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Effect of Differences in Specimen Processing and Passage Technique on Recovery of *Chlamydia trachomatis*

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We have previously found that optimum recovery of *Chlamydia trachomatis* in microdilution plate culture required multiple blind passages. However, others have found this not to be the case for culture in vials. In the present study, the effect on recovery of the use of vials (as opposed to microdilution plates) and the effect of vortexing, sonication, or both were compared. Three different passage techniques were also evaluated. Vortexing or sonication resulted in equivalent recoveries. However, compared with vortexing alone, a combination of vortexing and sonication increased recovery from 95 (78%) to 114 (94%) of 121 positive specimens ($P = 0.002$). In multiple-passage experiments, the combination of vortexing and sonication, compared with vortexing only, increased the proportion of isolates recovered with no more than a single passage from 81 to 96%. Substitution of vials for microdilution plates increased recovery with only a single passage to >96%, irrespective of whether sonication was employed. The most sensitive technique for single-passage technique was one using blunt scraping of cell monolayers with passage of two monolayers to one. The sensitivity of cell culture for *C. trachomatis* is highly dependent on the technique(s) employed. However, the combination of sonication and vortexing of clinical specimens enhanced recovery in microdilution plates, and a single blind passage did so in both microdilution plates and vials. Consideration should be given to their use for routine clinical cultures.

Although it is generally accepted that the sensitivity of culture for *Chlamydia trachomatis* is less than 100% (8, 13), absolute estimates have been difficult to obtain. Factors which affect culture include the type of swab used to obtain the culture, the transport conditions, the number of sites sampled, and the culture conditions themselves (3, 4, 8, 11, 13). Moreover, culture is the standard against which other diagnostic tests are measured, and in most instances it appears to be more sensitive (6, 7, 10, 16, 17). We have observed a substantial increase in the sensitivity of tissue culture in microdilution plates when one or more blind passages are included as part of the procedure (8). However, others have not observed this phenomenon to the same degree for microdilution plate or vial cultures (15). This led us to reexamine several aspects of our culture technique and to identify two factors not previously described which affect the recovery of *C. trachomatis* from clinical specimens. These were (i) the specific methods employed for blind passage and (ii) the effect of vortexing or sonication (or both) of clinical specimens prior to tissue culture inoculation. The former was evaluated because of the above-cited discrepancies between results obtained in our laboratory and elsewhere, and the latter was evaluated on the basis of the premise that while vortexing might be effective in dislodging elementary bodies or cells from a swab and in suspending them, sonication was more likely to be effective in rupturing infected cells and in breaking up clumps of elementary bodies.

MATERIALS AND METHODS

Specimen collection and processing. Clinical specimens were obtained from men and women considered to be at high risk for chlamydial infection (12). Endourethral swabs were obtained from men, and endourethral and endocervical swabs were obtained from women. Each swab was placed in a screw-cap glass vial containing 1.5 ml of transport medium and two 3-mm glass beads (Curtin Matheson Scientific, Inc., Indianapolis, Ind.) and was maintained at 4°C until tissue culture inoculation or processing for storage at −70°C. The transport medium used was phosphate-buffered sucrose (5% with respect to fetal calf serum) (7). Each transport vial was vortexed for 5 s on a desktop Vortex Genie Mixer (American Scientific Products, Evanston, Ill.) at a speed and control setting of 10. When sonication was employed, a sonic dismembrater (model 300; Artek Systems Corp., Farmingdale, N.Y.) with a 50-ml titanium cup tip at 60% power output was used. Five specimen vials at a time were placed in ice-water in the cup tip of the sonicator and sonicated for 20 s. When vortexing followed by sonication was compared with vortexing alone, the specimen was first vortexed and then divided into two samples of approximately equal volume; the specimen vial was recapped, and the sample still containing the swab was sonicated. Samples of these divided specimens were then used to inoculate cell monolayers for tissue culture.

Culture technique. Culture in 96-well microdilution plates was performed as previously described (8). Each of three McCoy cell monolayers in microdilution wells was inoculated with 0.1 ml of specimen, and then 0.2 ml of tissue culture medium was added. Centrifugation was performed at 1,750 × *g* at 30°C for 1 h. Plates were then incubated at 35°C for 72 h, after which one monolayer from each specimen was assessed for inclusion formation by indirect immunofluorescence with a genus-reactive monoclonal antibody (8). The medium from the two unstained monolayers was aspirated, and 0.05 ml of transport medium was added to each. The cells were scraped and disrupted with a Pasteur pipette which had been sealed and blunted by heating in a flame. These two suspensions were then aspirated, pooled, and used to inoculate a fresh monolayer of McCoy cells in a

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TABLE 1. Summary of experimental conditions

Expt no.	Comparison(s)[a]	Specimen handling	Culture vessel	Passage multiplicity	Passage ratio	No. of specimens
1	V vs S	V or S	Plates	Single	2:1	276
2	V vs V and S	V or V and S	Plates	Single	2:1	677
3	V vs V and S in plates and vials	V or V and S	Plates or vials	Multiple	2:3	448
4	Passage methods (stored specimens)	V and S, freeze and thaw	Vials	Single	2:1, 1:1, or 0.5:1	50
5	Passage methods (fresh specimens)	V and S	Plates	Single	2:1 or 1:1	669

[a] Abbreviations: V, specimen vortexed; S, specimen sonicated.

single well. Next, 0.2 ml of tissue culture medium was added, and centrifugation was performed as described above. After an additional 72 h of incubation, this monolayer was also stained and examined for inclusion formation.

For multiple passage, the procedure was modified as follows: three monolayers were initially inoculated, one was stained, and the tissue culture medium was aspirated from the remaining two. Transport medium (0.15 ml) was added to each well, the contents were scraped as before, and the suspensions from two wells were pooled and used to inoculate three fresh monolayers. These monolayers were incubated for 72 h, one was stained, and the suspensions from the other two were passaged to three fresh monolayers as described above. This procedure was repeated once more for a total of three blind passages.

Vial cultures were handled identically except that the tissue culture monolayers which were inoculated were on 13-mm glass cover slips in flat-bottom centrifuge tubes (vials) (7), and each monolayer was inoculated with 0.1 ml of specimen, after which 1.0 ml of culture medium was added. Triplicate vials were inoculated for each specimen. The vials were centrifuged at 3,000 × *g* for 1 h at 30°C, incubated at 35°C for 72 h, and one monolayer was stained for inclusions. The monolayers on the remaining two cover slips were scraped, suspended, pooled in 0.1 ml of medium if there was to be only one passage or in 0.3 ml of medium if there were to be multiple passages, and used to inoculate fresh monolayers.

To control for possible cross contamination in the multiple-passage experiment, control monolayers were inoculated with sterile transport medium and randomly interspersed with specimen monolayers such that there was a ratio of approximately one control monolayer for each two specimen monolayers. Also, the initial inoculation was done by a different technician than the one who subsequently passaged and read the specimens, and the technician who performed the passages and evaluated the stained monolayers was blinded as to which wells had been inoculated with sterile medium and which ones had been inoculated with patient specimens. No inclusions were identified in any of these control monolayers or in monolayers inoculated with material from them during passage.

Experimental conditions. Experimental conditions are summarized in Table 1 and involved comparisons of recovery of organisms from samples of specimens which had been vortexed, sonicated, or both prior to inoculation of monolayers in either microdilution plate wells or vials as well as comparisons of passage conditions. Except for experiment 4, in which stored specimens were used, all specimens were inoculated onto monolayers within 4 h from the time they were obtained from patients.

Three different methods of conducting blind passage were evaluated in experiment 4 with aliquots of clinical specimens which had been placed in storage at −70°C at the same time as another sample had been cultured in microdilution plates with a single passage. All specimens had been vortexed and sonicated before being divided for culture or storage at −70°C. Samples of 50 specimens, 30 of which had been culture positive and 20 of which had been culture negative, were thawed, divided, and used to inoculate monolayers in vials. Technique A was our standard technique as described above for vials. For technique B, vials were inoculated in duplicate. After 48 h of incubation, one monolayer was stained and the other monolayer was scraped for passage with a P-1000 Pipetman (Rainin Instrument Co., Woburn, Mass.) without aspirating the overlying medium. The resulting suspension then was transferred onto a new monolayer. Centrifugation was performed, overlying medium was aspirated, fresh tissue culture medium was added, and the monolayer was incubated for an additional 48 h and stained. Technique C also involved the inoculation of two monolayers, one of which was stained after 72 h of incubation. The vial containing the other monolayer was incubated for an additional 24 h. It was then vortexed for 60 s in order to disrupt the monolayer and suspend it, and 0.5 ml of the resulting suspension was used to inoculate a fresh monolayer. The inoculum was centrifuged, the medium was aspirated, new medium was added, and incubation continued for 72 h, after which time the monolayer was stained and evaluated. The technician evaluating monolayers for presence or absence of inclusions was blinded as to the technique employed.

Cell rupture. In order to obtain a crude estimate of the relative efficiency of rupturing cells of each of the different methods used for passage, a comparative study was performed. Uninfected McCoy cell monolayers in 10 vials were processed as for passage by each of the techniques described above. Monolayers in 10 control vials were incubated with trypsin for 3 min, the reaction was stopped by the addition of a Ca^{2+}-containing solution (7), and the cells were suspended by gentle swirling. Each suspension was then evaluated microscopically in a hemacytometer chamber, and the number of cells which appeared to be intact was enumerated.

Statistical methods. Comparisons between culture methods for numbers of isolates recovered were performed by using McNemar's test for marginal homogeneity. In addition, comparisons between methods involving three categories of results (negative, positive initially or after only one passage, and positive only after more than one passage) were performed by using a test for marginal homogeneity (5). Fisher's exact test (two-tailed) was used to compare the proportion of isolates recovered at later as opposed to earlier passages. Comparisons of cell rupture by different techniques were performed by using a one-way analysis of

TABLE 2. Effect of vortexing and sonicating specimens and culture in vials versus plates on passage number at which *C. trachomatis* was first identified

Specimen treatment and culture type	No. (%) of specimens first positive at passage no.:			
	0	1	2	3
Vortexing				
Plates (n = 98)	57 (58)	22 (22)	12 (12)	7 (7)
Vortexing and sonication				
Plates (n = 108)	72 (67)	32 (30)	3 (3)	1 (1)
Vials (n = 106)	81 (76)	24 (23)	1 (1)	0

variance followed by a Tukey multiple comparison procedure to test for pair-wise differences.

RESULTS

Comparison of vortexing with sonication and with vortexing followed by sonication. Duplicate endocervical specimens were obtained from 276 women, with one specimen being vortexed and the other being sonicated (experiment 1). Although we observed previously that sampling order does not affect recovery from the endocervix (8), the order of specimen collection was alternated each week such that one week the first specimen was vortexed and the second specimen was sonicated, and the order was reversed the next week. A total of 72 chlamydia-positive women were identified, with 67 specimens positive after vortexing and 69 specimens positive after sonication. Specimens from five women were positive after sonication but not after vortexing, and specimens from three women were positive after vortexing but not after sonication. Eighty-eight percent of specimens were positive on initial reading and twelve percent were positive only after passage by both techniques. Subsequently, we compared vortexing alone with vortexing followed by sonication on 677 urogenital specimens from 295 men (all urethral specimens) and 240 women (143 endocervical, 227 urethral, and 12 combined specimens) (experiment 2). A total of 121 specimens were positive by one or both techniques, with 114 positive after vortexing and sonication versus 95 positive after vortexing alone (P = 0.002).

Passage in vials and plates after vortexing or after vortexing and sonication. A total of 448 urogenital specimens and 216 controls were processed as indicated in Table 2. They consisted of 268 urethral specimens from men, and 88 endocervical and 92 urethral specimens from 94 women. Male and female urethral specimens tend to have lower numbers of inclusion-forming units and to become positive later than endocervical specimens (8). However, for the purposes of the present study, specimen source was not considered germane and data for endocervical and female urethral specimens are not presented separately. A total of 114 specimens were positive by one or more of the techniques used; 108 (95% of 114) were detected in microdilution plate culture after vortexing and sonicating versus 98 (86% of 114) after vortexing only (P = 0.009). Moreover, vortexing and sonicating resulted in a higher proportion (104 of 108 [96%]) of isolates being recovered in plate culture with no more than one passage than was observed when only vortexing was performed (79 of 98 [81%]; $P < 0.001$). For samples on which both vortexing and sonication were performed, essentially the same number of isolates were recovered with subsequent culture in plates as in vials. Also, with vortexing and sonication followed by vial culture, 105 of 106 (99%) of isolates were recovered with no more than one passage. This was not significantly different from the result observed with the vortexed and sonicated specimens in plates (P = 0.37). In a separate experiment (data not shown), culture in vials and plates was compared for samples of 124 urogenital specimens which were vortexed only. Forty of these were positive in both culture systems. However, only one (2.5%) vial culture was first positive after more than one passage, in contrast to seven (17.5%) plate cultures positive after more than one passage (P = 0.041).

TABLE 3. Effect of passage technique on recovery of *C. trachomatis* from stored specimens

Technique[a]	No. (%) of specimens first positive:	
	Without passage	After one passage
A (n = 30)	18 (60)	11 (37)
B (n = 30)	16 (53)	7 (23)
C (n = 30)	16 (53)	7 (23)

[a] See Materials and Methods for details.

In addition to the above comparisons, we also assessed the passage number at which each sample of a given specimen first became positive as a function of the manner in which the sample was processed or cultured (experiment 3). When results were compared for samples of specimens that had been cultured in plates but had been vortexed only or both vortexed and sonicated, 62 of the samples from the same specimen first became positive on the same passage. However, 30 samples that were vortexed only were positive at a later passage than was the sample from the same specimen that was both vortexed and sonicated. Conversely, only five samples that were vortexed and sonicated were positive at a later passage than the sample from the same specimen that was vortexed only ($P < 0.001$). Samples from 11 specimens were positive after vortexing and sonicating but negative after vortexing only. In contrast, for only one specimen was the vortexed-only sample positive and the vortexed-and-sonicated sample negative, and that one was positive only after three passages. When culture in vials was compared with culture in plates for samples all of which had been vortexed and sonicated, 79 were positive on the same passage, 16 were positive first in vials, and 4 were positive first in plates (P = 0.39). There were seven samples positive in vials but not in plates and nine samples positive in plates but not in vials.

Comparison of passage techniques. In experiment 4 (Table 3), 29 of the 30 previously positive specimens were again positive by technique A. Techniques B and C yielded both a lower recovery and a lower proportion positive after passage. Five of the six specimens that were detected by technique A but were missed by techniques B and C were the same specimens, and all five were detected by technique A only after passage. Techniques B and C each missed two specimens that the other technique of the two detected, with technique C detecting one specimen missed by both techniques A and B. None of the 20 specimens which were negative before storage were positive after storage. The recovery observed with technique A was different from that observed with technique B (P = 0.041) and marginally different from that observed with technique C (P = 0.077) Technique A was also compared with technique B by using samples from freshly obtained clinical specimens in microdilution plates (experiment 5). Both 48- and 72-h incubation periods were used with technique A, and a 48-h incubation period was used with technique B. However, the incubation

period did not affect recovery. Of 669 urogenital specimens, 95 were positive by technique A as opposed to 80 by technique B ($P < 0.001$). Twenty-one positive specimens were identified only after passage by technique A versus ten positive by technique B ($P = 0.11$).

Cell rupture. When separately assessed, the number of intact-appearing uninfected cells remaining after suspension by each of the techniques used for passage was as follows (in 10^5 cells per ml [mean ± standard error]): technique A, 6.0 ± 1.8; technique B, 11.4 ± 4.7; technique C, 9.7 ± 2.8; and controls, 29.4 ± 5.9. The number of intact cells remaining after technique A was significantly ($P < 0.05$) lower than that remaining after technique B but was not significantly lower than that remaining after technique C.

DISCUSSION

Until recently, sonication was only employed in selected circumstances to suspend elementary bodies (1, 9), whereas vortexing of clinical specimens was the recommended (2) and most widely used method. The failure to employ sonication more frequently has been due in large part to the difficulty of sonicating large numbers of specimens with available probe tips. However, cup tips have recently become available, allowing the contents of specimen vials to be effectively sonicated without opening the vial and introducing a probe. Using such a system, Warford et al. (19) found that sonication produced an increase in inclusion counts but not an increase in isolation rates. Likewise, in the present study we found that sonication, when used alone, gave recovery rates equivalent to those observed with vortexing. However, when clinical specimens were subjected to vortexing followed by sonication, the recovery of *C. trachomatis* was clearly enhanced and the proportion of isolates recovered without passage or after only a single passage was greatly increased. In addition, the proportion of isolates recovered without passage or after only a single passage was greatly increased when culture was performed in vials as opposed to in microdilution plates, regardless of whether or not the specimen had been sonicated. Thus, the results obtained are consistent both with our previous observations on enhanced recovery with microdilution plate culture and multiple blind passages (8) and with those of Schachter and Martin (15), who reported that multiple passage in vial culture does not materially affect recovery from urogenital specimens.

Although vial culture and vortexing in combination with sonication greatly reduced the number of specimens identified as positive after more than one passage, they did not reduce the number or proportion that were positive after a single passage. Because others (15, 18) using comparable culture techniques have not seen as high a proportion positive after passage, we attempted to compare different passage techniques. Those chosen were the one employed in our laboratory (technique A), another technique commonly used for microdilution plate culture (technique B), and one of the techniques described by Schachter and Martin (15) (technique C). Technique A resulted in recovery of more organisms from known positive specimens than did either of the other two techniques, and in a separate comparison with technique B, gave greater recovery from fresh clinical specimens. Moreover, much of the increased recovery was a result of additional positive specimens being detected after passage. The major differences between technique A and the other two is the use of a blunt scraping device to both detach and disrupt the cells before passage and the fact that two monolayers are passaged to one as opposed to one to one in technique B and 0.5 to one in technique C. Both factors are probably important. Visual inspection indicated effective removal of the monolayer by all three techniques. However, the blunt scraping was more effective in disrupting cells (and probably inclusions) than either of the other two techniques, although in uninfected cells this difference reached statistical significance only when it was compared with technique B.

Still, the mechanisms by which some of these differences in technique affect the recovery of *C. trachomatis* are not entirely clear. Presumably, the increased disruption of cells in the clinical specimen by vortexing and sonication causes increased release and dispersion of viable elementary bodies, and by this mechanism enhances recovery in the initial monolayer. Similarly, vial culture offers a substantially larger surface area than does microdilution plate culture and permits a higher speed of centrifugation, both of which enhance recovery (14). However, the mechanism by which the increased disruptive force applied to the specimen would result in increased recovery after passage is more difficult to explain. It is quite likely that any given clinical specimen which contains *C. trachomatis* contains a mixed population of organisms, some of which are capable of producing detectable inclusions, while others of which are unable to do so until after one or more passages. Perhaps the latter have been damaged by host factors such that they require a recovery period before they are able to replicate rapidly enough to produce a visible inclusion. In any case, to the extent that vortexing and sonication increase the release and dispersion of both infective and damaged organisms, they would increase the likelihood of recovery both on the initial monolayer, and, failing that, after passage. The observation that specimens which were sonicated tended to be positive on the same or earlier passages than specimens which were not is consistent with such a hypothesis.

These data further demonstrate that recovery of *C. trachomatis* after passage is a highly reproducible phenomenon in our laboratory, and they illustrate the degree to which the sensitivity of tissue culture for *C. trachomatis* is dependent upon minor variations in laboratory technique. They also show that substitution of vials for microdilution plates or subjecting clinical specimens to both vortexing and sonication prior to microdilution plate tissue culture inoculation greatly reduces the enhanced recovery associated with multiple passage. Under either circumstance a single blind passage is sufficient to identify >96% of specimens that would be positive even with multiple passages. Consequently, more than one blind passage is not likely to be cost-effective. Because vortexing and sonication with a cup tip are easily done, as is a single blind passage, laboratories performing chlamydial cultures should consider using them, at least to the extent of evaluating them in their own culture system.

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