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PCR and Direct Fluorescent-Antibody Staining Confirm *Chlamydia trachomatis* Antigens in Swabs and Urine below the Detection Threshold of Chlamydiazyme Enzyme Immunoassay

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In order to test the hypothesis that specimens blocking with a neutralizing reagent below the cutoff of the Chlamydiazyme enzyme immunoassay represent infected patients, we used direct fluorescent-antibody staining for elementary bodies (EBs) and PCR to confirm results for cervical swabs collected from 55,963 women and urethral swabs or first-void urine (FVU) samples collected from 5,781 men attending physicians' offices in the Toronto, Canada, area. Within a grey zone arbitrarily selected to represent values up to 40% below the positive threshold of the test run, 134 cervical swabs, 44 urethral swabs, and 39 FVU specimens exhibited a blocking response (>50% reduction in signal). Three or more EBs were observed in each of 98 cervical swabs (73.1%), 38 urethral swabs (86.4%), and 21 FVU specimens (53.8%). Of the 36 cervical swabs with fewer than three EBs, 33 were PCR positive; the positive PCR results for male specimens were 6 of 6 urethral swabs and 17 of 18 FVU samples. Application of the blocking test to specimens negative in the Chlamydiazyme enzyme immunoassay but having optical densities within 40% of the cutoff added 14.2% (217 of 1,531 specimens) more positive results to the survey. A total of 213 of 217 samples (98.2%) were reconfirmed as having EBs or DNA.

Although amplification probes such as those used in PCR or ligase chain reaction have demonstrated great accuracy in diagnosing *Chlamydia trachomatis* genitourinary infections in men (6, 8) and women (2, 11, 14), antigen detection assays have become established in many laboratories and because of low costs and ease of performance will probably continue to be used for screening. Before the development of confirmatory tests for positive antigen screening results, the Chlamydiazyme assay (Abbott Laboratories, North Chicago, Ill.), the first commercially available enzyme immunoassay (EIA), had a high positive cutoff value established in order to avoid the risk of reporting false-positive results. Since the manufacturer's introduction of a neutralizing blocking test (12), studies have shown variable false-positive rates (5, 13, 15), and a proportion of specimens have been shown to block below the cutoff value (3, 7, 9, 17). Confirmation of positivity for these grey zone low positive results has been shown by using direct fluorescent-antibody (DFA) staining (7, 9) or PCR (17).

Our study included a large number of cervical swabs from women and urethral swabs and first-void urine (FVU) samples from men. A substantial number of infected specimens would have been reported negative in this routine clinical laboratory if specimens with results 40% below the cutoff in the initial screening test had not been further examined in the blocking test and confirmed by DFA staining or PCR. The study reinforces the need for antigen detection confirmatory testing and provides an approach for increasing EIA sensitivity.

MATERIALS AND METHODS

Subjects. From 1 June to 30 September 1993, a total of 5,781 men and 55,963 women attending doctors' offices were entered in the study. The subjects were patients being examined for signs or symptoms of cervicitis or urethritis, contacts of symptomatic patients, patients without symptoms but with positive tests, or patients receiving routine gynecological examinations. In each case, a cervical swab (for females) or urethral swab or FVU sample (for males) was collected. The patients ranged from 15 to 40 years old. Ninety percent of the women were between the ages of 15 and 25, and 95% of the men were between 21 and 35 years old.

Specimen collection. Cervical swabs, urethral swabs, and FVU samples were collected as described previously (4, 16). The swabs (STD-EZE for women and STD-PEN for men) were supplied by Abbott Laboratories and after collection were placed in Chlamydiazyme transport tubes. The first 20 to 30 ml of FVU was also collected in the doctor's office and transported the same day, at 4°C, to the laboratory. Specimens were processed as described previously (4).

Chlamydiazyme. The Chlamydiazyme assay was performed according to the manufacturer's instructions, and readings were done with the Commander System (Abbott Laboratories). All samples found to be positive (specimen-to-control optical density [OD] ratio $\geq$ 1.0) or negative in a grey zone (an OD within 40% of the positive threshold) were retested immediately by using the blocking test (Abbott Laboratories) reagents and format. If the OD of a sample with the blocking reagent was reduced by 50% or more compared with that of the unblocked sample, the specimen was recorded as a confirmed positive. All specimens which were confirmed positive by the blocking test in the grey zone were subjected to DFA staining and/or PCR testing as described below.

DFA staining. The DFA staining test was performed on material left in the Chlamydiazyme transport tubes. The DFA test (Microtrak-Syva) was performed according to the manufacturer's instructions after the specimens were centrifuged at 2,000 $\times$ *g* for 15 min. A specimen was considered positive if three or more elementary bodies (EBs) were observed in a fluorescence microscope at $\times$400 and confirmed under oil immersion. Specimens with zero to two EBs were processed for PCR as described below.

PCR. PCR was performed as described previously (10), using a KL1-KL2 plasmid primer set and 5 μl of Chlamydiazyme sample added to 45 μl of PCR master mix, with 40 cycles of amplification. If the initial sample was negative, it was diluted 1:10 to reduce PCR inhibition and retested. Specimens still negative at a 1:10 dilution were retested to check whether the negative result was due to a sampling error or persistent inhibition.

RESULTS

From 1 June to 30 September 1993, a total of 61,744 clinical specimens were submitted to Gamma North Peel Laboratory by physicians in the Toronto, Canada, area. Table 1 summarizes the specimen types and the results of screening with the Chlamydiazyme EIA. Following the screening of 55,963 cervical swabs, 1,876 (3.4%) were found positive and an additional 1,000 (1.8%) were in the grey zone. Although there were

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TABLE 1. Distribution of clinical specimens submitted to a private laboratory for *C. trachomatis* testing between June and September 1993[a]

Sex of patients	Type of specimen	No. screened	No. (%) positive	No. (%) in grey zone[b]
Female	Cervical swab	55,963	1,876 (3.4)	1,000 (1.8)
Male	Urethral swab	3,035	333 (11.0)	42 (1.4)
	FVU	2,746	241 (8.8)	34 (1.2)

[a] All specimens were screened by the Chlamydiazyme EIA.
[b] Grey zone specimens had ODs which ranged from the cutoff of the screen to 40% below that level.

higher screening positivity rates for urethral swabs and FVU specimens (11.0 and 8.8%, respectively) from males, the rates in the grey zone were similar (1.4 and 1.2%).

Table 2 summarizes the outcomes of blocking of the 1,876 cervical swab specimens that were positive and the additional 1,000 swabs that were in the established grey zone after screening. The blocking test was able to confirm 57.0% (1,070 of 1,876) of the original positive results by reducing by 50% or more the OD above the cutoff. An additional 3.0% (56 of 1,876 samples) blocked within the grey zone. An additional 7.8% (78 of 1,000 samples) of the original grey-zone specimens also blocked. Further examination by DFA staining of the 134 cervical swabs found 98 (73.1%) with three or more EBs. An additional 33 were positive by PCR, and 3 others were PCR negative. One of the three PCR-negative specimens had two EBs as determined by DFA staining, and the other two had none. Table 2 also shows that the confirmatory rate for specimens above the positive threshold for male urethral swabs was higher than that for cervical swabs (87.4 versus 57.0%). An additional 4.2% (14 of 333) of the original positive urethral swabs and 71.4% (30 of 42) of the original grey zone urethral swab specimens were blocked below the cutoff. Thus, the grey zone confirmation procedure added an additional 44 positive urethral swabs, of which 38 (86.4%) were DFA staining positive and the other 6 were positive by PCR. Similar results were achieved for FVU samples (Table 2), with 70.5% (170 of 241 specimens) clearly confirmed by blocking above the cutoff and an additional 39 blocked in the grey zone (21 were DFA staining positive, 17 were PCR positive, and 1 was PCR negative).

There were 60 specimens with fewer than three EBs which neutralized in the grey zone, and PCR identified 56 of them as positive. Ten of the 56 (17.9%) required 1:10 dilution to be PCR positive. Five of these were urine specimens, four were cervical swabs, and one was a urethral swab.

TABLE 2. Testing of outcomes for various sample types by Chlamydiazyme blocking assay, DFA test, and PCR

Specimen type and category[a] (*n*)	No. (%) of specimens blocking[b]		No. of confirmed specimens blocked below cutoff		
			DFA test[c]	PCR	
	Above cutoff	Below cutoff		Positive	Negative
Cervical swab (55,963)					
Screened positive (1,876)	1,070 (57.0)	56 (3.0)	43 (76.7)	11	2
Grey zone (1,000)		78 (7.8)	55 (70.5)	22	1
Total (2,876)	1,070	134	98 (73.1)	33	3
Urethral swab (3,035)					
Screened positive (333)	291 (87.4)	14 (4.2)	13 (92.8)	1	0
Grey zone (42)		30 (71.4)	25 (83.3)	5	0
Total (375)	291	44	38 (86.3)	6	0
FVU (2,746)					
Screened positive (241)	170 (70.5)	23 (9.5)	13 (56.5)	10	0
Grey zone (34)		16 (47.0)	8 (50.0)	7	1
Total (275)	170	39	21 (53.8)	17	1

[a] Grey zone specimens had ODs which ranged from the cutoff of the screen to 40% below.
[b] Blocking confirmation required a 50% or more reduction in the OD between the specimen mixed with blocking reagent and the specimen mixed with an equal volume of control reagent.
[c] Considered positive if more than three EBs present.

DISCUSSION

In this study, a large number of specimens sent to a clinical laboratory were used to examine several aspects of screening and confirmatory testing with the Chlamydiazyme assay. The higher initial positivity rates for specimens from males than for those from females may be a reflection of swab specimens not normally being collected from men unless the men have symptoms. Since this was an internal laboratory study, we were unable to document the presence or absence of symptoms in a sufficient number of cases to allow meaningful analysis regarding symptoms.

Performing the blocking test on specimens above the cutoff and all specimens 40% below the cutoff in the Chlamydiazyme EIA is similar to lowering the threshold for positivity. This maneuver increased the number of specimens that required confirmation from 2,450 to 3,526. The extra positives that were identified totalled 217, an increase of 14.2% (217 of 1,531). Kellogg et al. (9) showed a comparably increased rate of 14.6% with cervical swabs from 0.03 to the cutoff in the Chlamydiazyme test. By lowering the cutoff to three times the negative control, Chan et al. (3) blocked an extra 104 male urethral swabs and increased the positivity rate by 12.9%; for cervical swabs, they blocked an additional 560 specimens and increased the positivity rate by 3.6%. Working with cervical swabs, Schwebke et al. (15) lowered the OD ratio of the Chlamydiazyme test from 1.0 to 0.3 and showed that DFA testing of 5% of the total number of specimens raised the sensitivity of the Chlamydiazyme test from 73 to 83%. In our study, blocking specimens occurring in a grey zone extending to 40% below the cutoff required an additional 1,076 specimens (1.7% of the total) to be tested. For cervical swabs, this raised the prevalence from 1.9% (1,070 of 55,963) to 2.2%, an increase of 134 more women (Table 2). For men, the prevalence rates increased from 9.6% (291 of 3,035 specimens) to 11.0% (335 of 3,035 specimens) for urethral swabs and from 6.2% (170 of 2,746) to 7.6% (209 of 2,746) for FVU specimens.

Counting EBs in specimens blocking below the cutoff, Chan et al. (3) showed that 65.7% of male urethral swabs (46 of 70) and 73.6% of cervical swabs (14 of 18) had at least one EB. In our grey zone, DFA staining (more than three EBs) confirmed the results for 73.1% of cervical swabs (98 of 134), 86.4% of urethral swabs (38 of 44), and 53.8% of FVU specimens (21 of 39) (Table 2). PCR was required to confirm positivity most often for FVU specimens and least often for urethral swabs.

Williams and coworkers (17), using a PCR with primers targeting a 16S rRNA gene of *C. trachomatis*, showed that 12

of 44 specimens (27.3%) with ODs between 0.5 and 0.9 were positive by PCR, and 8 of these (66.6%) were positive by the Microtrak-Syva DFA test. In our study, we performed a plasmid-based PCR on a total of 60 clinical specimens which were in the grey zone and had fewer than three EBs as determined by DFA staining. All but four were PCR positive, and 44.6% (25 of 56) had one or two EBs. Although PCR has been reported to be more sensitive than DFA testing or EIA (1, 8), the issue of inhibitors of PCR, some of which may disappear after freezing and thawing, has also been raised (2, 11). All of our specimens were frozen and thawed before PCR testing, but 10 needed to be diluted 1:10 before becoming positive in the PCR.

The Chlamydiazyme false-positive rates caused by blocking above the cutoff in this study were 42.9% (804 of 1,876 specimens) for cervical swabs, 12.6% (42 of 333) for male urethral swabs, and 29.5% (71 of 241) for male FVU samples. Chan et al. (3) reported false-positive rates of 20.5% for cervical swabs and 6.5% for male urethral swabs. These two rates are higher than those reported previously (5) but agree with differences according to gender and specimen type. Contaminating bacteria with lipopolysaccharides are thought to be found more often in female specimens.

Lowering the cutoff value in the Chlamydiazyme EIA increased the number of specimens requiring confirmatory testing. Using the blocking test in the grey zone also increased the number of infections identified. When the validity of the blocking which took place in the grey zone was tested with DFA staining as the only confirmatory test (with more than three EBs), the DFA procedure missed 26.9% of the cervical swabs (36 of 134), 13.6% of male urethral swabs (6 of 44), and 46.2% of male FVU samples (18 of 39). All of these specimens were tested by PCR, and 56 were found positive. This 40% lower threshold level can be used to determine which specimens should be selected for the Chlamydiazyme blocking assay, with the assurance that those blocking in the grey zone are truly positive 98.2% of the time.

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