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Pooling of Urine Samples for Screening for *Neisseria gonorrhoeae* by Ligase Chain Reaction: Accuracy and Application

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The accuracy of detection of genital *Neisseria gonorrhoeae* infection in pooled urine samples by ligase chain reaction (LCR) was examined in three populations. Firstly, urine specimens from 300 female military recruits (FMR) were tested by LCR individually and in pools of four and six. Secondly, 300 urine specimens from middle-school students (MSS) were tested individually by LCR, and then the processed specimens were stored frozen for subsequent testing in pools of 4 and 10. Thirdly, 600 frozen urine specimens from high-school students (HSS) were tested by using the LCR pooling algorithm, i.e., testing processed specimens in pools of four in one test unit dose, and retesting individual specimens from positive pools. Finally, the pooling algorithm results were compared to culture results for a subset of 344 students from the original 600 HSS from whom cervical or urethral samples were taken at the discretion of the school nurse practitioners. Compared to individual testing of specimens by LCR in the FMR population, the pooling-by-four algorithm was 100% sensitive (5 of 5) and 100% pool specific (70 of 70), and the pool-by-six algorithm was 100% sensitive (5 of 5) and 100% pool specific (45 of 45). In the MSS population, the pool-by-4 algorithm was 95.8% sensitive (23 of 24) and 100% (52 of 52) pool specific, and the pool-by-10 algorithm was 95.8% sensitive (23 of 24) and 100% (17 of 17) pool specific. In the subset of 344 HSS from whom endocervical or urethral specimens were collected for culture, 31 were positive by LCR in urine and 26 were positive by culture. After results discrepant between culture and LCR were adjudicated by a confirmatory LCR test, the pooling algorithm was 93.8% (30 of 32) sensitive and 99.7% (311 of 312) specific. Culture from these 344 HSS was 81.3% (26 of 32) sensitive. The pooling algorithm reduced the cost of the *N. gonorrhoeae* LCR assay by 60% compared to individual testing of the HSS specimens and was both sensitive and specific.

Twenty-two states in the United States still report gonorrhea rates above the Healthy People 2000 national objective of 100 cases or fewer per 100,000 persons (3). Furthermore, in certain geographic regions and among non-Hispanic blacks, the gonorrhea rate is up to 10 times higher than the national goal (3). It is estimated that annually there are 62 million incident gonorrhea cases worldwide (15), of which 800,000 occur in the United States (5).

Neisseria gonorrhoeae infections are frequently asymptomatic, particularly in women (5). Women are at risk of developing long-term sequelae from the infection, including pelvic inflammatory disease, chronic pelvic pain, ectopic pregnancy, and infertility. Perinatal transmission can lead to ophthalmia neonatorum (5). Treatment of gonorrhea reduces genital shedding of human immunodeficiency virus type 1 (HIV-1) in coinfected males (2), and there is epidemiologic evidence of decreased HIV transmission efficiency when coinfected subjects receive gonorrhea treatment (9). Routine screening and treatment of high-risk individuals could prevent transmission to sexual partners, sequelae due to infection, and perinatal transmission and could reduce sexual transmission of HIV.

The traditional "gold standard" for detection of *N. gonorrhoeae* has been culture; however, culture requires clinician-obtained cervical or urethral specimens and strictly controlled conditions during transportation of specimens (10, 14). The ligase chain reaction (LCR) test has been shown to be highly sensitive and specific for the detection of *N. gonorrhoeae* infection (1, 13). One advantage of LCR is that it can be used to detect gonorrhea in first-catch urine (FCU), which avoids invasive sample collection procedures. The sensitivities and specificities of LCR of FCU are 98.0 and 100% in men and 94 to 95 and 100% in women, respectively (12, 13). The reported sensitivity of culture of cervical- or urethral-swab samples compared to LCR of FCU for the detection of *N. gonorrhoeae* was 95.9% in men but only 84 to 95% in women (12, 13). The LCR test of FCU targets the *opa* gene, which has as many as 11 copies per *N. gonorrhoeae* genome, thus increasing sensitivity (11).

Although the cost of LCR is higher than that of culture, an algorithm of testing processed specimens in pools and then retesting specimens from presumptively positive pools individually could significantly decrease the assay cost of this expensive diagnostic test, as has been shown for *Chlamydia trachomatis* LCR (6). In this study, we examined the accuracy of procedures involving pooling of FCU specimens for the detection of *N. gonorrhoeae* by LCR compared to individual testing of specimens by LCR. We then applied the pooling algorithm, i.e., testing processed specimens which had been collectively pooled into one single test unit dose and retesting individual specimens from each positive pool, to determine the prevalence of gonorrhea in a high-school population. Additionally, we compared the results of the urine LCR pooling algorithm for the detection of *N. gonorrhoeae* with those of culture for a subset of patients from whom cervical- or urethral-swab samples had been taken for culture.

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TABLE 1. Characteristics of study specimens tested

Population	No. tested by gender		Pool size(s) tested and testing order	Specimen tested
	Female	Male		
FMR	300	0	1	Never frozen
			4	Never frozen
			6	Never frozen
MSS	260	40	1	Never frozen
			4	Frozen processed
			10	Frozen processed
HSS[a]	483	117	Pool by 4 algorithm[b]	Frozen unprocessed

[a] Of these, 344 (322 females and 22 males) were also cultured for *N. gonorrhoeae*.

[b] The pooling algorithm is a procedure in which processed specimens are tested by pooling them into one unit test dose and retesting individual specimens from positive pools only. All specimens from negative pools are considered to be negative.

MATERIALS AND METHODS

Study populations, specimen handling, and testing. As part of ongoing studies designed to prevent pelvic inflammatory disease, FCU specimens were collected from three study populations. Subjects were asked to collect 15 to 20 ml of urine from the first part of the urine stream, after having not urinated for at least 2 h. A variety of pretesting storage conditions and populations were studied to determine the effect of freezing of both unprocessed urine and processed urine on the accuracy of the pooling algorithm, as well as the effect of the prevalence of *N. gonorrhoeae* infection (Table 1).

FMR. FCU specimens ($n = 300$) from young adult female military recruits (FMR) were processed and tested individually for detection of *N. gonorrhoeae* by LCR. Processed samples were stored at 4°C and were retested 2 to 3 days later in 75 pools of four and in 50 pools of six. Thus, these samples were never frozen (Table 1).

MSS. Three hundred (never frozen) FCU specimens from female and male middle-school students (MSS) were processed and tested individually during 1996 to 1997, and the remaining processed specimens were stored at −70°C. After that school year's collection was completed, the frozen processed specimens were thawed and retested in 75 pools of 4 and 30 pools of 10. Thus, these samples were frozen after processing (Table 1).

HSS. FCU specimens ($n = 600$) from sexually active female and male high-school students (HSS) were collected during the 1996-to-1997 school year and stored as unprocessed urine at −70°C. For a subset of 344 subjects, at the time of the visit for urine specimen collection, a cervical or urethral swab was obtained, at the discretion of the school nurse practitioners, for culture of *N. gonorrhoeae* by standard methods (10, 14). After the school year's collection was completed, the urine samples were thawed, processed, and tested for gonorrhea by LCR in 150 pools of four. Processed urine specimens from presumptively positive pools (i.e., pools with LCR sample/cutoff ratios [S/CO] of ≥0.8) were retested individually to complete the pooling algorithm. Thus, these urine samples were frozen before the processing step (Table 1).

Pooling algorithm definition. The pooling algorithm is a two-step testing procedure whereby specimens are first tested in pools of two or more in a single test unit dose. Specimens from pools which test negative (S/CO $<$ 0.8) are all considered negative. Specimens from positive pools are retested individually to determine which specimen(s) in the pool is (are) positive. The number of specimens tested in each pool is dependent on the prevalence of the organism in the population and may range from 2 to 10.

Urine specimen processing for LCR. Urine specimens from the three study populations were collected, transported, and processed according to the manufacturer's instructions for the urine-based LCR assay for *N. gonorrhoeae* (Abbott Laboratories, Abbott Park, Ill.). One milliliter of each urine specimen was centrifuged at $\geq$9,000 $\times$ *g* for 15 ± 2 min at room temperature. The supernatant was removed, and the pellet was resuspended into 1.0 ml of LCR urine specimen resuspension buffer and vortexed. Preparations were then heated at 97 ± 2°C for 15 ± 1 min to extract the DNA.

LCR assay setup, DNA amplification, and detection. Specimens were amplified individually by LCR, according to the manufacturer's instructions. When specimens were tested individually, a volume of 100 μl of each processed urine specimen was placed into its own LCR gonorrhea amplification vial (unit dose). For pooling by four, 25 μl of each of the four processed specimens was placed into a single unit dose. For pools of six, 17 μl of each of the six processed specimens was placed into a single unit dose. For each pool of 10, 10 μl of each of 10 processed specimens was placed into a single unit dose. The total volume of the specimen(s) was then 100 μl for each unit dose. Two negative controls, two positive calibrators, and a positive processing control were included in every amplification run in accordance with the manufacturer's instructions.

Unit dose tubes containing DNA preparations were amplified in an LCR thermocycler (Abbott Laboratories) under the following conditions: 40 cycles of denaturation (at 93°C for 1 s), annealing (at 59°C for 1 s), ligation (at 62°C for 1 min 10 s), and soaking (at 25°C). Amplified DNA was detected in an LCR automated machine which performed a particle-based enzyme immunoassay with a fluorescent signal. For individually tested samples, an S/CO of ≥1.2 was considered positive, and borderline-negative samples (S/CO of ≥0.8 and $<$1.2) were retested, as specified by the manufacturer. Retested specimens were considered positive if the S/CO was ≥1.2. For the pooling algorithm, specimens from pools with S/CO of ≥0.8 were retested individually as described above.

Culture. Urethral swabs from males and endocervical swabs from females were cultured at the Maryland State Health Laboratory on selective medium (Thayer-Martin agar) by standard techniques (8). Suspicious colonies were tested by an oxidase assay and a Gram stain. Oxidase-positive, gram-negative diplococci were confirmed by a direct fluorescent antibody (DFA) test (Syva, San Jose, Calif.) as *N. gonorrhoeae*. If the DFA was negative, two additional tests, a latex agglutination test (Gonogen; Becton Dickinson, Cockeysville, Md.) and a carbohydrate fermentation test (Quadraferm; Biomerieux, Marcy l'Etoile, France), were used to identify *N. gonorrhoeae* isolates.

Adjudication of discordant pooling algorithm LCR and culture results for HSS. Culture-positive specimens for *N. gonorrhoeae* were considered to be true positives. Discrepant specimens which were culture negative and urine LCR positive were retested by a second urine LCR using different probes (targeting the pilin gene) by Abbott Laboratories. If the pilin gene LCR was positive, the discrepant specimen was resolved as a true positive. If the pilin gene LCR was negative, the original positive LCR was considered to be a false positive. The positive gold standard or positive "patient infection status" was considered to be either a positive culture or a culture-negative, LCR-positive result that was resolved as a true positive by the pilin gene LCR test. Discrepant specimens which were culture positive and LCR negative were retested by the original *opa* gene LCR and were also tested by the pilin gene LCR, although the results were not used for adjudication, i.e., the original LCR-negative results were considered false negatives.

Cost analysis. A model was developed to determine the pool size that yielded the greatest cost savings. Binomial distribution was used to estimate the number of pools that would be likely to be positive given a selected pool size and population disease prevalence. Next, the optimal pooling number for a range of disease prevalences was calculated. For a dichotomous outcome (i.e., a positive or negative test result for a genital *N. gonorrhoeae* infection), independence was assumed (i.e., the order of the samples received was random with regard to the distribution of the positive or negative samples in the population). The expected percentage of positive pooled assays was determined by the equation $s = [1 - (1 - r/n)^c] \times 100\%$, where s is the expected number of positive pools, r is the number of positive samples tested, n is the total number of samples tested, r/n is the prevalence of disease, and c is the number of specimens pooled (7). This equation accounted for the probability that 1 to c samples in the pool were positive. The calculated cost of the amplification unit dose per individual LCR test was $6.32, which included the cost of positive calibrators, negative controls, and positive processing controls (6).

RESULTS

Sensitivity and specificity of the pooled assays in FMR and MSS populations. Testing of processed (never frozen) FMR specimens pooled by four was 100% sensitive (5 of 5) and 100% pool specific (70 of 70) compared to individual testing. Testing of processed FMR specimens pooled by six was also 100% sensitive (5 of 5) and 100% specific (45 of 45). Testing of MSS specimens which were stored frozen and pooled by four was 95.8% sensitive (23 of 24) and 100% (52 of 52) pool specific. Testing of MSS specimens pooled by 10 was 95.8% sensitive (23 of 24) and 100% (13 of 13) specific (Table 2). Although in the MSS group the pool-by-4 and pool-by-10 testing algorithms each missed one positive specimen (1 of 24), each algorithm missed a different specimen.

Comparison of testing of specimens by using the LCR pooling algorithm with culture for HSS. The prevalence of *N. gonorrhoeae* in the subset of 344 HSS by the pooling algorithm was 7.1% (23 of 322) in females and 36.4% (8 of 22) in males (Table 3). The prevalence of *N. gonorrhoeae* by culture was 5.9% (19 of 322) in females and 31.8% (7 of 22) in males. Two female subjects who had culture-positive specimens had urine specimens which were negative by the LCR pooling algorithm. For the two female subjects who were culture positive and LCR negative, the repeat testing by LCR targeting the *opa* gene was positive, as was the LCR targeting the pilin gene.

TABLE 2. Accuracy of detection of genital *N. gonorrhoeae* infection by LCR testing of pooled urine samples compared to individual testing of specimens

Parameter	Result for:			
	Fresh urine specimens[a]		Frozen processed urine specimens[b]	
	Pool by 4	Pool by 6	Pool by 4	Pool by 10
Total no. of specimens	300	300	300	300
No. of pools	75	50	75	30
No. of positive specimens/ total specimens (%)	5/300 (1.7)	5/300 (1.7)	24/300 (8.0)	24/300 (8.0)
Sensitivity of pooling algorithm[c]	5/5 (100)	5/5 (100)	23/24 (95.8)	23/24 (95.8)
Specificity[d] of pooled assays	70/70 (100)	45/45 (100)	55/55 (100)	13/13 (100)

[a] From FMR.
[b] From MSS.
[c] The pooling algorithm tests all samples pooled and tests samples from presumptively positive pools individually. Sensitivity is defined as the number of confirmed positive results/total number of positive specimens (percentage).
[d] Number of confirmed negative pools/total number of negative pools (percentage).

Seven subjects (six female and one male) had urine specimens that were positive by the LCR algorithm but had negative culture specimens. Six of the seven discrepant results (from five females and one male) were adjudicated as true positives, and one was not confirmed and was considered to be a false positive after testing by the LCR targeting the pilin gene. The specimen was also negative when retested by the original LCR targeting the *opa* gene. After the resolution of discrepant results, the performance characteristics of the pooling algorithm for the subset of 344 HSS specimens cultured for gonorrhea were 91.7% (22 of 24) sensitivity and 99.7% specificity for females and 100% (8 of 8) sensitivity and 100% specificity for males (Table 4). Culture from these 344 HSS was 79.2% (19 of 24) and 87.5% (7 of 8) sensitive for females and males, respectively (Table 4).

Cost savings and public-health implications. By using only the LCR pooling algorithm, the prevalence of *N. gonorrhoeae* in the entire HSS population was 6.5% (39 of 600). In the subset of 344 specimens which were also cultured, 30 of 31 (96.8%) LCR-positive specimens were adjudicated as true positives. However, screening of the 256 students who were not tested by culture detected eight additional LCR-positive specimens, seven of which were confirmed by a second LCR test targeting a different gene (i.e., the pilin gene). The unconfirmed positive specimen was considered to be a false positive.

Thus, the pooling algorithm identified 37 positives which could be confirmed, detected 2 positives which could not be confirmed, and failed to detect 2 confirmed positives. Cultures performed in the HSS population at the discretion of the nurse practitioners detected only 26 positives among 344 students cultured. Thus, screening everyone by using the urine LCR pooling algorithm detected 11 more of the 39 confirmed positives than culturing selected patients only. When the pooling algorithm was used, 306 assays were performed to test 600 specimens, including the retesting of positive pools. The overall assay cost per specimen tested with the pooling algorithm was half (51%) the assay cost per specimen tested individually. Figure 1 demonstrates the expected unit dose cost savings at different population prevalences.

DISCUSSION

Pooling of processed urine specimens for detection of *N. gonorrhoeae* by LCR produced accurate results compared to results of individual testing of specimens by LCR. The cutoff ratio of the LCR test for the pooled samples was not reduced from the cutoff for individual specimens, as was necessary in the pooled chlamydia testing described previously (6). The high sensitivity and specificity of LCR were not affected by pooling of as many as 10 samples, whether they were stored frozen or were never frozen. Freezing of processed or unprocessed urine samples had no effect on the accuracy of testing of pooled samples. Consequently, the pooling algorithm could be used in laboratories with a high volume of samples or in laboratories conducting epidemiological research where specimens are stored frozen and tested at a later time.

It is unknown why one LCR pooling algorithm-positive specimen was unable to be adjudicated as a true positive. This specimen was pool positive and individual test positive but negative when tested later for adjudication by both the *opa* and the pilin gene LCR. *N. gonorrhoeae* from cervical or urethral swabs of patients with low levels of infection seems less likely to be cultured successfully. Similarly, low levels of target DNA in processed specimens could be less likely to be able to be amplified over time and after multiple cycles of freezing and thawing. Although it cannot be determined, it is possible that this specimen was from a patient with a low organism load. Because it was unable to be adjudicated, it was considered to be a false-positive result in this study according to our definition of a true positive.

A theoretical concern is that pooling would dilute the low-level positive sample below the limit of detection of the assay. However, review of the manufacturer's data presented in the package insert from individually tested specimens ($n = 3,362$) indicated that "low-positive" samples (with S/CO of <2.0) constituted only 3.3% of positive specimens. In the FMR and MSS data presented in this paper, none of the specimens were low positives.

A potential limitation of the pooling algorithm is the chance

TABLE 3. Comparison of pooled algorithm testing of urine by LCR with culture for the detection of *N. gonorrhoeae* after analysis of discrepancies for 344 HSS

Test, specimen, and result	No. (%) of HSS with result	No. of HSS with the following resolved infection status[a]	
		Positive	Negative
Females ($n = 322$)			
Urine LCR			
Positive	23 (7.1)	22	1
Negative	299 (92.9)	2	297
Cervical culture			
Positive	19 (5.9)	19	0
Negative	303 (94.1)	5	298
Males ($n = 22$)			
Urine LCR			
Positive	8 (36.4)	8	0
Negative	14 (63.6)	0	14
Urethral culture			
Positive	7 (31.8)	7	0
Negative	15 (68.2)	1	14

[a] A student was considered positive if the culture was positive, or if a culture-negative and LCR-positive specimen was confirmed as positive by repeat LCR testing of the urine with probes for the pilin gene.

TABLE 4. Resolved performance characteristics of the LCR urine pooling algorithm and culture for the detection of *N. gonorrhoeae* after analysis of discrepant results[a]

Reference standard[b]	Sensitivity[c] (%)	Specificity[d] (%)	PPV[e] (%)	NPV[f] (%)
LCR pooling algorithm				
Female urine specimens	22/24 (91.7)	297/298 (99.7)	22/23 (95.7)	297/299 (99.3)
Male urine specimens	8/8 (100)	14/14 (100)	8/8 (100)	14/14 (100)
Culture				
Female cervical swabs	19/24 (79.2)	298/298 (100)	19/19 (100)	298/303 (98.3)
Male urethral swabs	7/8 (87.5)	14/14 (100)	7/7 (100)	14/15 (93.3)

[a] n = 344 (322 females and 22 males).
[b] A student was considered positive if the culture was positive, or if there was a negative culture and a positive urine LCR test which was confirmed positive by repeat LCR testing of the urine with probes for the pilin gene.
[c] Number of confirmed positive results/total number of positive specimens.
[d] Number of confirmed negative results/total number of negative specimens.
[e] Positive predictive value, defined as number of confirmed positive results/number of specimens testing positive.
[f] Negative predictive value, defined as number of confirmed negative results/number of specimens testing negative.

for technician error in the pooling of processed samples in the LCR run. The use of tray maps simplifies this process. We have used the following process for eliminating technician error. Samples are be organized by skipping a space after each pool group in the specimen rack. Thus, pooling adds no significant complexity to the process of setting up individual unit dose assays. Additional technician error can be avoided when samples from presumptively positive pools (detected in the previous run) are retested individually at the beginning of the batch before the routine testing of the new pool groups. Therefore, each run has a combination of samples that are retested individually and new pooled samples from the next group of specimens.

Pooling is a technique which could be used in high-volume laboratories such as state public-health labs and reference labs for significant cost savings. Public-health screening programs which are currently using culture can benefit from the ease of specimen collection, higher sensitivity, and lower cost of pooled LCR. Specific populations or laboratories that might benefit from pooling include any laboratory where, as a minimum, both turnaround time and volume allow for a combination of 19 pools and retests per day. With 96 specimens at a population prevalence of about 4%, pooling by 6 would allow for the completion of one full run (38 test unit doses) per day. The run would theoretically include, on average, 16 pools of 6 and 22 individual retests.

Use of the pooling algorithm could benefit investigators and program planners in two ways: (i) money saved by using the pooling algorithm could be applied to other areas of disease prevention, and/or (ii) the amount of money allocated to screening would allow more specimens to be tested for the same total cost. Pooling of urine samples for the detection of genital *N. gonorrhoeae* infection is a cost-saving strategy, simple to perform, and could be applicable in screening programs in the United States and in population-based research worldwide. In addition, a combined chlamydia and gonorrhea detection program which uses pooling of processed urine specimens for LCR testing could be used in populations at significant risk for both pathogens and would detect most infections for less cost, since the same processed urine specimen can be used for both the chlamydia and gonorrhea LCR tests. Although not considered here, technician cost can be estimated as previously described in detail for LCR pooling for the detection of chlamydia (12). Running specimens pooled for both chlamydia and gonorrhea testing by LCR would most significantly reduce technician time, specimen processing costs, and LCR assay costs.

Laboratory managers should consider two points before using pooling. First, processed specimens from presumptive positively pools need to be amplified and detected individually. This additional step adds a minimum delay of 3 h to the laboratory turnaround time until individual test results on specimens in presumptively positive pools are known. Second, the estimated cost savings to be gained for a particular laboratory depend on a combination of the salaries of technicians and their benefits, institutional overhead, and the prevalence of gonorrhea in the populations the laboratory serves. Pooling of samples from patients in a population where the prevalence of gonorrhea may be 20% or greater is not advised and would be minimally cost saving. The pooling algorithm would be cost saving at lower prevalences of infection.

The study laboratory has met Clinical Laboratory Improvement Act requirements for the modification of a manufacturer's package insert directions for performance of a test by a clinical laboratory using a diagnostic kit cleared by the Food and Drug Administration. The investigators considered the performance and documentation of the required study adequate for using the pooling algorithm protocol in testing of clinical specimens in the study laboratory. Each laboratory that wishes to introduce pooling must meet the requirements set forth to modify the package insert from a test cleared by the

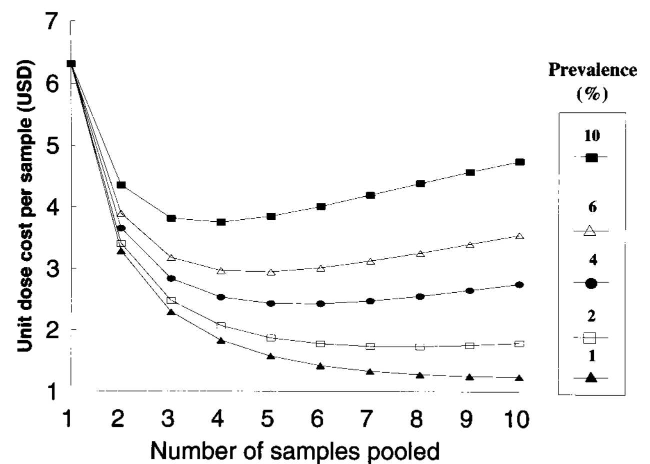

FIG. 1. Cost-saving ability of pooling of processed urine specimens before performance of the urine LCR test for the detection of *N. gonorrhoeae* infections. The graph shows the cost in U.S. dollars (USD) per amplification unit dose when the pooling algorithm was used, depending on the number of specimens per pool and taking into account various prevalences of infection in the population screened. A baseline total cost of $6.32 per unit dose was used.

Food and Drug Administration. These requirements are explained more fully as regulations set forth in the Federal Register (4).

Pooling of processed urine samples for LCR testing of *N. gonorrhoeae* will decrease the cost of screening, providing more evidence to health planners that screening programs can and should be implemented. An additional application of pooling of urine specimens by LCR is the detection of genital *C. trachomatis* infections (6). The cost savings of pooling of urine for both *N. gonorrhoeae* and *C. trachomatis* should also be considered. Although LCR failed to detect two cervical culture-positive specimens, this strategy of screening everyone in a population by testing urine specimens detected 11 more of the 39 true positives (28.2%) than the strategy of performing culture on specimens collected from the portion of females who received pelvic examinations where cervical swabs were taken or on specimens from males where urethral swabs were obtained due to their clinical presentation of signs and symptoms. Screening of urine from sexually active students by using the pooling algorithm was more sensitive (92 and 100%) than culture (79 and 88%) in women and men, respectively, and more cost saving than performing individual LCR assays for *N. gonorrhoeae*.

In conclusion, the LCR urine pooling algorithm for the detection of *N. gonorrhoeae* was accurate compared to testing of specimens individually and selective culturing of specimens, and it could be used as a cost-saving public-health measure for screening of populations at risk for gonorrhea, especially when a cervical or urethral swab cannot be obtained.

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