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INVESTIGATIVE REPORT

Pooling Samples: the Key to Sensitive, Specific and Cost-effective Genetic Diagnosis of *Chlamydia trachomatis* in Low-Resource Countries

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The aims of this study were to compare the performance characteristics and cost-effectiveness of pooling endocervical samples for screening and diagnosis of *Chlamydia trachomatis*, and to investigate the prevalence of *C. trachomatis* infection in women in Leningrad Oblast, Russia. A total of 1500 endocervical samples were tested individually and when pooled in groups of 5 and 10 samples, respectively. A previously evaluated in-house diagnostic polymerase chain reaction (PCR) assay was utilized. The sensitivity and specificity of the PCR were not affected by either pooling strategy. The estimated prevalence of genital *C. trachomatis* infection was 6.6%, 6.1% and 6.0% based on individually tested samples, and pools of 5 and 10, respectively. For diagnosis of individual samples, the pooling strategies resulted in cost savings of 53.3% (5 samples per pool) and 44.0% (10 samples per pool). Pooling samples for PCR detection of *C. trachomatis* is an accurate and cost-saving approach for diagnosis and large-scale prevalence studies in St Petersburg, Russia. ***Key words: Chlamydia trachomatis; diagnostics; PCR; NAAT; pooling; Russia.***

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In 1999, approximately six million people in Eastern Europe were infected with genital *Chlamydia trachomatis* (1). In Russia, the reported incidences of chlamydial infections vary significantly between different regions. However, due to suboptimal laboratory diagnostics and incomplete case reporting and epidemiological surveillance, the reported incidences are presumably not reliable (2).

More than half of chlamydial infections in women may be asymptomatic and these individuals may not be identified without specially designed screening programs. Furthermore, improved control of genital chlamydial infections can also reduce the transmission of other sexually transmitted infections (STIs) including human immunodeficiency virus (HIV) (3).

In Russia, there are no local, regional or national screening programs for genital chlamydial infections. It is recommended to test pregnant women, infants, children, and socially unsupported persons attending outpatient gynaecological, paediatric, or skin and venereal disease departments for STIs. However, because of the lack of resources, the implementation of this decision is limited. The cheapest test systems, such as Russian produced, sub-optimally evaluated and quality assured antigen and/or nucleic acid detection assays, may be funded by the state. Consequently, the laboratory assays used for diagnosis of genital chlamydial infection in Russia have to be optimized, standardized and quality assured (2) to be utilized effectively in comprehensive and broad screening programs.

Pooling can be a cost beneficial approach for screening and diagnosis of *C. trachomatis* in both male and female samples (4–6). It allows estimation of population-based prevalence, which is needed to design screening programs for specific core or target groups and for individual-based prevalence, which form the basis for implementing effective intervention strategies. Pooling strategies could be highly applicable in resource-poor countries, such as the post-Soviet countries, e.g. Russia.

The objectives of the present study were: (*i*) to evaluate the sensitivity and specificity of an in-house polymerase chain reaction (PCR) using pooled cervical samples in comparison with testing the samples individually; (*ii*) to investigate the prevalence of *C. trachomatis* infections in the general female population of Leningrad Oblast, Russia; and (*iii*) to estimate the cost-effectiveness of pooling strategies.

MATERIALS AND METHODS

Study population

Leningrad Oblast is a region comprising a territory of 83,900 km^2 that surrounds the city of St Petersburg, Russia. Leningrad Oblast has approximately 1.6 million inhabitants, out of which approximately 387,000 are females of reproductive age.

In the present study, endocervical samples obtained from 1500 asymptomatic females attending 16 gynaecological departments in Leningrad Oblast, Russia for ordinary gynaecological check-

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ups were analysed at the Laboratory of Microbiology, DO Ott Research Institute of Obstetrics and Gynecology RAMS, St Petersburg, Russia. The inclusion criteria for enrolment in the study were: (*i*) not being pregnant; (*ii*) not having used antibiotics during the last 6 months; and (*iii*) being of reproductive age. Informed consent was obtained from all patients.

The study was approved by the Local Institutional Review Board at DO Ott Institute of Obstetrics and Gynecology and the Russian Academy of Medical Sciences (RAMS), St Petersburg, Russia.

Specimen collection and storage

Endocervical samples were collected using Dacron swabs, which were placed in empty 5-ml plastic tubes. Specimens were kept at 4°C for up to 4 days before they were shipped to the laboratory. At the laboratory, samples were stored at 4°C and tested within 1–3 days.

Isolation of genomic DNA

Processing of individual samples. Isolation of genomic DNA was performed according to the manufacturer's instructions (DNA-express, Lytech, Moscow, Russia) with minor modifications. Briefly, 1 ml of 0.9% saline was added to the specimen, the specimen was vortexed and 100 µl aliquot was centrifuged at 13,000 × g for 10 min. Subsequently, the supernatant was discarded and 200 µl of lysis buffer was added. After being vortexed for 15 s, the samples were incubated at 95°C for 10 min, centrifuged at 13,000 × g for 15 s, and finally the supernatant was used as template in the PCR.

Processing of pooled samples. The pooling of samples was carried out by combining 100 µl aliquots from 5 and 10 consecutive samples to yield a total volume of 500 and 1000 µl, respectively. After centrifugation of the pooled samples at 13,000 ×g for 10 min, the subsequent steps of the procedure were performed as described above for the individual samples.

Polymerase chain reaction

A previously evaluated in-house PCR (2), which amplifies a 273-bp fragment of the *C. trachomatis* cryptic plasmid, was used in accordance with the manufacturer's instructions (Lytech). In brief, 5 µl DNA template was amplified in a total reaction volume of 25 µl using a thermocycler Thercyc (DNA-technology, Moscow, Russia) and the following parameters: 94°C for 5 min, followed by 35 cycles of 94°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The amplification was ended with an extension step at 72°C for 5 min. To visualize amplified products, 1.5% agarose gel electrophoresis with ethidium bromide staining was performed. DNA extracted from a *C. trachomatis* reference strain D/UW-3/Cx (ATCC VR-885) was included in each PCR run as a positive control, and distilled water as a negative control. To determine the size of the amplicons, Gene Ruler DNA Molecular Weight Marker (Fermentas, Kaunas, Lithuania) was used.

In order to ascertain the validity of negative results, i.e. identify the presence of PCR inhibitors, an internal control (IC) comprising a 227-bp sequence with annealing sites for the *C. trachomatis* primers was included in the PCR master mix. In case of inhibition, dilution of the sample was performed according to the manufacturer's instructions (Lytech). In brief, 100 µl of the processed sample was mixed with 200 µl of lysis buffer; the sample was vortexed for 15 s, incubated at 95°C for 10 min, centrifuged at 13,000 × g for 15 s, and finally the supernatant was used as template in a new PCR. For validation of positive results, all positive samples and pools were re-tested.

Estimation of population prevalence based on pooled samples

The estimation of the population prevalence was performed as described previously (7). Consequently, the following equations were used: (*i*) $p = \{1 - [1 - (s/n)]^{1/c}\} \times 100\%$; (*ii*) $SD = \{\{s/n[1 - (s/n)]^{(2/c)-1}\}/c^2n\}^{0,5} \times 100\%$; (*iii*) 95% CI = $p \pm (2\sqrt{SD})$, where p is the estimated prevalence, SD is standard deviation, 95% CI is 95% confidence interval, s is the total number of positive pools, n is the total number of pools, and c is the number of specimens in each pool.

Cost analysis

At the Laboratory of Microbiology, DO Ott Institute of Obstetrics and Gynecology, RAMS, the cost of diagnostic *C. trachomatis* PCR of one sample including test kits, consumables, technician's salary and other related costs is US$2.6. In the present study, the potential cost saving percentages (CS) associated with a reduction of the number of tests due to the pooling strategies were calculated as previously described (5), i.e. $CS = n - (P + pp)/n \times 100\%$, where P is the number of tests required to screen all the pools, pp is the number of tests required to identify the positive samples in each of the positive pools, and n is the total number of tests.

RESULTS

Sensitivity and specificity using pooled cervical samples

Out of the 1500 cervical samples, 99 were positive for *C. trachomatis*. Two individual samples were repeatedly negative in the IC; however, these samples were not inhibited after being diluted.

Out of the 300 pools (5 samples per pool), 80 were positive in the *C. trachomatis* PCR. Out of these 80 pools, 65 contained 1, 11 contained 2, and 4 contained 3 PCR-positive samples. Out of the 150 pools (10 samples per pool), 69 were positive in the PCRs. Out of these 69 pools, 46 contained 1, 18 contained 2, 3 contained 3, and 2 contained 4 PCR-positive samples. Furthermore, none of the pools were negative in the IC.

All positive individual samples and pools were confirmed in the re-testing. Consequently, PCR of pooled cervical samples (pools of 5 or 10) comprised 100% sensitivity in comparison with individual testing, i.e. all the pools containing positive samples were positive and 100% specific, and no false positive pools were detected.

Population prevalence

The prevalence of *C. trachomatis* in asymptomatic women in Leningrad Oblast, Russia determined by testing individual samples was 6.6% (99 out of 1500). The estimated prevalence when specimens were tested pooled in groups of 5 and 10 was highly similar, i.e. 6.1% (95% CI 4.5–7.7) and 6.0% (95% CI 4.3–7.7), respectively (Table I).

Cost analysis

For the estimation of the population prevalence based on pooled samples, without further testing of the posi-

tive pools, pooling endocervical samples by 5 reduced the costs by 80% (300 vs. 1500), and pooling by 10 decreased the costs by 90% (150 vs. 1500). For the diagnosis of individual cases, the pooling strategy resulted in cost savings of 53.3% and 44.0% when samples were pooled by 5 and by 10, respectively (Table I).

DISCUSSION

Molecular diagnostic methods for genital chlamydial infections are increasingly utilized in the countries of Eastern Europe (2, 8, 9). The costs for such analyses and performance quality of the different assays used significantly vary in these countries, mainly depending on the origin of the test kits, i.e. whether they are national diagnostic products or imported kits. Genital chlamydial infections are, to a great extent, asymptomatic, especially in women, and these infections can be detected mainly by actively looking for the infected individuals in the entire population or in defined target groups. However, screening programs do not exist in most East European countries, mainly because effective test kits are too expensive. Most importantly, the chlamydial control programs that have been developed and implemented in some other countries are considered to have a significant effect on the prevalence of *C. trachomatis* infections (10–13).

Recently, highly sensitive and specific molecular methods for diagnosis of *C. trachomatis* have opened the door for large-scale low-cost screening. In the present study, pooling of cervical samples proved to have identical sensitivity and specificity compared with individual testing using an in-house PCR assay for detection of *C. trachomatis*, which is in concordance with several previous studies (6, 14–16).

Table I. *Characteristics of pooling endocervical samples for detection of Chlamydia trachomatis using an in-house PCR in St Petersburg, Russia*

Parameter	Individual samples	Pooled by 5	Pooled by 10
Individual cervical samples or pools tested (*n*)	1500	300	150
Positive pools (*n*)		80	69
Samples or pools inhibited (*n*)	2	0	0
Samples to be tested totally (*n*)	1502	700[a]	840[b]
Positive samples revealed (*n*)	99	99	99
Cost to detect one positive[c] (US$)	39.5	18.4	22.1
Estimated prevalence (95% CI)	6.6	6.1 (4.5–7.7%)	6.0 (4.3–7.7%)
Cost savings for population-based screening (%)		73.4%	54.0%
Cost savings for diagnosis of individual samples (%)		53.3	44.0
Sensitivity (%)		100	100
Specificity (%)		100	100

[a]Sum of 300 pools and individual samples from positive pools (80 × 5).
[b]Sum of 150 pools and individual samples from positive pools (69 × 10).
[c]Number of samples tested × price per test/number of positive detected.
CI: confidence interval

Interestingly, two samples seemed to contain PCR inhibitory substances, i.e. they were repeatedly negative in the IC when individually tested. However, none of the pools of samples was inhibited. This has earlier been observed using urine samples (17). As previously described (18–21), pre-treatment procedures, such as dilution of the samples, storage in refrigerator/freezer, resuspending dried endocervical swabs in saline, delayed testing, etc., can decrease the number of PCR-inhibited samples.

Population-based *C. trachomatis* prevalence is considered to be a prerequisite for making a cost analysis to determine whether a screening program should be initiated in a certain population. Pooling urine specimens has been shown to give an accurate estimate of the population prevalence (22, 23). In the present study using endocervical specimens, the *C. trachomatis* prevalence determined by individual testing (6.6%) was very similar to, and clearly within 95% CI of, the estimated prevalence, i.e. 4.5–7.7 and 4.3–7.7, when pooling samples by 5 and by 10, respectively. In addition, the cost savings associated with using the pooling strategies for the estimation of the population prevalence were 73.4% and 54.0% when samples were pooled by 5 and by 10, respectively. Furthermore, pooling endocervical samples by 5 and by 10 in combination with subsequent individual re-testing for diagnosis of individual samples reduced the test costs by 53.3% and 44.0%, respectively. Given that the cost of the diagnostic in-house PCR used for *C. trachomatis* at the microbiology laboratory at Ott Institute, St Petersburg, Russia is US$2.6 per individual sample, pooling samples by 5 reduces the cost of the PCR assay to US$1.2 per individual sample.

Consequently, the present pooling strategy would be most useful and cost saving for diagnostics, especially at laboratories that process a large number of specimens and for large-scale studies aiming to estimate population-based prevalence of *C. trachomatis* infection. However, the application of this approach depends on: (*i*) the acceptance of the delay of the test result, i.e. the additional time needed to test individual samples from the positive pool and (*ii*) the prevalence of the population (4, 22, 23). Thus, the cost-effectiveness of utilizing pooling strategies is highly dependent on the prevalence of infection and the pool size used. As shown earlier (4), pooling is not cost-effective when the prevalence of disease is 20% or higher. However, when the prevalence is approximately 5%, a cost saving of more than 50% is possible. Overall, the introduction of pooling strategies into laboratory practice may require a revision of the laboratory logistics (22, 23) in order to avoid technical mistakes (22). Another possible drawback might be that a positive specimen is pooled with specimens containing inhibitors, which might require additional time to resolve.

In conclusion, the introduction of effective programs for control of genital chlamydial infections, especially in resource-poor countries, relies on the introduction of targeted screening of high-risk core groups or populations. Pooling of genital samples could help to overcome budgetary constrains in such countries, e.g. Russia, and make control programs for genital *C. trachomatis* infection feasible.

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