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Rapid screening for *Chlamydia trachomatis* infection by detecting α-mannosidase activity in urogenital tract specimens

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Abstract

Background: *Chlamydia trachomatis* may cause multiple different urogenital tract disorders, but current non-culture assays for rapid screening of *C. trachomatis* typically use immunochromatography-based methods. We established another new rapid non-culture method for detection of *C. trachomatis* based on the measurement of α-mannosidase enzymatic activity in urogenital tract specimens.

Method: To evaluate the performance of this method, α-mannosidase activities of *C. trachomatis* serotype D strain 、 and 29 standard strains related to clinical urogenital pathogens were investigated. Furthermore, 553 urogenital tract specimens were used for clinical assays via cell culture method and ligase chain reaction method (LCR), adopting an expanded gold standard.

Results: Only *C. trachomatis* was positive for α-mannosidase activity among different types of microbes tested in the research. When prostate fluid specimens, which have some interfering activity, were excluded, the sensitivity and specificity of the enzymatic method were 91.8% (78/85) and 98.3% (409/416), respectively. There were no significant differences ($P > 0.05$).

Conclusions: These results showed that α-mannosidase activity could be utilised as a screening marker of *C. trachomatis* infection.

Keywords: *Chlamydia trachomatis*, α-mannosidase, Activity, Gold standard, Marker

Background

C. trachomatis infection is the most common sexually transmitted disease (STD) in the United States [1]. Mounting evidence has indicated that it not only evokes nongonococcal urethritis (NGU), cervicitis, pelvic inflammatory disease (PID), salpingitis, orchitis, and epididymitis, but also increases risk of invasive cervical cancer [2,3] and gives rise to serious reproductive disorders such as infertility [4,5], miscarriage/premature birth/missed miscarriage [6-9], and neonatal conjunctivitis [10].

A large number of methods have been established for screening and diagnosis of *C. trachomatis* infection [11]. Nevertheless, few of these assays meet the requirements of outpatient diagnosis, especially in terms of sensitivity, specificity, time, and simplicity of operation. Currently, non-culture assays for *C. trachomatis* screening typically adopt immunochromatography-based methods. Technologies based on chromogenic reactions of specific microbial enzymes have been widely applied in bacterial identification systems and chromogenic media [12-15]. However, no chromogenic assay for detecting *C. trachomatis* has been made available to date.

Our previous findings [16] suggested that *C. trachomatis* might have very high α-mannosidase activity. The purpose of the study was to establish a novel screening method for *C. trachomatis* infection without culture that would be rapid and convenient for use in outpatient clinics.

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Methods

Ethics statement

All patients were treated in accordance with the Helsinki Declaration on the participation of human subjects in medical research. Ethics approval for the study was obtained from the First Affiliated Hospital Ethics Committee of Zhengzhou University (Approved No. 20100802) and Henan Provincial People's Hospital Ethics Committee (Approved No. 20100901).

Organisms

Reference strains and cell lines were obtained from the organisations shown in Table 1.

Specimens

This study evaluated 553 specimens from clinical patients attending the STD (257, 46.47%) and Gynaecology (296, 53.53%) clinics at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) and the Henan Provincial People's Hospital (Zhengzhou, China), respectively. For the 203 male cases, three urethral discharge specimens (151 outpatients, 74.38%) or three prostate massage liquid specimens (52 outpatients, 25.62%) were collected with sterile rayon swabs (Copan, Brescia, Italy). Meanwhile, for the 350 female cases, three cervical secretion specimens (232 outpatients, 66.29%) or three vaginal secretion specimens (118 outpatients, 33.71%) were collected with sterile rayon swabs using vaginal forceps.

Table 1 Reference strains and cell line

Strains or cell line	Accession numbers	Organizations
Acinetobacter baumannii	ATCC19606	Harmony Biotechnology Co., Ltd (Shanghai, China)
Candida albicans	ATCC10231	Harmony Biotechnology Co., Ltd (Shanghai, China)
Candida glabrata	ATCC15126	Harmony Biotechnology Co., Ltd (Shanghai, China)
Candida guilliermondii	ATCC6260	Harmony Biotechnology Co., Ltd (Shanghai, China)
Candida krusei	ATCC14243	Harmony Biotechnology Co., Ltd (Shanghai, China)
Cryptococcus neoformans	CMCC(F)D2q	China Medical Microbiological Culture Collection Center (fungi)(Nanjing, China)
Candida parapsilosis	CGMCC2.1846	China General Microbiological Culture Collection Center (Beijing, China)
Candida tropicalis	ATCC750	Harmony Biotechnology Co., Ltd (Shanghai, China)
Chlamydia trachomatis Serovar D	VR-885	American Type Culture Collection(Manassas, USA)
Enterococcus faecalis	ATCC29212	Huankai Microbial Sci & Tech. Co., Ltd (Guangzhou, China)
Enterococcus faecium	ATCC700221	Harmony Biotechnology Co., Ltd (Shanghai, China)
Escherichia coli	ATCC25922	Henan Provincial Institute of Food and Drug Control (Zhengzhou, China)
Gardnerella vaginalis	ATCC14018	Harmony Biotechnology Co., Ltd (Shanghai, China)
Haemophilus influenzae	ATCC10211	Harmony Biotechnology Co., Ltd (Shanghai, China)
Klebsiella pneumoniae	CMCC46117	Tianhe Microorganism Reagent Co., Ltd (Hangzhou, China)
McCony	CRL-1696	American Type Culture Collection(Manassas, USA)
Mycoplasma hominis	ATCC15488	Harmony Biotechnology Co., Ltd (Shanghai, China)
Neisseria gonorrhoeae	ATCC19424	Henan Provincial Center for Disease Control and Prevention (Zhengzhou, China)
Pseudomonas aeruginosa	ATCC25619	Land Bridge Biotechnology Co., Ltd (Beijing, China)
Proteus mirabilis	CMCC(B)49005	Huankai Microbial Sci & Tech. Co., Ltd (Guangzhou, China)
Salmonella enteritidis	ATCC13076	Land Bridge Biotechnology Co., Ltd (Beijing, China)
Staphylococus aureus	ATCC25923	Henan Provincial Institute of Food and Drug Control (Zhengzhou, China)
Staphylococcus aureus	ATCC29213	Capital Institute of Pediatrics (Beijing, China)
Staphylococcus epidermidis	ATCC12228	Huankai Microbial Sci & Tech. Co., Ltd (Guangzhou, China)
Staphylococus saprophyticus	ATCC49453	Land Bridge Biotechnology Co., Ltd (Beijing, China)
Stenotrophomonas maltophilia	ATCC17666	Land Bridge Biotechnology Co., Ltd (Beijing, China)
Streptococus agalactiae	ATCC13813	Harmony Biotechnology Co., Ltd (Shanghai, China)
Streptococus pneumoniae	ATCC49619	National Center for Clinical Laboratory (Beijing, China)
Streptocus pyogenes	ATCC19615	Harmony Biotechnology Co., Ltd (Shanghai, China)
Trichomonas vaginalis	ATCC30001	Harmony Biotechnology Co., Ltd (Shanghai, China)
Ureaplasma urealyticum	ATCC15531	Harmony Biotechnology Co., Ltd (Shanghai, China)

Three swabs collected for each specimen, were no significant differences in sampling link and randomly used with three methods. None of the patients received any antibiotics one week before sample collection, when samples were taken before diagnosis.

Media, culture and inoculation

Liquid media A (LMA) and liquid media B (LMB) were prepared for mycoplasma culture. The components of LMA were shown in Table 2, but LMB consisted of 50 mg/l phenol red besides LMA components. The media were inoculated with both *Ureaplasma urealyticum* and *Mycoplasma hominis* and analyzed by using the colour-changing unit (CCU) method, as previously reported [17]. Once the concentrations of mycoplasma reached 10^6 CCU/ml in LMB, the cultures of LMA should be immediately stored at 4°C.

Trichomonas vaginalis was obtained after 24 h incubation at 37°C in Trichomonas medium (Oxoid, Basingstoke, UK) supplemented with 8% horse blood and 1,000 units/ml penicillin sodium and 500 mg/ml streptomycin. The collection was stored at 4°C.

All other micro-organisms except *C. trachomatis* used in the study were inoculated and cultured as described in Table 3. The collections described in Table 3 were resuspended to 0.5 McFarland standards in a sterile solution of 0.9% NaCl and then stored at 4°C.

Enzymatic method

The enzymatic method was based on the substrate of α-D-mannosidase. The substrate solution contained 1.5 mg/ml 6-chloro-3-indolyl-α-D-mannoside (J&K, Shanghai, China), 100 mM citrate buffer (pH 4.0), and 1% Triton X-100. The sample diluent was 0.9% NaCl. The chromogenic reagent contained 0.08% fast violet B salt (J&K, Shanghai, China). Aliquots (50 μl) of substrate solution and chromogenic reagent were added sequentially into sample solutions (extracted from every swab sample with 500 μl sample diluent) or the aforementioned microbial suspensions as well as the chlamydial suspension mentioned in the section called limit of detection (LOD) of the enzymatic method. The result was considered to be positive ($OD_{512} \geqq 0.150$) if the colour changed from colourless to red or brown after 15 min of incubation at 37°C; otherwise, the result was considered to be negative ($OD_{512} < 0.150$). The suspensions containing bacteria or cells was centrifuged at 5,000 rpm for 5 min, and then OD_{512} value of the supernatants were measured.

Table 2 Composition of LMA for Mycoplasma (per liter) *

Ingredient	Concentration	Ingredient	Concentration
NaCl	6.4 g	Beef heart extract power	7.2 g
$CaCl_2$	112 mg	Yeast extract power	2.72 g
$MgCl_2 \cdot 6H_2O$	80 mg	Peptone	8.0 g
$MgSO_4 \cdot 7H_2O$	80 mg	Horse serum	200 ml
KCl	320 mg	Penicillin sodium	1,000,000 units
$Na_2HPO_4 \cdot 12H_2O$	122 mg	Ampicillin sodium	375 mg
KH_2PO_4	48 mg	Vancomycin	40 mg
Cysteine hydrochloride	0.8 g	Polymyxin B	40,000 units
Arginine hydrochloride	4.0 g	Nystatin	15,000 USP units
Urea	4.0 g		

*Adjusted to pH 6.25.

LOD of the enzymatic method

McCoy cells infected with *C. trachomatis* serovar D were stored at −80°C, and frozen-thawed twice to obtain chlamydial suspension before use. Serial 10-fold dilutions of the chlamydial suspension were inoculated in six duplicate into wells (100 μl/well) of cycloheximide-treated McCoy cell monolayers that had been incubated with MEM medium (Gibco, Grand Island, NY, USA) in a 96-well flat-bottom microtiter plate (Nunc Inc., Roskilde, Denmark) at 37°C under 5% CO_2 for 48 h. Inclusion body titers of the chlamydial supernatant were quantified by titrating the number of inclusion-forming units (IFU). The contents of each well were stained with a *C. trachomatis* direct fluorescent antibody kit (Academy of Military Medical Science, Beijing, China) and examined by microscopy for IFU counts. The average IFU of each dilution culture of three replicate wells was taken as the concentration of *C. trachomatis* in the corresponding dilution of the chlamydia suspension. Each dilution culture of the other three replicate wells was examined via the enzymatic method after collection to determine the LOD for *C. trachomatis* of the enzymatic method. 10^{-1} u/ml α-D-mannosidase (EC3.2.1.24; Sigma, USA) solution was 5-fold serially diluted to 10^{-4} u/ml. These enzyme solutions fold-diluted were detected via the enzymatic method to determine the LOD for α-D-mannosidase.

Reference method

Cell culture and LCR method were used to evaluate the clinical performance of the enzymatic method. The swabs used for culture were dipped directly into incidental transport medium (Copan, Brescia, Italy) and cultured according to the aforementioned method. The cultures were tested using a *C. trachomatis* direct fluorescent antibody kit. LCR was carried out using the LCx *C. trachomatis* assay (Abbott Laboratories, Abbott Park, Israel) according to the manufacturer's instructions. Although culture method has a good specificity, its sensitivity may be influenced by various factors.

Table 3 The media and culture methods for bacteria and candida

Strains	Media	Temp.	Time
Bacteria			
A. baumannii, *E. faecalis*,	Blood agar base [a]	37°C	24 h
E. faecium, *E. coli*, *K. pneumoniae*,			
P. mirabilis, *P. aeruginosa*,			
S. enteritidis, *S. aureus*,			
S. epidermidis, *S. saprophyticus*,			
S. maltophilia, *S. agalactiae*,			
S. pyogenes, *S. pneumoniae*			
G. vaginalis *	Blood agar base	37°C	48 h
H. influenzae *	Thayer Martin media [b]		24 h
N. gonorrhoeae *			48 h
Candida			
C. albicans, *C. tropicalis*,	Sabouraud dextrose agar [c]	37°C	24 h
C. glabrata, *C. parapsilosis*,			
C. guilliermondii, *C. krusei*,			
C. neoformans		30°C	48 h

[a,b,c] Oxoid, Basingstoke, UK; [a] supplemented with 5% sheep blood; [b] supplemented with 5% horse blood; * cultured in a candle jar.

Therefore, the research adopted an "expanded gold standard" [18-20] described as follows: any positive by either culture or LCR was classified as a true positive, whatever the result of the enzymatic method.

Results

In our developed assay, only *C. trachomatis* samples tested was positive for α-D-mannosidase activity; but OD_{512} of both other organisms and cell cultures which were not inoculated with *C. trachomatis* used in the study was all below 0.100, which fell into the range of negative results negative with 0.150 (OD_{512}) as the cut-off value, even if the reactions were allowed to proceed for 1 h at 37°C. Of the 553 clinical samples, 132 samples were positive with OD_{512} ranging from 0.161 to 1.955, and 421 samples were negative with OD_{512} ranging from 0.013 to 0.142. *C. trachomatis* detection results of 553 cases with culture, LCR and enzymatic method used an expanded gold standard as the reference standard were showed in Table 4. The enzymatic method was least reliable when prostate specimens were used. The sensitivity of the enzymatic method was 91.5% (95% confidence interval [CI], 85.9% to 97.1%), and the specificity of this method was 90.0% (95% CI, 87.3% to 92.7%) (Table 5). The sensitivity and specificity of the LCR were 94.7% (95% CI, 90.2% to 99.2%) and 100% (95% CI, 100.0% to 100.0%), respectively. However, in those specimens other than prostate fluid samples, the sensitivity and specificity of the enzymatic method were 91.8% (95% CI, 86.0% to 97.6%) and 98.3% (95% CI, 97.1% to 99.5%), in the meantime the sensitivity and specificity of the LCR were 95.3% (95% CI, 90.8% to 99.8%) and 100% (95% CI, 100% to 100%) (Table 6), respectively. There were no significant differences in performance between the enzymatic method and the expanded gold standard ($P > 0.05$).

The result of the chlamydial suspension quantified with 617 IFU/ml was light pink, and might be considered positive (OD_{512} = 0.162). Meanwhile, the result of the chlamydial suspension was colourless if quantified with 126 IFU/ml, and might be considered negative (OD_{512} = 0.098). Therefore, the LOD was 617 IFU/ml for *C. trachomatis*. In addition, the LOD was 10^{-3} u/ml (OD_{512} = 0.155) for α-D-mannosidase.

Discussion

There are various well-known methods for detecting *C. trachomatis*, including cell culture-, immunology-, molecular biology-, and biochemistry-based methods. Cell culture is complicated to perform and requires experience to produce accurate results, and it also has more stringent requirements for the sampling swabs and transporting before inoculation [21,22]. Therefore, cell culture is rarely used in clinics. Among the available immunological methods, serological tests for the *C. trachomatis* antibody have significant limitations [23], but methods for *C. trachomatis* antigen detection (mainly referring to the lipopolysaccharide, LPS), especially immunochromatography-based methods, have been widely used due to their simplicity of operation. There are two ways to extract the LPS antigen for *C. trachomatis* immunochromatographic assays: heat extraction and acid extraction. Neither of these methods guarantees the full extraction of LPS as an intact antigen, which influences the sensitivity of this method. The biochemical detection of glycogen in *C. trachomatis* inclusions [24] is greatly affected by *Candida spp*. that often exist in these specimens and are especially common during the female menstrual cycle and pregnancy. With the increasing glycogen in vaginal epithelial cells, this method may also cause false positives. The detection of *C. trachomatis* in the United States and Europe has mainly focused on molecular biology methods [25-30]. Although these methods are both high sensitivity and high specificity, it can be challenging for molecular biology methods to meet the requirements of the actual application in clinical screening.

Enzymatic studies of *C. trachomatis,* especially for enzymes with diagnostic significance, have not been reported in the literature. Previous studies [31] and our research have shown that *C. trachomatis* secretes extracellular enzymes with high α-D-mannosidase activity. Although some organisms used in the study such as *C. albicans* have genes encoding α-1,2-mannosidase

Table 4 *C. trachomatis* detection results of 553 cases with culture, LCR and enzymatic method used an expanded gold standard as the reference standard

Method	No. of true positives/positives	No. of true negatives/negatives
Enzymatic method	86/132	413/421
	Urethral discharge 22/24	Urethral discharge 126/127
	Prostate massage liquid 8/47	Prostate massage liquid 4/5
	Cervical secretion 31/33	Cervical secretion 196/199
	Vaginal secretion 25/28	Vaginal secretion 87/90
Cell culture	60/60	459/493
	Urethral discharge 18/18	Urethral discharge 128/133
	Prostate massage liquid 7/7	Prostate massage liquid 43/45
	Cervical secretion 21/21	Cervical secretion 198/211
	Vaginal secretion 14/14	Vaginal secretion 90/104
LCR	85/89	459/464
	Urethral discharge 22/22	Urethral discharge 128/133
	Prostate massage liquid 8/8	Prostate massage liquid 43/45
	Cervical secretion 32/32	Cervical secretion 198/211
	Vaginal secretion 27/27	Vaginal secretion 90/104
Expanded gold standard	94	459
	Urethral discharge 23	Urethral discharge 128
	Prostate massage liquid 9	Prostate massage liquid 43
	Cervical secretion 34	Cervical secretion 198
	Vaginal secretion 28	Vaginal secretion 90

[32], α-D-mannosidase activity was invisible by naked eyes with 6-chloro-3-indolyl-α-D-mannoside as substrate. This may be because the extracellular α-D-mannosidase from these organisms is much less or the enzyme activity is relatively low. Previous records [31,33-35] on substrates of α-D-mannosidase mainly involved p-nitrophenol-α-D-mannoside and 4-methylumbelliferyl-α-D-mannoside. However, 6-chloro-3-indolyl-α-D-mannoside is a novel chromogenic substrate, and colour reaction of its chromogen is much more sensitive especially in the case of the presence of an azo reagent such as fast violet B salt.

Our results showed that clinical specimens such as urethral discharge, cervical secretions, and vaginal secretions did not interfere with the chromogenic detection of α-D-mannosidase activity to screen for *C. trachomatis*, although prostate massage liquid produced more false positive results. Some human sperm surface proteins possess α-D-mannosidase activity [36], which may be the reason that prostate specimens produce less reliable results. Serotype D was only one of the most prevalent (11.1%), and no serovar L2 was found in China [37]. Although the results of this study suggested that other serotypes, such as serotypes E, F, G, K, H, J, I, and Ba, at least most of them, might have α-D-mannosidase activity, there seems to be some limitation of tests on *C. trachomatis* cultures based on only one strain of serotype D. Further studies and more comprehensive clinical evaluations should be conducted due to little research on α-D-mannosidase activity of *C. trachomatis*. In addition, our studies did not evaluate *C. pneumoniae* or *C. psittaci*; the α-D-mannosidase activities of these species should be studied as well.

Table 5 Clinical performances of three assays for *C. trachomatis* using specimens included prostate massage liquid

Methods	% Sensitivity (95% CI)	% Specificity (95% CI)
Enzymatic method[a]	91.5 (85.9, 97.1)	90.0 (87.3, 92.7)
Cell culture[b]	63.8 (54.1, 73.5)	100.0 (100.0, 100.0)
LCR[c]	94.7 (90.2, 99.2)	100.0 (100.0, 100.0)

[a] $X^2 = 8.030$, $P = 0.005$, $P < 0.05$; [b] $X^2 = 8.721$, $P = 0.003$, $P < 0.05$; [c] $X^2 = 0.164$, $P = 0.685$, $P > 0.05$.

Table 6 Clinical performances of three assays for *C. trachomatis* using specimens excluded prostate massage liquid

Methods	% Sensitivity (95% CI)	% Specificity (95% CI)
Enzymatic method[a]	91.8 (86.0, 97.6)	98.3 (97.1, 99.5)
Cell culture[b]	64.6 (54.3, 74.9)	100.0 (100.0, 100.0)
LCR[c]	95.3 (90.8, 99.8)	100.0 (100.0, 100.0)

[a] $X^2 = 0$, $P = 1$, $P > 0.05$; [b] $X^2 = 8.605$, $P = 0.003$, $P < 0.05$; [c] $X^2 = 0.116$, $P = 0.734$, $P > 0.05$.

Conclusions

The present study demonstrated that there were no significant differences between the enzymatic method and the reference method when prostate specimens were excluded. Therefore, α-D-mannosidase activity may be a useful marker for *C. trachomatis* in urogenital tract specimens, with many advantages, such as its speed, ease of use, convenience, and need for no special equipment. Taken together, these data show that the enzymatic method has great potential as a clinical method for *C. trachomatis* screening.

Competing interests

The authors declare that they have no competing interests. We are applying for one patent (CN patent 102286608A) relating to the content of the manuscript, and the authors do not have any objection on the patent right of authorship and ownership.

Authors' contributions

ZYW conceived of the study and designed all the experiments and drafted the manuscript. ZYW, GYF, SMW and DCQ performed the experiments. ZYW and ZQW performed the statistical analysis. ZQW and JC provided valuable insight for designing the study and revising the manuscript. All authors read and approved the final manuscript.

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