. A further four laboratories each used their own 'in-house' assay. Detailed methodologies and evaluation data for these EIAs have been published elsewhere [5,10–12].

The Biotest EIA kit (Biotest AG, Dreieich, Germany) is a standard 'sandwich' ELISA which uses a polyclonal rabbit antiserum to capture soluble antigen and peroxidase-labeled rabbit antibodies to detect immobilized antigen. The kit was used by all participants according to the manufacturer's instructions.

To avoid any possible discrepancy in results of the two tests due to the effects of storage on specimens, all urine samples were examined for this study in parallel by both the Biotest EIA and the laboratories' 'current assay'.

Inter-laboratory agreement

This was determined using two panels of urine specimens which were coded and distributed 'blind' to each participating laboratory. Laboratories were asked to record the optical density (OD) values obtained for each specimen, together with the calculated cut-off values for each batch of specimens tested.

Panel I was prepared by Biotest AG, was dispatched frozen to participants, and comprised 12 urine samples obtained from patients with culture-proven *L. pneumophila* infection (nine samples) or healthy donors (three samples). In some cases the urine samples had been diluted in 'negative urine' to achieve the desired antigen concentration. Thus two specimens were intended to

be strongly positive, five to be positive, two to be weakly positive and three to be negative.

Panel II was prepared by the Respiratory and Systemic Infection Laboratory, Colindale from specimens submitted by participating laboratories. It was dispatched unfrozen to participating laboratories as part of the external quality assurance (EQA) scheme recently established by EWGLI laboratories. This panel comprised 10 urine samples. The intended results for these were: positive (four samples), equivocal (defined as an OD ± 10% of the originating laboratory's EIA cut-off value, two samples) and negative (four samples).

Table 1 Estimate of the specificity of the Biotest EIA, compared with the 'current assay', determined using urine specimens from patients with lower respiratory tract infections of known etiology

Causative organism[a]	Number of samples positive using: Biotest	'Current assay'
Streptococcus pneumoniae	0/48	0/48
Mycoplasma pneumoniae	0/29	0/29
Chlamydia spp.	0/13	0/13
Haemophilus influenzae	0/6	0/6
Pseudomonas aeruginosa	0/2	0/2
Moraxella catarrhalis	0/1	0/1
Coxiella burnetii	0/1	0/1
Mycobacterium tuberculosis	0/1	0/1
Escherichia coli	0/1	0/1
Respiratory syncytial virus (RSV)	0/17	0/17
Influenza A virus	0/2	0/2
Candida albicans	0/1	0/1
Plasmodium falciparum	0/1	0/1
Totals	0/123	0/123

[a]Evidence for the etiology: *S. pneumoniae*—antigen in urine/and or cultured from blood (38), cultured from sputum (9), not stated (1); *Mycoplasma pneumoniae*—positive serology (28), two also PCR positive, one PCR positive only; *Chlamydia* spp.—serology (11), DFA (2); *H. influenzae*—culture from tracheal aspirate (3), from sputum (3); *Pseudomonas aeruginosa*—culture from tracheal aspirate (1), from sputum (1); *Moraxella catarrhalis*—culture from sputum; *E. coli* and *Candida albicans*—cultured from tracheal aspirate; *Coxiella burnetii*—serology; influenza A—direct fluorescent antibody (DFA) in nasopharyngeal aspirates (NPA) (1), serology (1); RSV—all DFA in NPAs; *Mycobacterium tuberculosis*—not stated; *Plasmodium falciparum*—positive blood film in malaria case complicated by adult respiratory distress syndrome (ARDS).

Performance of the Biotest EIA—combined results from all participants

Each participant selected and examined urine specimens from a variable number of patients either with evidence of legionella infection, or with evidence of infection due to another specific microorganism. The combined data were used to estimate the specificity and sensitivity as follows. Specificity was determined using single urine samples from 123 patients with pneumonia or lower respiratory tract infection of known etiology (Table 1). Performance was assessed against single urine samples from 189 patients with evidence of legionella infection (Tables 2 and 3). Urine samples from 143 (76%) of these patients had been previously found to be positive in the 'current assays' (Table 2), and these were used to estimate the comparative sensitivity of the Biotest EIA. Urine samples from 46 of the patients, which had been previously found to be negative, were also included to determine whether the Biotest assay could detect antigen 'missed' by current assays (Table 3).

To assess the utility of the Biotest kit in general use, unselected urine samples from 228 patients submitted for routine *L. pneumophila* antigen detection were examined in various laboratories using both the Biotest kit and their current assay.

Table 2 Comparative performance of the Biotest EIA, estimated using urine specimens shown to be positive with the 'current assays' obtained from patients with proven *Legionella* spp. infection

Evidence of etiology	Number of samples positive using: Biotest	'Current assay'
Culture-proven *L. pneumophila* serogroup 1	47/49	49/49
Four-fold rise in titer to *L. pneumophila* serogroup 1	41/44	44/44
Total for *L. pneumophila* serogroup 1	88/93 (94.6%)	93/93 (100%)
Culture-proven *L. pneumophila* nonserogroup 1[a]	0/8	8/8
DFA (*L. pneumophila*)	6/6	6/6
Four-fold rise in titer to *L. pneumophila* nonserogroup 1[b]	2/3	3/3
PCR positive	9/9	9/9
'Current assay' positive with no other evidence	18/24	24/24
Total for *Legionella* spp.	123/143 (86%)	143/143 (100%)

[a]*L. pneumophila* serogroup 2 (1), serogroup 3 (3), serogroup 4 (1), serogroup 6 (2), serogroup 10 (1).
[b]*L. pneumophila* serogroup 3 (2), serogroup 6 (2).

Table 3 Ability of the Biotest EIA to detect legionella urinary antigen in samples shown to be negative with the 'current assays' from patients with proven *Legionella* spp. infection

Evidence of etiology	Number of samples positive using:	
	Biotest	'Current assay'
Culture-proven *L. pneumophila* serogroup 1	2/7	0/7
Four-fold rise in titer to *L. pneumophila* serogroup 1	2/20	0/20
DFA (*L. pneumophila*)	0/6	0/6
Culture-proven *L. pneumophila* nonserogroup 1	1/6	0/6
Four-fold rise in titer to *L. pneumophila* nonserogroup 1	0/6	0/6
PCR positive	0/1	0/1
Totals	5/46 (11%)	0/46 (0%)

A selection of 40 urine samples, including some from known positive and known negative patients, were examined with and without a 5-min boiling step before the EIA.

RESULTS

Inter-laboratory agreement

Panel I

Because of difficulties with the international postal authorities, one laboratory did not receive Panel I. The reported results for the remaining 13 participants were in complete agreement for all the samples using the Biotest kit. Reported results for the two weakly positive samples (B3 and B4) were between the cut-off value and the cut-off ± 0.2 OD in the cases of three and two laboratories respectively. The manufacturer's instructions recommend that such results are repeated to confirm the positive result. Results for the 'current assay' were very similar to those obtained with the Biotest kit, except in the cases of B3 and B4, where two laboratories obtained negative results for both (Table 4).

Panel II

All 14 participants reported complete agreement for the four positive and four negative samples using the Biotest EIA. For the two 'equivocal' specimens (E3 and E8), both were found to be positive by 12 laboratories and negative by two. Again, results with current assays were very similar, except for E3 and E8, where seven and five laboratories, respectively, found these to be negative (Table 4).

Thus overall for 20 of 22 coded specimens the Biotest kit results were in complete agreement among all laboratories. For 18 of 22 specimens, the results were in agreement for both the Biotest and current assays among all laboratories. The four specimens which were intended to give weakly positive or equivocal results were reported by most laboratories to be positive, but one laboratory found all to be negative in its current assay (Table 4).

Performance of the Biotest EIA—combined results from all participants

All 123 urine specimens from patients with non-legionella infections gave a negative result with both the Biotest and current assays (Table 2). Thus specificity is estimated to be 100% for this series of patients.

The comparative performance of the Biotest EIA was estimated using single urine specimens from 143 patients which had been previously shown to be positive in participants' 'current assays'. It was found that 123 (86%) of these were also positive in the Biotest

Table 4 Number of participating laboratories with positive and negative results for the four urine samples giving weakly positive (B3 and B4) or equivocal (E3 and E8) results

	Urine sample							
	B3[a]		B4[a]		E3		E8	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Biotest	13	0	13	0	12	2	12	2
Current assays	11	2	11	2	7	7	9	5

[a]Only 13 laboratories reported results with this panel.

EIA (Table 3). Of these 143 specimens, 93 were obtained from patients with proven (49) or likely (44) *L. pneumophila* serogroup 1 infection, and 88 (94.6%) of these were positive by the Biotest EIA. Urine specimens previously found to be negative in participants' 'current assays' were also available from 46 patients with good microbiological evidence of legionella infection (Table 3). Of these, five (11%) were found to be positive in the Biotest EIA. These comprised specimens from: two patients with culture-proven *L. pneumophila* serogroup 1 infection, a patient with culture-proven *L. pneumophila* serogroup 6 infection and two patients with serologic evidence of a *L. pneumophila* serogroup 1 infections.

The Biotest kit was found to be simple to use and generally gave clearcut results. Of the 228 unselected urine samples examined, nine (4%) gave positive results in the Biotest EIA. One of these was not confirmed by the 'current assay'. This was obtained from a patient in whom the diagnosis was subsequently confirmed by a four-fold rise in titer (against *L. pneumophila* serogroup 1) in paired sera together with a positive PCR result for a respiratory specimen. One sample was found to be positive using the 'current assay' but was Biotest negative. This was obtained from a patient who died shortly after admission to hospital, and no other specimens were available for examination.

The 40 specimens examined with and without a boiling step prior to testing showed no obvious difference in the OD values obtained (data not shown); all positive results remained positive and all negative results remained negative.

DISCUSSION

LD is an uncommon form of pneumonia, probably accounting for less than 5% of all pneumonia cases requiring hospital admission [13]. However, the diagnosis and detection of cases of LD is important for several reasons. First, although uncommon overall, legionella infections are much more significant in the context of severe community-acquired pneumonias, possibly being the second commonest form, accounting for 14–37% of cases, with an associated mortality rate in excess of 25% [14]. Second, the early recognition of cases of LD allows the source of actual, or potential, outbreaks to be identified and hastens the implementation of appropriate control measures. As LD has no particular clinical features that clearly distinguish it from other pneumonias, laboratory investigations must be relied upon if a diagnosis is to be established with any confidence.

The advantages of diagnosing LD by urinary antigen detection, such as early detection, rapidity of testing and the ease of specimen collection, were recognized soon after LD was first described [15,16]. However, until recently the widespread application of this method has been inhibited by the lack of reliable and widely available diagnostic reagents.

The first commercially available EIA was the Binax kit. Although it has not been evaluated comprehensively, studies using this assay have found it to be specific and to be reasonably sensitive [9,17]. Unfortunately, at present, this kit is expensive, particularly where specimen throughput is low [9]. Obviously, the availability of other commercially produced kits is likely to lead to overall reductions in costs per test. However, it is clearly important that, in addition to being economic to use, any new kits also show good performance characteristics. By combining the resources of 14 European laboratories active in the study of legionella infections, we were able to obtain sufficient material to make a rapid and valid assessment of the Biotest EIA.

For an infection of low prevalence, such as LD, test specificity is of paramount importance. The data presented indicate that, at least for patients with lower respiratory tract infections, the specificity of the Biotest and 'current assays' is excellent, with no false-positive results being found among the 123 non-LD patients examined. Although not encountered in this study, it has been reported that some urine samples give false-positive results, due to non-specific reactions, which can be removed by boiling the sample prior to testing [5,18]. Although it is not a stated prerequisite in the manufacturer's instructions, it is noted that boiling samples does not interfere with the Biotest assay. The limited data presented here confirm this claim, at least for *L. pneumophila* serogroup 1, and indicate that if it is considered desirable, positive results can be confirmed after boiling.

As there is no reliable standard test for the diagnosis of LD and, in this study, the precise timing of specimen collection in relation to the appearance of symptoms was unknown, it was not possible to determine the diagnostic sensitivity of the Biotest EIA with any confidence. Rather, the performance was assessed in comparison with those assays already in use. However, patients with culture-proven, or a four-fold or greater rise in antibody titer against, *L. pneumophila* serogroup 1 might be reasonably considered as confirmed cases of *L. pneumophila* serogroup 1 infection. Combination of the results for these patients in Tables 3 and 4 results in the overall sensitivity for the detection of *L. pneumophila* serogroup 1 antigen being 76.7% (92/120) and 77.5% (93/120) for the Biotest and 'current assay' respectively. These values, which are very close, match well with other reports of the sensitivity for diagnosing

L. pneumophila serogroup 1 infection by detecting urinary antigen [5,8,12]. However, it is quite likely that they are underestimates of the real diagnostic sensitivity, as some samples might well have been collected after cessation of antigen excretion. Although 10 laboratories used the Binax kit as their 'current assay', 64% of the specimens used in the estimation of sensitivity were contributed by the four laboratories that used in-house assays. Thus the sensitivities of these two commercial kits could not be meaningfully compared.

Overall, in urine samples previously shown to be positive in current assays, the Biotest kit was positive in 94.6% for *L. pneumophila* serogroup 1 infections and in 86% for *L. pneumophila* of any serogroup. Evidence that the kit could detect *L. pneumophila* nonserogroup 1 infections was found, as positive results were obtained with urine samples from two patients with evidence of *L. pneumophila* serogroup 6 infection (one culture proven) and one with evidence of *L. pneumophila* serogroup 3 infection. However, none of the eight 'current-assay'-positive specimens from patients with culture-proven *L. pneumophila* nonserogroup 1 infection was positive with the Biotest kit (Table 2). Positive results for these latter specimens were obtained using either of two broadly reacting and sensitive in-house EIAs [12,19]. Whether or not the Biotest EIA's failure to detect the antigen was due to the sensitivity (in terms of ability to detect very small quantities of antigen), compared with these assays, or due to the specificity of the assay (in terms of ability to detect a broad range of *L. pneumophila* serogroups), is not clear.

A large proportion of cases of LD are now recognized to be travel-associated, particularly in patients returning from holidays in other countries, and a sophisticated surveillance scheme has been developed to detect associated cases even though they may be widely dispersed within Europe [1]. The implication that a particular hotel or resort is the source of LD infections can have severe economic consequences. Hence, central to the EWGLI scheme are the reliability of the diagnostic tests used, and the uniformity with which diagnostic criteria are applied when defining cases. The data presented here show that, overall, the reproducibility of the EIAs both within and between laboratories was excellent, indicating that considerable confidence can be placed on the results obtained using them. Not surprisingly, the reproducibility for those specimens considered 'equivocal' showed some variability and there is clearly an important role for EQA schemes to help ensure consistency of reporting. For international surveillance purposes, a positive urinary antigen result is currently considered as presumptive evidence of legionella infection rather than diagnostic [20]. In the light of earlier reports [21] and the findings reported here, it is clear that this classification should be reviewed.

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