External Quality Assessment Program for *Chlamydia trachomatis* Diagnostic Testing by Nucleic Acid Amplification Assays

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We report the results from 57 Australian diagnostic laboratories testing two external quality assessment panels using either the Roche Amplicor *Chlamydia trachomatis* test (R-PCR) or the Abbott LCx *Chlamydia trachomatis* assay (A-ligase chain reaction [LCR]). Panel samples were either normal urine spiked with *Chlamydia trachomatis* antigen or clinical urine specimens. There was no significant difference between laboratories or between assays in detection of *C. trachomatis*-positive clinical samples. Only at the lower limit of detection of the assays did the R-PCR demonstrate increased sensitivity over the A-LCR in the detection of *C. trachomatis* antigen. However, it was found that single-sample testing could lead to decreased test sensitivity. Detection of the presence of inhibitors of nucleic acid amplification differed between laboratories.

Diagnosis of genital *Chlamydia trachomatis* infection by nucleic acid amplification (NAA) technologies using urine specimens is presently the most effective, sensitive, and noninvasive method available (2, 3, 11, 16, 18, 20, 21, 28). NAA technologies and nucleic acid probe and antigen detection methods have virtually replaced isolation in cell culture, once considered the “gold standard” for the detection of *C. trachomatis* (25). NAA is a suitable technique for testing urine specimens. Australian diagnostic laboratories have readily adopted NAA assays for chlamydial diagnosis, and over 90% include first-void urine in their specimen repertoires.

The mechanism for quality assurance of pathology testing is two pronged. First, it requires the inclusion of internal quality control samples in assays to check test reliability on a run-by-run basis, and second, the laboratories participate in external quality assessment (EQA) programs (12). EQA programs are conducted by an organization independent of the testing laboratories. Prior to distribution, panel samples are characterized or tested numerous times by at least one reference laboratory. Reference laboratories are chosen for their recognized expertise with and high testing throughput of the particular analyte involved. The laboratories are required to process panels of samples using their standard protocols and to return results for analysis to the quality assurance provider. Participation in EQA thereby provides retrospective analysis of the laboratory's testing procedure at a particular point in time and comparison with other testing laboratories and a reference laboratory. Participation in regular EQA programs has been shown to be one of the best methods of maintaining and improving the quality of laboratory diagnosis, as well as detecting testing problems and deficiencies (24).

We report the design of and results from an EQA program

conducted in Australian diagnostic laboratories testing for *C. trachomatis* using either the Roche Diagnostics Systems, Inc. (Branchburg, N.J.) Amplicor *Chlamydia trachomatis* test (R-PCR) or the Abbott Laboratories (Diagnostic Division, Abbott Park, Ill.) LCx *Chlamydia trachomatis* assay (A-ligase chain reaction [LCR]). Participation in the program was voluntary. The aims of the program were to assess the sensitivities of NAA assays at the lower limit of detection and in the detection of *C. trachomatis* in clinical urine samples and to raise awareness of the possibility of inhibitors of NAA in clinical specimens that can mask detection of the organism (15, 23).

To our knowledge, this is the first report of field assessment of *C. trachomatis* NAA testing. Previous reports have compared the performance characteristics of the R-PCR and A-LCR for the detection of chlamydial infection (4, 5, 6, 10, 19, 26, 27). For all studies to date, specimen testing was carried out at a central laboratory under idealized conditions. Therefore, the results generated by these studies do not accurately represent field results. EQA programs can be used to compare the field performances of diagnostic assays. Under field conditions, operator and laboratory variation are uncontrolled. However, the significance of the results lies in the fact that the performance of the assays is evaluated under the conditions in which they are used to generate diagnostic test results. The present report compares the performances of the two most widely used commercial assays for the detection of *C. trachomatis*, R-PCR and A-LCR, under field conditions.

While NAA testing offers increased sensitivity over isolation in cell culture and antigen detection methods, it has been shown in several studies that inhibitors of NAA can mask detection of positive specimens (1, 9, 15, 19, 22). The proportion of specimens containing NAA inhibitors is estimated to range from 1 to 7%, depending on the specimen type and patient population. A large study showed that the prevalence of inhibitors in urine specimens collected from women ($n = 388$) was 7.0% for R-PCR and 3.9% for A-LCR (15). Substances implicated in inhibition include beta-human chorionic

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TABLE 1. Detection of *C. trachomatis* in panel 1 samples by laboratories using R-PCR or A-LCR

<i>C. trachomatis</i> status	No. of samples per panel	Endpoint concentration ^a	Detection rate			
			R-PCR		A-LCR	
			No. of tests	No. positive (%)	No. of tests	No. positive (%)
Positive	2	100×	80	78 (98)	22	20 (91)
Positive	2	10×	80	48 (60)	22	8 (36)
Positive	4	1×	160	15 (9) ^b	44	0
Negative	2	0	80	0	22	0

^a Derived by repeat R-PCR and A-LCR testing of 10-fold dilutions of *C. trachomatis* at three reference laboratories.

^b Between-assay detection rate was significantly different ($P < 0.05$).

gonadotropin, crystals, nitrates, and hemoglobin. Different assays may be susceptible to different inhibitors (4, 5). Furthermore, the sensitivity of multiplex PCRs, which are becoming more common in diagnostic testing, can be compromised due to competition for reagents in the reaction (23). Users of the R-PCR have the option to test in parallel for the amplification of an internal control so that suboptimal amplification of the test analyte can be detected. The present EQA program was used to investigate the proportion of laboratories that incorporate an internal amplification control into their testing algorithms.

MATERIALS AND METHODS

Both the R-PCR and the A-LCR are qualitative and amplify a conserved region in a cryptic plasmid present in all serovars of *C. trachomatis* (17). The apparent analytical sensitivity is 1 inclusion-forming unit per amplification reaction, according to the manufacturers' data.

The R-PCR requires a sample volume of 0.5 ml of urine and uses PCR technology to amplify *C. trachomatis* DNA. An internal control of PCR amplification, in the form of a plasmid that is coamplified with the target DNA, is included in each reaction. After hybridization to a solid phase, the products of amplification are detected in an enzyme immunoassay (EIA)-based system. A generic EIA kit, purchased separately from the *C. trachomatis* kit, is used for detection of the internal control product. After sample preparation, the detection component of the assay may be automated using the Roche COBAS amplification and detection system technology (COBAS).

The A-LCR requires 1.0 ml of urine and relies on LCR for amplification. Probes anneal at specific sites on the target DNA; the small gaps between the probes are filled by the action of DNA polymerase and closed by ligase. The ligated probes then become the templates for future rounds of amplification. The LCx analyzer is used to label and detect the product via an immunocolorimetric bead capture system. The A-LCR does not have the capability to monitor inhibition of nucleic acid amplification.

The first of two *C. trachomatis* EQA panels (panel 1) was designed to contain low levels of target material to enable evaluation of sensitivity at the lower limits of detection of NAA assays (Table 1). *C. trachomatis*, cultivated in HeLa cells, was sonicated (three 10-s pulses at 30% duty cycle with 30-s breaks on ice), serial 10-fold dilutions were prepared, and aliquots were frozen at -70°C . Four aliquots of each dilution were tested at two reference laboratories using R-PCR and at one reference laboratory using A-LCR ($n = 12$ per dilution). The endpoint was defined as the concentration at which 50% of the collective test results were positive (Table 1). This method is analogous to the method used to define infectivity of chlamydial, rickettsial, and viral cultures (7). Samples for inclusion in the panel were prepared in duplicate by spiking *C. trachomatis* antigen into *C. trachomatis*-negative urine at the endpoint concentration and at 10 and 100 times the endpoint concentration. One negative urine sample was included in the panel of samples in duplicate.

Eight months later, the second panel (panel 2), containing five samples in duplicate, was distributed to laboratories. In the second panel, each sample was prepared from a single micturition specimen; they were collected from individ-

uals presenting with overt symptomatic genital *C. trachomatis* infection at a local sexually transmitted disease clinic. One sample was diluted 1:1,000 in *C. trachomatis*-negative urine to make a low-level positive sample. Another sample was spiked with humic acid (final concentration, 0.0006% [wt/vol]) to simulate clinical specimens that contain inhibitors of NAA (29, 30). Each panel sample was characterized by being tested at three reference laboratories using R-PCR and A-LCR between 20 and 40 times. The results were 100% concordant within each assay for all replicates.

All panel samples were prepared and aliquotted aseptically, randomized as is usual in blinded panel testing, frozen at -70°C , and shipped to testing laboratories on dry ice. Participating laboratories were instructed to process panel samples as part of their routine testing procedure. The results were analyzed by the EQA provider. Results from the two methods of detection of the R-PCR, COBAS and manual EIA, were considered together. The chi-square test was used for all statistical calculations for both panels.

RESULTS

Panel 1: detection at the lower limit of sensitivity. The majority of laboratories that tested panel 1 (40 of 51) used R-PCR (with detection by either manual EIA or COBAS). A-LCR was used by 11 laboratories. The manufacturers' cutoff levels for detection were applied. For the purpose of analysis, results reported as equivocal were considered negative and low positives were called positive. Laboratories were not provided with sufficient sample volume to repeat test.

Counting each test as one result, the detection rate for each assay was calculated (Table 1). At 100 and 10 times the endpoint concentrations, there was no significant difference in the detection rates between assays. At the endpoint concentration, 15 of 162 (9%) samples were detected by R-PCR compared with none by A-LCR ($P < 0.05$). In samples that were negative by only one assay system, there was no correlation with optical density, A_{660} , or sample-to-cutoff ratio. For all *C. trachomatis*-spiked samples, R-PCR showed a higher level of detection than A-LCR.

There was no interlaboratory difference in the detection of *C. trachomatis* at 100 times the endpoint concentration. The detection rate between laboratories differed at 10-fold endpoint and endpoint concentrations. Using R-PCR, 33 of 40 laboratories (83%) reported one or both of the 10-fold endpoint duplicate samples as positive. Using A-LCR, 6 of 11 laboratories detected at this level. At the endpoint concentration, *C. trachomatis* was detected in two of the quadruplicates by 1 and at one of the quadruplicates by 13 of the 40 laboratories using R-PCR. None of the laboratories using A-LCR reported positive results at this concentration. All laboratories correctly reported unspiked samples as negative.

Panel 2: detection of clinical *C. trachomatis*-positive samples and effects of amplification inhibitors. Fifty-seven laboratories tested panel 2. Again, most laboratories (47 of 57) used R-PCR. Of those, 33 of 47 used COBAS and 14 used the microwell plate format EIA for detection postamplification. A-LCR was used by 10 laboratories.

The level of concordance with the reference laboratories' results was calculated (Table 2). Results were only considered concordant if duplicate samples were detected correctly by participating laboratories. For the *C. trachomatis*-positive samples (samples 1 to 3) and the *C. trachomatis*-negative sample (sample 5) for laboratories using R-PCR (both COBAS and microwell plate EIA) and A-LCR, there was no significant interassay difference in the levels of detection of *C. trachoma-*

TABLE 2. Concordance among 51 laboratories testing panel 2 samples using either R-PCR or A-LCR

Sample no.	<i>C. trachomatis</i> status	Laboratory concordance with reference results (%)		
		R-PCR COBAS (<i>n</i> = 33) ^a	R-PCR MWP (<i>n</i> = 14) ^b	A-LCR (<i>n</i> = 10)
1	Positive	32 (97)	13 (94)	9 (90)
2	Positive	22 (68)	7 (50)	9 (90)
3	Positive (low level)	21 (62)	10 (71)	9 (90)
4	Positive; inhibitors detected	23 (70)	8 (57)	0
5	Negative	32 (97)	14 (100)	10 (100)

^a Number of laboratories using assay.^b MWP, microwell plate EIA.

tis. The decreased level of detection for samples 2 and 3 correlates with the lower optical density values these samples yielded in reference laboratory testing (data not shown). However, a significant number of testing sites using R-PCR (14 of 47) detected only one of the duplicates of both samples 2 and 3 as *C. trachomatis* positive, demonstrating that single-sample testing can lead to false-negative results ($P < 0.001$).

There was significant difference in the reporting of an inhibitor of amplification in sample 4 between the users of the R-PCR and A-LCR ($P < 0.05$). Thirty-one of 47 laboratories using the R-PCR reported the presence of inhibitors because they assayed for amplification of the internal control. The remaining 16 laboratories using the R-PCR and all laboratories using the A-LCR found this sample negative, while the correct result was inhibitors detected and/or positive. None of these laboratories assayed for the presence of inhibitors; for those using the A-LCR, it was not an option.

DISCUSSION

Results from an EQA program for *C. trachomatis* created a unique opportunity to compare the field performances of two commercial assays. While outcomes of field research are valuable, investigators cannot control for certain aspects, such as assay usage. In this program, 80% of the laboratories used R-PCR and the remaining 20% used A-LCR. This imbalance may bias the significance of the results. Nevertheless, this should not preclude the comparison of field data.

Studies have shown that a significant proportion of clinical urine specimens collected from infected individuals in high- and low-prevalence areas have low organism loads (<10 chlamydial elementary bodies per 15 to 20 ml of first-void urine) (8, 13, 14). The present EQA panel was designed to evaluate the sensitivities of assays at low antigen concentrations.

There was no significant difference between the detection rates of two commercial NAA assays (R-PCR and A-LCR) at concentrations 100- and 10-fold higher than the endpoint of detection, as determined by reference laboratory testing. However, R-PCR was more sensitive than A-LCR in the detection of samples spiked with lower concentrations of antigen. The interlaboratory variation in detection limit was significant. Some laboratories using R-PCR detected down to the endpoint. In contrast, one laboratory using A-LCR failed to detect at 100 times the endpoint. The sensitivities of the two assays have been compared in previous studies, where testing was

conducted at central laboratories (4, 5, 6, 10, 19, 26, 27). The results from these studies differ with respect to which assay had greater sensitivity and the level of sensitivity achieved by each assay. The reported sensitivity levels ranged from 100 to 62% for the R-PCR and 94 to 75% for the A-LCR when testing urine specimens. The report of 62% sensitivity for the R-PCR found that on freezing and thawing samples, the sensitivity of the R-PCR increased to the same level as the A-LCR, 93% (27). The present field study found that both assays were highly and equivalently sensitive at very low antigen concentrations. However, R-PCR was more sensitive than A-LCR at the detection limits of the assays.

The second panel was designed to assess the sensitivities of NAA assays by using clinical urine specimens. The use of clinical specimens in an EQA panel can be problematic. A large volume of specimen is required for a panel. Usually, clinical specimens are dispatched at ambient temperature to testing laboratories and tested without prior storage. However, to permit evaluation of its suitability for inclusion in the panel and subsequent reference testing, the potential EQA specimen must be frozen. This may not be standard laboratory practice and therefore deviates from the actual testing process. Furthermore, it is often difficult to pitch EQA samples at a meaningful level. Strong positive and negative samples are easier to produce, but results from testing these will have little value for most testing laboratories. Weaker positive samples, on the other hand, may help detect problems in test sensitivity.

The results showed no significant interassay difference between R-PCR and A-LCR in detection of positive clinical urine specimens. However, only half of the laboratories detected all duplicate samples as positive, indicating that, in particular, lower-level positive samples may not be consistently detected when single-sample testing is used, a common testing procedure in many diagnostic laboratories.

The presence of naturally occurring inhibitors of NAA in some clinical specimens can mask detection of positive specimens (9, 11, 15, 18, 23, 31). Laboratories using the R-PCR kit have the option to include an internal control in their testing algorithms, thereby verifying that amplification was not compromised by inhibition. The A-LCR kit does not have this facility. However, if inhibition of amplification is suspected on the basis of clinical history or sample type or appearance, further processing of the sample prior to repeat testing can remove the effects of amplification inhibitors (5, 15). Furthermore, *C. trachomatis*-spiked specimens can be tested in parallel if there is concern about inhibition. A significant number of laboratories did not report the presence of an inhibitor in *C. trachomatis*-positive samples that had been spiked with an inhibitor, and they incorrectly reported these samples as negative. Previous studies have shown that the frequency of inhibitors varies in different sample types and within different patient populations but is sufficiently common to have a diagnostic impact (15, 31). The inclusion of an inhibitor-containing sample in the EQA panel prompted laboratories that did not consider the possibility that amplification inhibitors may be present in a proportion of clinical samples to examine their testing procedures (with respect to specimen type and source).

Analysis of the results of an EQA program has shown that both of the commercial NAA assays used widely in Australia for the detection of *C. trachomatis* (R-PCR and A-LCR) de-

tected nucleic acid in clinical urine samples with equal efficacy in field testing. The vast majority of laboratories detected organism concentrations down to 100-fold above the lowest limit of detection. R-PCR demonstrated increased sensitivity at lower *C. trachomatis* concentrations compared to A-LCR. The results of this program have highlighted the importance of the possibility of inhibitors of NAA leading to false-negative results.

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