Lowering the cut off value of an automated chlamydia enzyme immunoassay and confirmation by PCR and direct immunofluorescent antibody test

C Y W Tong, C Donnelly, N Hood

Abstract

Aims—To increase the sensitivity of an automated chlamydia enzyme immunoassay by significantly lowering its cut off value, and to maintain specificity by confirmation with polymerase chain reaction (PCR) and direct immunofluorescent antibody test (DFA).

Methods—Over five months, the cut off value of the enzyme immunoassay used to screen urogenital samples for chlamydia antigen was reduced from 80 to 10. Samples with a test value of 10 or above were further tested with a commercial PCR assay. All samples during the first three months and discrepant samples during the last two months of the study were also tested with the DFA.

Results—3250 urogenital swabs (1246 urethral, 1335 endocervical, 669 pooled urethral/endocervical) from 1246 males and 2004 females were processed. Using the manufacturer’s recommended cut off of 80, the enzyme immunoassay identified chlamydia antigen in 134 samples (4.1%). Using the lower cut off value of 10 and either PCR or DFA as the confirmatory test, Chlamydia trachomatis was identified in 178 samples (5.5%). Thus, 45 additional positive samples were identified and the confirmed detection rate was increased by 33.8% (45/133). Excluding equivocal PCR results, the concordance between DFA and PCR was 91.8%. This strategy increased the detection rate by 2.1% in men and 0.9% in women (significant only in men). In female patients, pooled urethral/endocervical swabs as a specimen gave a significantly higher yield than endocervical swabs regardless of whether the lower cut off strategy was used.

Conclusions—This strategy of significantly lowering the cut off test value with confirmation on the same specimen by either PCR or DFA is feasible and cost effective. The use of pooled urethral/endocervical specimens in females should be considered routinely as detection rate was significantly improved.

Enzyme immunoassay (EIA) is one of the most frequently used commercial methods for the screening of Chlamydia trachomatis antigen in genitourinary specimens. Automation allows testing with flexible batch sizes and the cost per test is low. Direct immunofluorescent antibody test (DFA) is more sensitive and is frequently used as a confirmatory test for EIA. DFA, however, is not suitable for large scale work as it is subjective and very labour intensive. Newer molecular techniques such as polymerase chain reaction (PCR) and ligase chain reaction have been proved to be significantly more sensitive than EIA. Our earlier studies comparing an automated EIA system (VIDAS; Biorieux, France) using an expanded gold standard including PCR and DFA suggested that the manufacturer’s recommended cut off value of the VIDAS was set too high and could be lowered without significant effect on its specificity.

Using the recommended cut off, the EIA was found to have a sensitivity of 76% with urethral swabs from high risk male patients comparing with the expanded gold standard. It was estimated that using VIDAS alone, the rate of underdiagnosis from endocervical swabs was more than 30%. Screening by molecular techniques, although promising, is costly and there are problems of contamination and inhibition. Instead of using PCR as a primary screening test, Østergaard et al have reported on the successful use of PCR in addition to DFA as confirmatory tests for another commercial EIA (MicroTrak EIA; Syva, USA) using a revised cut off value. We applied the same principle in our laboratory by using a significantly lower cut off value in the VIDAS system and confirming the results with a commercial PCR (Amplicor; Roche, Switzerland) and DFA (MicroTrak direct specimen; Syva, USA). The changes in prevalence rates in males and females and of different specimen types were studied.

Methods

SPECIMENS

Over five months, 3250 genitourinary specimens from 1246 male patients (38%) and 2004 female patients (62%) were analysed. There were 1335 endocervical swabs and 669 pooled urethral/endocervical swabs from women, and 1246 urethral swabs from men. Eye, pharyngeal, and rectal swabs, and urine samples were excluded from the study. The majority of samples (> 90%) came from patients at risk for
sexually transmitted diseases who attended the genitourinary clinic in the Royal Liverpool University Hospital. The other specimens were submitted by general practitioners, generally from symptomatic females. All specimens were collected and sent using the VIDAS chlamydia collection kit according to the manufacturer’s instructions. The routine specimens taken from female patients in the hospital clinic during the first three months of the study were endocervical swabs. This policy was changed in the last two months so that a urethral swab was taken in addition to the standard endocervical swab and the two swabs were sent in the same transport tube as a single pooled specimen. The standard investigation for male patients was urethral swabs throughout the study.

ENZYME IMMUNOASSAY

All specimens were analysed following the manufacturer’s instructions. Briefly, 2 ml of sample treatment reagent was added to the specimen tube containing the swab(s) and incubated for 15–30 minutes at room temperature. It was then vortexed for 30 seconds and the swabs were discarded. The fluid in the tube was boiled for 15 minutes after which 350 μl were added to a VIDAS reagent strip. The strip together with a plastic spur coated with antibody to the chlamydia lipopolysaccharide, which acted as both the solid phase and a pipetting device, were loaded into the VIDAS analyser programmed for the chlamydia assay. The test value was obtained by subtracting the fluorescent reading of a standard from that of the sample. The VIDAS protocol recommends a test value of 80 or above as a positive result. In this study, the cut off was lowered to 10. Any sample with a test value of 10 or above was further tested by PCR.

POLYMERASE CHAIN REACTION

The PCR assay was performed within 48 to 72 hours after the EIA. An adapted procedure of specimen preparation was used so that the same specimen prepared for EIA, stored at 4°C, could be used in the PCR assay. Briefly, 200 μl of urine resuspension buffer (Roche) was added to 50 μl of sample in VIDAS sample treatment reagent after the boiling step and incubated at room temperature for one hour. One millilitre of urine diluent (Roche) was then added and the mixture vortexed and incubated for 10 minutes at room temperature. Amplification of the C trachomatis cryptic plasmid DNA was performed on 50 μl of the prepared sample following the usual Amplicor procedure, and PCR product was detected in the supplied microtitre plates following the manufacturer’s instruction. An absorbency reading at 450 nm of 0.5 or above was considered as positive and a reading of between 0.2 and 0.5 as equivocal.

DIRECT IMMUNOFLUORESCENT ANTIBODY

During the first three months of the study, all samples with an EIA reading above the lowered cut off value of 10 were further tested by DFA. During the last two months of the study, only samples with discrepant EIA and PCR results or samples that gave equivocal PCR results were further tested by DFA. Specimen preparation for DFA was similar to that previously described. Briefly, residue samples were centrifuged at 12 000 ×g for 30 minutes in a microfuge and the pellets were resuspended in 25 μl of phosphate buffered saline, air dried and fixed to microscope slides with the fluorescein labelled monoclonal antibody against C trachomatis major outer membrane protein supplied by the manufacturer. The presence of three or more elementary bodies was taken as a positive result. The quality of each slide was also assessed by recording the presence of epithelial cells. The DFA test was read independently by two microscopists who then agreed on a result without knowledge of the EIA test values.

DEFINITIONS

Samples with EIA test values below 10 were discarded as negative with no further tests. As the sensitivity and specificity of both PCR and DFA have been evaluated previously and found to be satisfactory for EIA confirmation, for the purpose of this study, samples with an EIA test value of 10 or above that were found positive by either PCR or DFA were reported as positive. Equivocal PCR results were not considered as positive unless they tested positive by DFA. There were no further attempts to resolve discrepant results because of depletion of samples.

STATISTICAL ANALYSIS

The change in prevalence of detection was analysed using the χ² test. A p value of less than 0.05 was taken as significant. The odds ratio (OR) and the 95% confidence intervals (CI) were calculated in cases of significance.

Results

Using the cut off value of 80 as recommended by the manufacturer of VIDAS, 134 of 3250 samples (4.1%) were identified as positive; 47 of 1246 (3.8%) from men and 87 of 2004 (4.3%) from women. Among female samples, positive results were obtained in 42 of 1335 (3.1%) endocervical swabs and 45 of 669 (6.7%) pooled urethral/endocervical swabs. There were 94 samples that had EIA test values of between 10 and 80. Thus, 228 samples were further tested with PCR. Of the 134 EIA positive samples, 133 were also positive by PCR. The single discrepant sample was an endocervical swab from a female patient. Of the 94 samples with EIA test values between 10 and 80, 43 (45.7%) were confirmed as positive by PCR (25 of 57 from men and 18 of 37 from women); seven samples, four from men and three from women, gave equivocal PCR results (table 1). Overall, positive PCR results were found in 72 of 1246 (5.8%) male specimens and 104 of 2004 (5.2%) female specimens. The positive detection rates in endocervical swabs and pooled urethral/endocervical swabs were 49 of 1335 (3.7%) and 55 of 669 (8.2%), respectively.

DFA confirmation was performed on 165 samples. Excluding the seven PCR equivocal
samples, overall agreement of DFA and PCR was 91.8% (145 of 158). The single sample that was EIA positive and PCR negative was also DFA negative, suggesting a false positive EIA result. Of the seven samples that were PCR equivocal, one was positive by DFA and six were negative. Discrepant DFA and PCR results were found in 13 samples (8.2%): 12 were PCR positive but DFA negative, and one was DFA positive but PCR negative (table 1). The sample that was DFA positive but PCR negative also had a relatively high EIA test value of 57 and more than 10 elementary bodies were seen by DFA. This suggested that it was a false negative result by PCR. With the 12 DFA negative/PCR positive samples, most had a very low EIA test value. Four of these samples appeared to have one visible elementary body by DFA, but was regarded as negative by definition. One sample gave a positive EIA test value of 93 and a positive PCR result but only one visible elementary body by DFA. The quality of the slide was judged to be poor as there were not many cells in the smear. There was no further attempt to resolve the discrepancy as samples were depleted after the three tests.

Using either PCR or DFA results as confirmation, the revised prevalence of *C trachomatis* detection was 178 of 3250 (5.5%), and the respective prevalence for male and female patients were 73 of 1286 (5.9%) and 105 of 2004 (5.2%). The revised rate of detection in endocervical swabs and pooled urethral/endocervical swabs were 3.7% (49 of 1335) and 8.4% (56 of 669), respectively. Thus, using the strategy of this study, 45 additional positive samples were identified and the confirmed positive rate was improved by 33.8% (45 of 133). The overall rate of detection was increased by 1.4%—an increase of 2.1% in men and 0.9% in women. Statistical analysis showed that this increase was significant in men (p = 0.015; OR = 1.59; 95% CI = 1.08 to 2.36) but not in women (p = 0.183). Study of the changes in detection rate according to specimen types in women also did not identify any significant increase in detection rate (endocervical swabs, p = 0.455; pooled urethral/endocervical swabs, p = 0.255). However, when endocervical swabs were compared with pooled urethral/endocervical swabs, the pooled samples gave a significantly higher yield regardless of whether the confirmation strategy was used (EIA only, p = 0.0002; OR = 2.22; 95% CI = 1.41 to 3.50; revised cut off with confirmation, p << 0.0001, OR = 2.4, 95% CI = 1.58 to 3.64). Apart from the specific change made in the specimen collection policy in female patients, there was no evidence of any change in consultation pattern in the hospital genito-urinary medicine clinic during the study period. These results suggested that the inclusion of urethral swabs in addition to endocervical swabs improved the detection of *C trachomatis* in female patients.

The percentage of confirmed positive samples in relation to the actual EIA test value was studied (table 2). When the EIA test value was cut off at 20, the overall percentage of confirmation was uniformly 80% or above and the percentages of confirmation in male patients were always 100%. This confirmed that the lack of sensitivity of the EIA test was partly because of the recommended cut off value being set too high. However, when the EIA test value was below 20, the percentage of positive confirmation was reduced to 25%. All five samples that had EIA test values above 20 but not confirmed as positive were from female patients (three endocervical swabs and two pooled urethral/endocervical swabs). When EIA reading was below 20, the percentage of confirmed positive were 26% in men and 24% in women (not significant, p = 0.838).

### Discussion

The use of PCR or DFA for confirmation of chlamydia EIA results has been published previously. Most studies involved the use of a revised cut off value for the EIA. Östergaard et al. reported the successful use of PCR (Amplico) and DFA to confirm EIA (MicroTrak EIA) using a revised cut off that was 70% below the recommended value and found a high concordance rate between PCR and DFA (98.2%). Here, we report a successful attempt to reproduce their method using another EIA system (VIDAS). As we have not tested samples that had an EIA reading of less than 10, it was not possible to say whether this approach has identified all the positive samples. However, our previous work using an expanded gold standard on the same patient population showed that EIA underdiagnosed by about one-third and this is similar to the figure of 33.8% obtained from the present study. Also, the drop in percentage of confirmed positive results from 80% to 25% when
the test value was below 20 suggested that the selected revised cut off value was appropriate.

It is of interest that the sample that was EIA positive but both PCR and DFA negative, and four other samples that had high EIA reading but not confirmed as positive, were all from female patients. This highlighted the need of a confirmatory test for female specimens. The role of cross reacting organisms in particular Gram negative bacteria in urogenital tracts of women and children causing false positive EIA has previously been described.10 11 Although female samples appeared to have the benefit of improved specificity using this confirmatory system, the improvement in yield was highest among male patients in this series. This was probably because male urethral samples had fewer chlamydiae and positivity was underestimated by the less sensitive EIA. The improved sensitivity of the revised cut off with confirmation thus allowed the detection of more positive cases in male specimens compared with female specimens. The inclusion of urethral swabs from female patients also had a significant effect in yield. Detection of C trachomatis in female urethral samples but not in concomitantly taken endocervical swabs has been described.12 13 During this study, we did not notice any significant difference in the quality of the endocervical smears, as judged by the presence of epithelial cells, to that from pooled urethral/endocervical specimens. Thus, the findings in this study that pooled urethral/endocervical swabs consistently gave a higher positive yield suggested that in infected females, the urethra may harbour organisms that are sometimes absent or only present in small quantities in the endocervix. It is not necessary in practice to determine the exact anatomical site of infection in most clinical situations, therefore, the use of pooled specimens should be recommended as it is more cost effective than testing swabs separately from different anatomical sites.

Most studies on the laboratory detection of C trachomatis have used more than one diagnostic test and defined true positive by expanded gold standard.4 14 15 However, in practice, exhaustive testing of individual samples is not possible. Most diagnostic laboratories rely on a combination of a screening assay plus one or two confirmatory tests. Repeat samples are usually requested when the results remain equivocal. The strategy adopted in this study appears to fit this purpose. EIA is ideal as a screening test but it is neither sensitive nor specific. Lowering of the cut off value may increase non-specificity, boost sensitivity, and allows confirmation by another method with a different principle.

The two confirmatory tests used in this study, however, have their own problems. DFA, if performed by experienced technicians is both sensitive and specific.7 16 Some studies comparing DFA and PCR have concluded that DFA could be as sensitive16 or more so than PCR.13 However, DFA is a subjective test and performance could be variable dependent on experience. The procedure is labour intensive and clearly not suitable for large volume work.

PCR, on the other hand, can be automated. The Amplicor system has a built-in enzyme (Amperase) that digests amplicons after reaction to prevent PCR product carryover, and gives an objective optical density reading. Amplicor was found in one study17 to be capable of detecting chlamydiae organisms at 10-fold higher concentrations than DFA. This may explain the 12 samples in the present series that were Amplicor positive but DFA negative. However, false negative results are possible because of PCR inhibitors18 or plasmid free organisms.19 This was illustrated by one of the samples in this series, which had a relatively high EIA reading of 57 and more than 10 elementary bodies by DFA, but was negative by Amplicor. If Amplicor were used as the primary screening test or the only confirmatory test, this sample would have been missed. The confirmation rate was uniformly high when the EIA reading was above 20, therefore, any samples with readings above 20 but negative by PCR should be further tested by DFA.

The results from this study suggest that it is possible to use EIA with a significantly lowered cut off as a primary screening test, and use PCR as the second line confirmatory test on the same specimen. DFA, being more labour intensive, could be used as a third test to confirm equivocal PCR results, and on samples that had a relatively high EIA reading but negative by PCR. Assuming a 10% confirmation rate in a laboratory that handles 10 000 patients, the laboratories will need confirmation per week and this is a suitable batch size for PCR. The overall cost will be significantly lower compared with direct screening of all samples by an amplification method. This strategy may not be the perfect solution to the current problem of underdiagnosis of chlamydia, but should be considered as an interim measure while funding for the use of better tests are sought. As the detection rate using pooled urethral/endocervical swabs was significantly higher than endocervical swabs, the use of pooled specimens in females should be considered.

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