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Original Article

Chlamydia trachomatis screening in resource-limited countries – Comparison of diagnostic accuracy of 3 different assays

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Abstract

Introduction: Commercially available assays were evaluated in order to determine diagnostic accuracy of *Chlamydia trachomatis* specific tests for screening.

Methods: The study included 225 sexually active men and women, who were tested for genital chlamydial infection in Institute of Public Health Kragujevac. Three screening tests were used: direct immunofluorescence (DIF) and rapid lateral immunochromatographic test (RT) for qualitative detection of chlamydial antigens and immunoenzyme (ELISA) test for detection of serum levels of anti-chlamydial IgA and IgG antibodies. Diagnostic efficiency of these tests were determined in relation to results obtained by RT-PCR method.

Results: Statistical significance between the results obtained by RT-PCR as a gold standard and DIF, RT and ELISA were analyzed using chi-square (χ^2) test. Statistical analysis showed a significant difference between RT-PCR and analyzed screening tests: DIF (χ^2 = 303; $p < 0.001$), RT (χ^2 = 4.19; $p = 0.041$), serum IgA (χ^2 = 4.19; $p = 0.041$) and serum IgG (χ^2 = 67; $p < 0.001$) which indicates poor agreement between these tests. Large numbers of false positive (FP) and false negative (FN) results were observed for all tested assays. According to Youden's index, serum IgG and DIF testing demonstrated the most-balanced sensitivity-specificity rate. RT assay exhibits the highest expanded Youden's index, as well as the best overall diagnostic accuracy.

Conclusions: None of evaluated screening tests can be recommended as individual method for the diagnosis of acute infection. We suppose that RT-PCR is unlikely to be a cost-effective screening strategy within the Serbian health system.

Key words: *Chlamydia trachomatis;* acute infection; diagnostic methods.

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Introduction

Chlamydia trachomatis infection is one of the most common sexually transmitted bacterial infections in the world [1,2]. In 2016, a total of 1,598,354 cases were reported to the Centers for Disease Control and Prevention (CDC) [3]. Rates of chlamydial infections raised in all the parts of the United States in the period 2015-2016 [3]. The global rate of reported chlamydial infections in Europe is high, but shows stability. In the period from 2010 to 2014, the number of cases from 26 countries that were reported to the European Centre for Disease Prevention and Control increased from 358,489 to 396,128; however, there is a large variation between the countries and their reported rates [4].

Most of the chlamydial infections are asymptomatic and therefore undiagnosed and consequently untreated [5,6]. If *C. trachomatis* infection remains untreated, it can lead to serious consequences in reproductive tract in women including pelvic inflammatory disease (PID), tubal factor infertility as well as ectopic pregnancy [7,8]. Because of high prevalence and severe complications, CDC recommends annual screening of *C. trachomatis* in all sexually active adolescents and young women under 25 years old, for all pregnant women and all women with increased risk of infection due to risky sexual behaviour. CDC also recommends rescreening for all previously infected women three months after being treated for chlamydial infection [9].

The asymptomatic nature of infection, as well as the specific developmental cycle of *C. trachomatis* is the real challenge for establishing the diagnosis of acute chlamydial infection. Significant progress has been made in the field of diagnosing chlamydial infection in the last thirty years. Multiple laboratory testing options can be used to detect *C. trachomatis,* although some of them cannot be recommended for routine use [9,10].

C. trachomatis is an obligate intracellular bacterium and isolation in cell culture is the only test which can prove the presence of viable chlamydia in a patient's sample. This bacterial culture method, although rarely used nowadays, is still needed in some circumstances to prove a patient has *C. trachomatis* infection [11]. Isolation of *C. trachomatis* in cell culture shows high specificity, but at the same time there are several disadvantages, such as technical complexity and relatively high costs with relatively low sensitivity [12].

On the other hand, a number of commercial tests that are not based on cell culture are available today. Polyclonal antibodies for the detection of chlamydial lipopolysaccharide (LPS) or monoclonal antibodies for the detection of major proteins of the external membrane (MOMP) are used in chlamydial antigen detection assays. With a high specificity of 97-100%, the sensitivity of commercially available enzyme immunoassays (EIA) and direct immunofluorescence assays (DIF) ranges from 60-75% in relation to nucleic acid amplification tests (NAATs) [13]. Despite the high specificity, the unsatisfactory sensitivity and subjectivity of these tests means they are not recommended for routine testing of genital tract samples. Rapid lateral immunochromatographic test (RT) have similar diagnostic performances so their application in ambulance screening is being considered [11]. Despite the lower sensitivity (50–85%), RT as an easy-to-use, low-cost method which may provide an easy and reliable alternative in the detection of chlamydial infections, particularly in a developing countries [14,15]. Serological tests for the detection of antibodies specific to chlamydial MOMP are not recommended for the detection of chlamydial infections, except for neonatal infections and patients with tubal factor infertility [11,13].

Over the past decades, tests based on nucleic acid amplification have become available. NAATs are highly sensitive with a specificity comparable to cell culture. With the additional advantages of time saving and ease of sampling, NAATs can be implemented on a variety of clinical specimens particulary on non-invasive specimens such as urine and vulval swabs [16]. Therefore NAATs are considered as the method of choice for detecting of chlamydial infections in the developed countries [17].

The American and European Center for Disease Control and Prevention recommends NAATs as the only tests for the detection of genital tract infections caused by *C. trachomatis* in both men and women, with or without symptoms, because of their superior sensitivity, specificity and speed [9,10]. According to the same sources, isolation of *C. trachomatis* in cell culture and direct immunofluorescence can be used to diagnose acute genital chlamydial infection only in case where nucleic acid amplification techniques are unavailable for any reason.

However, the high costs of these tests along with the technical complexity related to the space, equipment and human resources means that most diagnostic laboratories in developing, low-income countries are unable to meet this standard. For these reasons, the majority of laboratories in Serbia are unable to use NAATs, and the diagnosis of acute chlamydial infection of the genital tract in more than 90% laboratories is based on direct immunofluorescence, serological or rapid tests.

The main objective of this study was to determine the diagnostic accuracy of direct immunofluorescence test, rapid lateral immunochromatogaphic test, and two serological tests for the detection of *C. trachomatis*.

Methodology

Study population

Prospective study was conducted from January 2015 to December 2016 in Kragujevac, Serbia. The study population included 225 sexually active individuals, both males and females, who were successively tested for genital chlamydial infection in Institute of Public Health Kragujevac. The study excluded all persons: (I) under the age of 18 years, (II) who had any illness, condition or other factor that could significantly affect the result of the assessment (pregnancy, menstruation, recent use of antibiotics or topical preparations during the previous 72 hours, co-infection with other pathogens, etc.), (III) who were already taking part in another clinical trial orrefused to participate in the study and (IV) who had any other circumstances that significantly inhibited their participation in the study. The study was approved by the Ethical Committee of the Institute of Public Health Kragujevac. In accordance with the Declaration of Helsinki, all the investigated patients signed the Ethical Committee approved informed consent and were in every respect informed about their examination.

Sampling and data collection

The samples were prepared under standard laboratory protocols. Two swabs were collected from all participants (cervical in women and urethral in men). The first swab was used for bacteriological and mycological examination, direct immunofluorescence (DIF) and rapid immunochromatographic (RT) tests for qualitative determination of anti-chlamydial antigens. The second swab was frozen at -20 °C for subsequent determination of specific sequences of chlamydia genome by RT-PCR test. A peripheral blood sample (3 mL) was also taken from all subjects, collected in polystyrene tubes, centrifuged at 400g and then serum samples were aliquoted and stored at −20 °C until further analysis. The serum samples were used to quantitate the serum levels of IgA and IgG antibodies to MOMP antigen of *C. trachomatis*.

Screening methods

Direct immunofluorescence test (DIF) for qualitative detection of chlamydial antigen

The Chlamydia Cel IF test is a commercially available rapid direct immunofluorescence test for the qualitative detection of chlamydial antigen in patients' samples (Cellabs Pty Lty, Brookvale, Australia). The samples were tested according to the manufacturer's instructions.

Rapid immunochromatogaphic test (RT) for qualitative detection of Chlamydia antigen

Chlamydia test card is a commercially available rapid chromatographic immunoassay for the qualitative detection of chlamydial antigen in patients' samples (ulti med Products GmbH, Ahrensburg, Germany). The samples were tested according to the manufacturer's instructions.

Determination of the serum level of the antibodies to the chlamydial MOMP antigen

The serum samples were used to quantitate the serum levels of IgA and IgG antibodies specific for MOMP antigens of *Chlamydia trachomatis.* Tested antibodies were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Euroimmun, Lubeck, Germany). Cut-off values were suggested by the manufacturer: RU/mlL ≥ 22 for IgG and S/Co ≥ 1.1 for IgA.

Diagnostic method

Real-time polymerase chain reaction (RT-PCR)

C. trachomatis Real-TM PCR kit is a commercially available nucleic acid amplification test for qualitative detection of *C. trachomatis* DNA in the clinical materials by means of real-time hybridization-fluorescence detection. The test was performed on a Sa Cycler-96 thermocycler according to the manufacturer's instructions (Sacace Biotechnologies, Como, Italy).

Diagnostic criteria

All patients were tested by all screening and diagnostic methods. In this study, the primary (independent) variable is the result obtained by RT-PCR test, whereas secondary (dependent) variables are the results obtained by DIF, RT and ELISA. Results obtained by screening tests were read by a researcher who was blinded to the results obtained from RT-PCR tests. The diagnostic accuracy of the screening tests was compared with the results obtained by the RT-PCR method representing the recommended diagnostic method (gold standard regarded as the best test under reasonable conditions) for the detection of acute chlamydial infection.

Statistical analysis

Variables were presented as frequencies of individual parameters (categories), and statistical significance of differences was evaluated by chi-squared test and Fisher exact test using free on-line calculator (http://www.physics.csbsju.edu/stats/). For diagnostic test evaluation MEDCALC statistical software was used. Statistical difference of $p < 0.05$ was considered significant.

Table 1. Percentage of positive and negative results in men and women by four chlamydial screening tests.

	Female (n = 146)		**Male (n = 55)**		**Total (n = 201)**		**Fisher Test**
	positive	**negative**	**positive**	**negative**	**positive**	**negative**	
DIF	45 (30.8%)	101 (69.2%)	15 (27.3%)	40 (72.7%)	60 (29.9%)	141 (70.1%)	0.379
RT	2 (1.4%)	144 (98.6%)	1 (1.8%)	54 (98.2%)	3 (1.5%)	198 (98.5%)	0.619
IgA	12 (8.2%)	134 (91.8%)	3 (5.5%)	52 (94.5%)	15 (7.5%)	186 (92.5%)	0.372
IgG	25 (17.1%)	121 (82.9%)	8 (14.5%)	47 (85.5%)	33 (16.4%)	168 (83.6%)	0.419
RT-PCR	6 (4.1%)	140 (95.9%)	3 (5.5%)	52 (94.5%)	9 (4.5%)	192 (95.5%)	0.467

n – number of patients; DIF - direct immunofluorescence; RT- rapid lateral immunochromatographic test; IgA and IgG – antibodies; PCR – polymerase chain reaction.

Results

Of the 225 subjects who were included in the study 55 were men aged 20-54 years (mean age 38.9 years) and 146 were women aged 20-62 years (mean age 35.4 years) had complete results for all screening and diagnostic tests. The statistical analysis of the results show that there is no statistically significant difference between testing results in men and women. All further analysis was performed in this cohort of 201 subjects since they had all diagnostic tests completed (while the other 24 subjects had not) (Table 1).

Using direct immunofluorescence test (DIF) we found 29.9% of patients tested positive for *C. trachomatis*, while 70.1% of the results were negative. By means of rapid immunochromatogaphic test (RT), 1.5% of patients were positive, while 98.5% were negative. The presence of serum IgA was found in 7.5% of cases, whereas 92.5% of tested patients were negative for IgA antibodies. Analyzing IgG we found that 16.4% of subjects were positive and 83.6% were negative for IgG antibodies. Using RT-PCR assay as gold standard we determined the presence of specific sequences of C. trachomatis gene in 4.5% cases, whereas in 95.5% the test result was negative.

For testing of statistical significance between the results obtained by RT-PCR as a gold standard and DIF, RT and ELISA chi-squared (χ^2) test were used. Statistical analysis showed a significant difference between RT-PCR and all four analyzed screening tests: DIF (χ^2 = 303; p < 0.001), RT (χ^2 = 4.19; p = 0.041), serum IgA (χ^2 = 4.19; p = 0.041) and serum IgG (χ^2 = 67; p < 0.001) which indicates poor agreement between these tests. As a consequence, a large number of false positive (FP) and false negative (FN) results were observed for all tested assays (Table 2). Analyzing DIF, we found 53/192 of FP results where RT-PCR negative findings were categorized by DIF as positive, and 2/9 of FN results where RT-PCR positive findings were negative by DIF. Considering RT we did not find any FP result since all RT-PCR negative findings were properly designated by RT; however, a large-scale of disagreement was observed against RT-PCR positive findings with 6/9 of FN results. Similarly, comparing the results of the RT-PCR with the results obtained by ELISA IgA, only 11/192 of FP, but 5/9 of FN results were found. Finally, ELISA IgG assay incorrectly classified 27/192 of RT-PCR negative findings as positive (FP), whereas 3/9 of RT-PCR positive findings were FN.

Table 2 also shows measures of diagnostic accuracy determined according to results obtained by RT-PCR as a gold standard. Generally, sensitivities associated with screening tests ranged from approximately 33% (RT) to almost 80% (DIF), with negative predictive value >95% for all assays. Although all evaluated assays have satisfactory high specificity RT demonstrated perfect sensitivity (100%) and convincingly showed the highest positive predictive value (100.0%), whereas other secreening test have much lower positive predictive values ranging from approximately 10-30%. To select the most relevant assay maintaining an accurate and well-balanced sensitivity–specificity value Youden’s index were calculated. According to Youden’s index, serum IgG and DIF testing demonstrate, although low, the most balanced sensitivity–specificity rate of ~50%. However, taking into account positive and negative predictive value as well, RT assay exhibit the highest expanded Youden’s index (130%), as well as the best overall diagnostic accuracy (97%).

Table 2. Diagnostic accuracy of four chlamydial screening tests.

Statistic	DIF	RT	IgA	IgG
True positive	77.8%	33.3%	44.4%	66.7%
True negative	72.4%	100%	94.3%	85.9%
False positive	27.6%	0%	5.7%	14.1%
False negative	22.2%	66.7%	55.6%	33.3%
Sensitivity (Sen)	77.8%	33.3%	44.4%	66.7%
Specificity (Spec)	72.4%	100.0%	94.3%	85.9%
Positive likelihood ratio	2.8	/	7.8	4.7
Negative likelihood ratio	0.3	0.7	0.6	0.4
Disease prevalence	4.5%	4.5%	4.5%	4.5%
Positive predictive value (PPV)	11.7%	100.0%	26.7%	18.2%
Negative predictive value (NPV)	98.6%	96.9%	97.3%	98.2%
Youden’s index	50.2%	33.3%	38.7%	52.6%
Youden’s index (exp.)	60.4%	130.3%	62.7%	69.0%
Accuracy	72.6%	97.0%	92.0%	85.1%

Youden’s index = (Sen+Spec)-100; Youden’s index (exp.) = (Sen+Spec+PPV+NPV)-200; DIF - direct immunofluorescence, RT- rapid lateral immunochromatographic test, IgA and IgG – antibodies.

Discussion

Undiagnosed and consequently untreated *C.trachomatis* infections can lead to serious complications and consequences in the reproductive tract [7, 8]. Early diagnostics of an acute chlamydial infection is exceptionally important, but highly challenging due to its asymptomatic nature and unique development cycle of *C.trachomatis*. A large number of tests for the diagnosis of chlamydial infection are available today. According to recommendations of American and European Center for Disease Control, NAATs are highly recommended for detection of genital tract infections caused by *C. trachomatis* due to their high sensitivity, specificity and performance speed.

Our study provides theoretical basis for practical recommendations regarding to the selection of screening tests in the detection of an acute chlamydial infection in cases where nucleic acid amplification techniques are inaccessible for any reason. One potential limitation of our study is detection bias. Namelly, NAATs are evidently better for urine samples than cervical/urethral swabs and first void urine is recommended first choice specimen for men. In this study we used urethral swabs for chlamydia detection in male patients which may influence chlamydia detection. However, our study was designed so the screening tests and gold standard (RT-PCR) are performed using the same sample. Although the number of positive cases may be underestimated, the diagnostic accuracy of the screening tests can be directly compared to the results obtained from the gold standard. Another obstacle could be sexual history of patients, as samples selected for chlamydia testing would depend on the sexual orientation of the patient. Yet, we assumed that one could doubt the validity of a sexual history as patients in Serbia may not be honest due to social norms and stigmas. While this part is interesting, we consider that the absence of these anamnestic data can not affect the diagnostic accuracy of the tests and validity of the study.

Based on a careful analysis of the results of individual tests, our study showed that the rapid test has demonstrated the best diagnostic accuracy. However, due to disappointing low sensitivity it cannot be recommended for the diagnosis of an acute chlamydial infection. Our findings are in accordance with findings from other studies where low sensitivity (20-60%) of rapid tests was observed [18-22]. Moreover, the study of Nateghi Rostami et al. suggest primary screening of chlamydial infection in women by the low-cost EIA, but confirmation of the negative results by a DNA amplification method is required because of low sensitivity of EIA assays [23]. Contrary to our results, some authors showed that currently available rapid tests show high sensitivity (60-99%) and offer the possibility of testing patients more difficult to access, which will also allow the treatment of more cases but still do not recommend the laboratory use of these tests [24,25].

We also showed that DIF has well balanced ratio of sensitivity and specificity, but extremely low positive predictive value, which is the main reason why this test cannot be recommended for the diagnosis of an acute chlamydial infection. Similar values of DIF sensitivity and specificity are also presented in other studies [13,26-28]. However, opposite to our findings, some of these earlier studies recommend DIF method as practical and inexpensive, relevant in routine laboratory practice and suitable for the early detection of infection because of its high sensitivity [26,27]. Nevertheless, false negative and false positive results should be prevented by taking quality clinical samples, evaluating of test by the experienced staff and using quality control samples [27]. When the expanded golden standard is used, percentage of DIF sensitivity coincides with the values from our study, with a slightly higher specificity, but contrary to our findings some authors recommend the use of this tests in low-prevalence population [28]. However, other doubt this statement assuming that DIF is not recommended in low prevalence conditions and should be used only in the laboratories that process a small number of samples [13,29].

Our results also provide satisfying results for serum IgA and IgG testing. These tests had specificity, negative predictive value, as well as Youden's index and overal accuracy that were quite high, but due to low sensitivity and positive predictive value these tests also cannot be recommended as sole methods for the diagnosis of chlamydial infection. The vast majority of previous studies indicate that, except in patients with suspected chronic CT infection of upper genital tract, serological IgA and IgG testing have no relevance in establishing the diagnosis of acute chlamydial infection [9-13,30]. The results of one study suggest that, although the correlation of the serology with the active infection of lower genital tract is very low, it can exclude the active infection with high reliability [31]. On the other hand, it has been shown that new, synthetic peptide-based, species specific, serological tests can detect IgA and IgG antibodies that strongly correlated with active infection [32,33]. Moreover, this and other findings suggest that only the serum IgA or secretory sIgA antibody may be present at early stages indicating active chlamydial infection [33,34]. However, in all of

these studies, authors suggest that in such cases of IgA seropositivity, confirmation via detection of *C. trachomatis* nucleic acid is still needed.

Our research did not include cost-effectivenes analysis of the three screening test strategies, because there are no national data are available about. The international studies are rare and primarily coming from the highest-income countries [20]. Taking into account the price tariffs for different health services within the Serbian health system as well as the prices for reimbursed drugs, both regulated by the national found for health care insurance, we could propose that the screening test for chlamydial infecton with the best diagnostic performances, RT-PCR, is highly unlikely to be cost-effective stratiegy here [35,36]. The RT-PCR test has very high price (4.2-5.4 times more exepsive than the other tests) and the physician services and drugs effective for chlamydial infections (tetracyclines, macrolides) have low-to-moderate tariffs. Therefore, the savings which would be achieved with RT-PCR testings, due to avoidance of unnecessary costs for diagnostics and treatments of the patients with false positive and false negative results on other screeneng test, probably could not compensate the lagre difference within initial test expenses.

One currently available screening strategy for controlling chlamydia infection, that is being considered in resource limited health care systems in developing countries, is the use of point-of-care (POC) tests [14,15,37-39]. Although some rapid POC tests are easy to perform, specific and affordable, currently there is a little confirmative evidence on POC tests in screening settings [20,40]. Another possibility, where NAATs could be good candidate for POC settings, is usage of pulled urine samples. Urine samples are noninvasive, acceptable in screening context especially in men, quick to analyse, do not require trained personnel and even when pooled, remain an accurate method for chlamydia detection [41,42]. Therefore, the use of pooled urine samples would make any global-based screening strategy easier to implement and more cost-effective, particularly in developing countries. Nevertheless, the most cost-effective method for diagnosis of chlamydia in Serbia and other countries with similar socio-economic status remain unknown until appropriate data of future, health-economic studies are published.

Conclusion

In conclusion, as in other cases of screenings, the diagnosis of acute chlamidial infections implies a delicate balance among benefits and risks. Increase in sensitivity came at cost of amplifying false positive and opposite enhancing specificity will lead to increase in false negative results. According to our results, none of evaluated screening tests can be recommended as individual methods for the diagnosis of acute chlamydial infection. When they are affordable, NAATs are still preferred as of their superior sensitivity and specificity. However, for the most diagnostic laboratories in countries with low incomes, such as Serbia, this standard will remain unavailable. Until there is a quite sophisticated cost benefit study or a change in the market pricing, it seems that DIF and RT will remain widely used tests for vast majority laboratories in Serbia and other countries with similar health care and socio-economic environments.

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