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A molecular screening assay to identify *Chlamydia trachomatis* and distinguish new variants of *C. trachomatis* from wild-type

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Summary

***Chlamydia trachomatis* is the most common sexually transmitted pathogen globally, causing serious health problems and representing a burden on public health. A new variant of *C. trachomatis* (nvCT) that carries mutations (C1514T, C1515T and G1523A) in the 23S rRNA gene has eluded detection in Aptima Combo 2 assays. This has led to false negatives in diagnostics tests and poses a challenge for *C. trachomatis* diagnostics on a global level. In this study, we developed a simple and cost-effective assay to identify *C. trachomatis*, with a potential application to screen for nvCT. We developed a screening assay based on high-resolution melting (HRM), targeting the 23S rRNA gene and cryptic plasmid. To evaluate the performance of the assay, 404 archived *C. trachomatis* DNA specimens and 570 extracted clinical specimens were analysed. Our HRM assay not only identified *C. trachomatis* in clinical specimens, but also correctly differentiated nvCT carrying C1514T, C1515T and G1523A mutations from the wild-type. We observed no cross-reactions with other clinically related agents, and the limit of detection was 11.26 (95% CI; 7.61–31.82) copies per reaction. Implementation of this screening assay could reduce detection times and costs for C. trachomatis diagnoses, and facilitate increased research on the presence and monitoring of nvCT.**

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Introduction

Worldwide, *Chlamydia trachomatis* is one of the most prevalent sexually infectious aetiological agents (Menon *et al*., 2015; Aung *et al*., 2019; Chi Wai Wong *et al*., 2019), causing approximately 131 million new infections each year (https://www.who.int/reproductivehealth/publications/stis-surveillance-2018/en/). *C. trachomatis* infections not only can lead to severe reproductive sequelae, including ectopic pregnancy, pelvic inflammatory disease and infertility, but also can facilitate the transmission of human immunodeficiency virus (Xiu *et al*., 2019; Durukan *et al*., 2020). However, in some populations, *C. trachomatis* infections are often asymptomatic, causing significant challenges (underestimations) for detection and epidemiological characterization.

Routine screening and accurate diagnosis for *C. trachomatis* infections are mainstays for deciding appropriate treatments and interrupting transmission. The current diagnostic algorithms for *C. trachomatis* are based on nucleic acid amplification tests (NAATs), which are highly sensitive and specific (Turingan *et al*., 2017). Currently, NAATs come in the form of regulatory-approved commercially available molecular kits, including Hologic Aptima Combo 2 assay (AC2), Becton Dickinson Molecular Diagnostics BD Max CT/GC/TV, Roche Cobas CT/NG Test, Abbott RealTime CT/NG assay and Cepheid GeneXpert Xpert CT/NG (Hokynar *et al*., 2019). However, in recent years, continued false-negative results have been reported during *C. trachomatis* diagnostics, suggesting the occurrence of intrinsic mutation or recombination events (Turingan *et al*., 2017), thus complicating diagnoses. One possible cause for the higher number of diagnostic discrepancies in the NAATs results for *C. trachomatis* may be due to genetic diversity for diagnostic methods with a single target. In 2006, the Swedish new

variant of *C. trachomatis* (S-nvCT) with a plasmid deletion (Ripa and Nilsson, 2006) was reported to have escaped detection in several commercial *C. trachomatis* diagnostic kits, including Cobas Amplicor/TaqMan48 (Roche Diagnostics, Basel, Switzerland) and the Abbott m2000 (Abbott Laboratories, Abbott Park, IL, USA; Herrmann *et al*., 2008). Consequently, the S-nvCT resulted in thousands of false-negative test results, resulting in an increase in sexually transmitted diseases across several Nordic countries (Unemo and Clarke, 2011). However, the ongoing evolution of *C. trachomatis* under the high diagnostic selective pressure (Unemo *et al*., 2019a) is still occurring.

The Finnish new variant of *C. trachomatis* (F-nvCT) was first reported in Finland in mid-February 2019 (Rantakokko-Jalava *et al*., 2019) and contained a C1515T mutation in its 23S rRNA gene. Notably, this mutation was located in the region originally utilized by the NAATs Aptima Combo 2 (AC2) (Hologic Inc., San Diego, CA, USA) to detect *C. trachomatis*, which were used in many countries worldwide. This new variant of *C. trachomatis* (nvCT) escaping detection in the AC2 assay has subsequently been detected in other European countries such as Sweden, England, Norway and Denmark (Johansen *et al*., 2019; Roberts *et al*., 2019; Unemo *et al*., 2019b; Hadad *et al*., 2020). Two additional *C. trachomatis* variants harbouring C1514T and G1523A mutations in the 23S rRNA target region, also escaped AC2 detection in England, Norway and Denmark (Johansen *et al*., 2019; Roberts *et al*., 2019; Hadad *et al*., 2020). False-negative results due to the continuously emerging mutants (Unemo *et al*., 2007; Clarke, 2011) have increased the risk of unrecognized transmission. Timely determination and surveillance of the nvCT mutants are, therefore, crucial for our understanding of presence and monitoring of the spread of the *C. trachomatis* variants (Johansen *et al*., 2019; Rantakokko-Jalava *et al*., 2019; Roberts *et al*., 2019; Unemo *et al*., 2019a,b). Here, we report a new molecular screening assay suitable for the identification *C. trachomatis* and surveillance of these new variants escaping detection by AC2.

Results

The molecular screening assay

After HRM analysis and standardization, melting curve clusters for different targets were generated. A cluster analysis of melting profiles was used to differentiate targets into two distinct clusters (Fig. 1). These two distinct clusters were used to identify and confirm the presence of *C. trachomatis* in clinical samples. Furthermore, in the 23S rRNA target region, the HRM assay successfully revealed four different cluster formations for the wide-type and three different point mutations, involving AC2 diagnostic-eluding nvCTs, C1514T, C1515T and G1523A. Twenty-eight wild-type *C. trachomatis* and three control plasmids harbouring individual mutations (those escaping detection by the AC2 assay) were used to develop and evaluate this HRM assay. Our assay yielded two clusters for the two targets (23S rRNA and the cryptic plasmid) from all 28 well-characterized *C. trachomatis* samples. Our data showed that the HRM assay accurately identified *C. trachomatis*, demonstrating a high specificity for the primers used in this study. Our optimization data also revealed that HRM analysis correctly differentiated C1514T, C1515T and G1523A mutations from the wide-type. However, plasmids containing C1515T or G1523A mutations were not distinguished from each other due to their similar melting temperatures (Tm) values (Table 1).

To assess the potential of the HRM assay to cross-react with other genetically or clinically related agents, we conducted a specificity test using microbial species DNA extracts from 20 confirmed clinical samples and isolates. No specific melting profiles, consistent with both reference controls, were observed for all organisms across all sample types (Table 1). The average Tm value for each DNA used in the HRM assay is summarized in Table 1.

To investigate the limit of detection (LOD) of our HRM assay, a sensitivity test was performed by testing a dilution series using plasmids containing two target regions at the following concentrations: 200, 100, 50, 20, 10, 5 and 1 gene copy per reaction mixture. Each concentration was tested in 12 replicates, and Probit analysis of the results was carried out using SPSS statistical software package version 17.0 (SPSS Inc., Chicago, IL, USA). Our Probit analysis data revealed a LOD of 11.26 (95% CI, 8.90–19.87) and 9.46 (95% CI, 7.58–14.57) copies per reaction for the *C. trachomatis* target 23S rRNA gene and the cryptic plasmid at 95% confidence, respectively (Table 2).

Evaluation of the performance on clinical samples

A total of 570 DNA samples were first analysed by the HRM assay for the validation of the ability to directly identify *C. trachomatis* in clinical specimens. Among 570 tested samples, 36 showed similar profiles and aligned with melting curves for both targets (i.e. 23S rRNA and the cryptic plasmid). No melting curves were observed for the remaining negative *C. trachomatis* specimens (Fig. 2). The test performance characteristics of HRM assay for the detection of *C. trachomatis* were compared with our reference STI-MS assay (Table 3). The overall per cent agreement between assays was 98.8% (95% confidence interval [CI], 97.4 to 99.5%), positive per cent agreement was 87.2% (95% CI, 71.8 to 95.2%), and

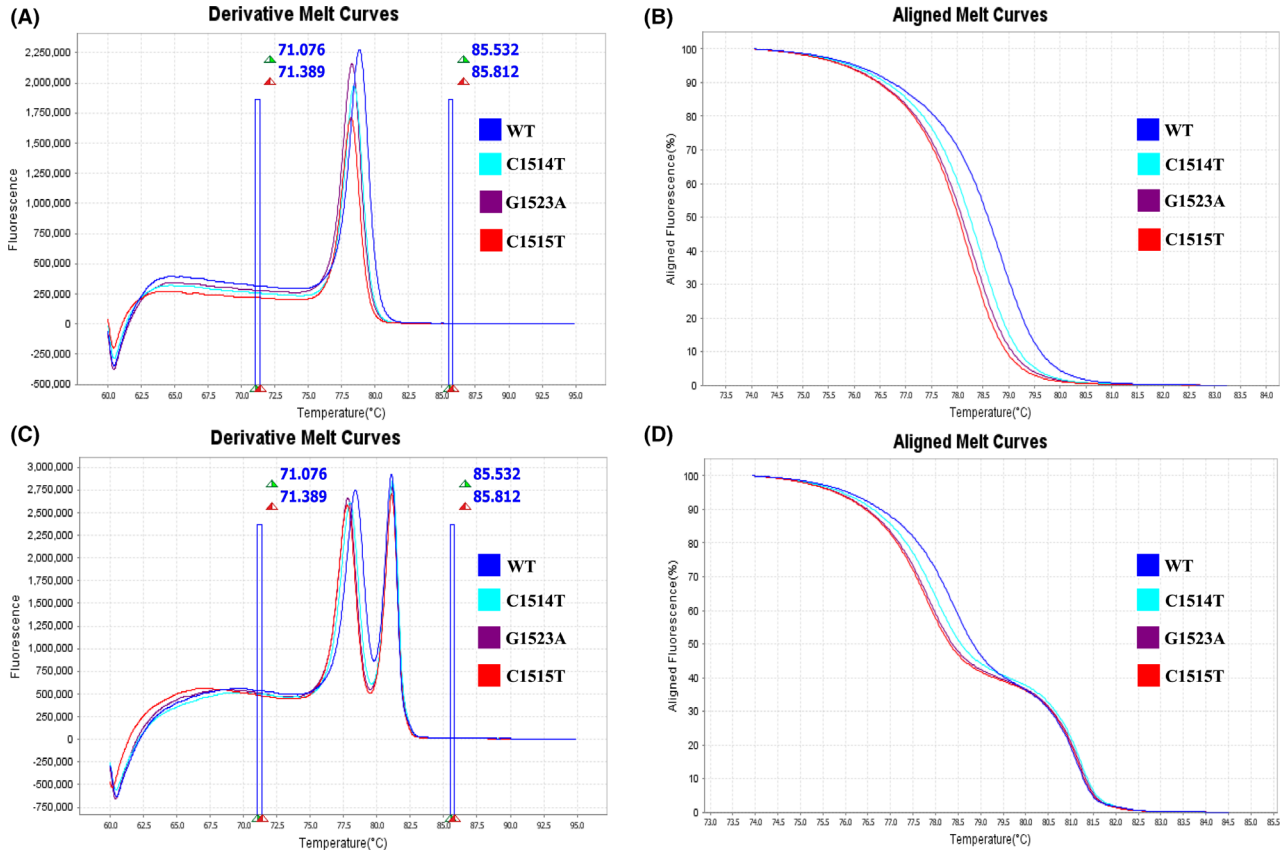

Fig. 1. The results of singleplex and multiplex HRM assay. (A) Derivative melt curves of the singleplex HRM assay. (B) Aligned melt curves of the singleplex HRM assay. (C) Derivative melt curves of the multiplex HRM assay. (D) Aligned melt curves of the multiplex HRM assay.

Table 1. Summary of Tm values for each of the organisms used in the HRM assay.

	Tm of target 23S rRNA		Tm of target cryptic plasmid	
Organisms	Range	Mean ± SD	Range	Mean ± SD
C. trachomatis (WT, *n* = 28)	78.40–78.48	78.43 ± 0.03	81.14–81.16	81.14 ± 0.01
Plasmid (C1514T mutation, *n* = 1)	78.00–78.06	78.02 ± 0.02	81.19–81.25	81.21 ± 0.02
Plasmid (C1515T mutation, *n* = 1)	77.84–77.94	77.90 ± 0.04	81.17–81.25	81.22 ± 0.04
Plasmid (C1523A mutation, *n* = 1)	77.84–77.86	77.85 ± 0.01	81.17–81.20	81.19 ± 0.01
Herpes simplex virus types 1 (*n* = 2)	NA	NA	NA	NA
Herpes simplex virus types 2 (*n* = 2)	NA	NA	NA	NA
Neisseria gonorrhoeae (*n* = 2)	NA	NA	NA	NA
Chlamydia pneumonia (*n* = 2)	77.69–77.70	77.69 ± 0.01	NA	NA
Treponema pallidum (*n* = 2)	NA	NA	NA	NA
Trichomonas vaginalis (*n* = 2)	NA	NA	NA	NA
Mycoplasma hominis (*n* = 2)	NA	NA	NA	NA
Ureaplasma urealyticum (*n* = 2)	NA	NA	NA	NA
Mycoplasma genitalium (*n* = 2)	NA	NA	NA	NA
Ureaplasma parvum (*n* = 2)	NA	NA	NA	NA

NA, no amplification was observed; WT, wild-type.

negative per cent agreement was 99.6 (95% CI, 98.5 to 99.9%). When compared with the STI-MS assay, the HRM assay showed excellent overall and negative agreement. For those positive specimens identified by the developed HRM assay, the results were confirmed using an *in house* real-time PCR assay (Mahony *et al.*, 1997). The HRM results for the 36 positive samples were 100% concordant with the results obtained by real-

Table 2. Limit of detection of each target gene using regression probit analysis.

Target	No. of replicates detected at each dilution/total no. of replicated at indicated no. of copies/reaction (% positive rate)							Final LOD, no. of copies/reaction
	200	100	50	20	10	5	1	
23S rRNA	12/12 (100)	12/12 (100)	12/12 (100)	12/12 (100)	10/12 (83.3)	7/12 (58.3)	0/12 (0)	11.26 (95% CI, 8.80–19.87)
Cryptic plasmid	12/12 (100)	12/12 (100)	12/12 (100)	12/12 (100)	11/12 (91.7)	8/12 (66.7)	0/12 (0)	9.46 (95% CI, 7.58–14.57)

time PCR. Therefore, our results showed that this HRM assay may be used for the detection and identification of *C. trachomatis* in clinical samples.

Screening samples for the presence of nvCT

To investigate the possible prevalence and spread of nvCT in China, we screened 404 archived DNA specimens that had previously tested positive for *C. trachomatis* using the Roche Cobas® 4800 CT/NG test. All samples were amplified and resulted in corresponding melting curves, meaning they were positive for *C. trachomatis* by HRM assay. The nvCT was not identified in any of the samples (Table 4). All tested archived DNA specimens were assigned as wide-type of *C. trachomatis* by the HRM assay (Fig. 2). Subsequent DNA sequencing analyses (Hokynar *et al*., 2019) from 364 specimens were confirmed as wide-type *C. trachomatis*. The remaining 40 samples were found to be positive by HRM assay, but their sequences of the 23S rRNA gene were not acquired. We speculate these discrepancies may have been caused by the lower LOD of the HRM assay.

Discussion

In the past decade, several PCR-based molecular diagnostics techniques have been successfully developed and widely applied for the detection of *C. trachomatis* (Meyer, 2016; Feodorova *et al*., 2019; Hokynar *et al*., 2019). However, some of these assays use only one target region for detection. Lessons from the Swedish nvCT (Ripa and Nilsson, 2006; Moller *et al*., 2010; Unemo *et al*., 2010; Unemo and Clarke, 2011) and the nvCT discovered in 2019 (Hokynar *et al*., 2019; Johansen *et al*., 2019; Rantakokko-Jalava *et al*., 2019; Roberts *et al*., 2019; Unemo *et al*., 2019a,b; Hadad *et al*., 2020) have suggested that false-negative results were generated due to genetic diversity in diagnostic methods using a single target. These findings suggest that the detection of more than one gene from *C. trachomatis* may be necessary to avoid false-negative results due to mutation within the target sequences. The implementation of a dual-target strategy represents an important improvement in molecular screening methods, ensuring the detection of new variants with mutations in one of the target regions (Meyer, 2016). To avoid such false-negative results, therefore, we used two targets from *C. trachomatis* to increase the sensitivity and specificity. Previous studies (Jalal *et al*., 2011; Turingan *et al*., 2017) reported that the organism load of *C. trachomatis* in genital swabs ranged from fewer than 10 to more than 10^6. Our results revealed that our assay was highly sensitive in detecting *C. trachomatis* to approximately 11.26 (95% CI: 7.61–31.82) copies per reaction. Furthermore, we observed no cross-reactions during pathogenic microbial species testing. These indicated that the assay had good specificity for detection of the *C. trachomatis* directly in the clinical specimens.

The mutational events in specific target binding sequences of *C. trachomatis* can raise the possibility of false-negative results, which led to increasing the spread of nvCT, especially in high-frequency transmitting populations (Unemo and Clarke, 2011). Though an updated AC2 assay containing dual-target detection that detects all known nvCT strains have been FDA-cleared in the United States and clinically validated (Unemo *et al*., 2020) and CE-Marked in Europe, knowledge regarding the spread of the nvCT is relatively limited (Unemo *et al*., 2019a) because the majority of laboratories are still not able to identify these nvCTs. Enhanced and continued surveillance based on screening *C. trachomatis* samples (negative results using the AC2 platform and positive results using other detection platforms based on targets without 23S rRNA) by this assay will be very useful in addressing these concerns. On the one hand, our HRM assay reduces the possibility that mutation or recombination results in false-negative results. On the other hand, this screening assay using HRM analysis allows for the precise assessment of sequences and can be used to identify variants with single point mutations without the need for sequencing, thereby significantly reducing turnaround time (Bernal-Martinez *et al*., 2017). Since no clinical samples or nvCT isolates were available, for this study, plasmids containing individual mutations within the 23S rRNA target region for AC2 were constructed as positive controls. Although this is a

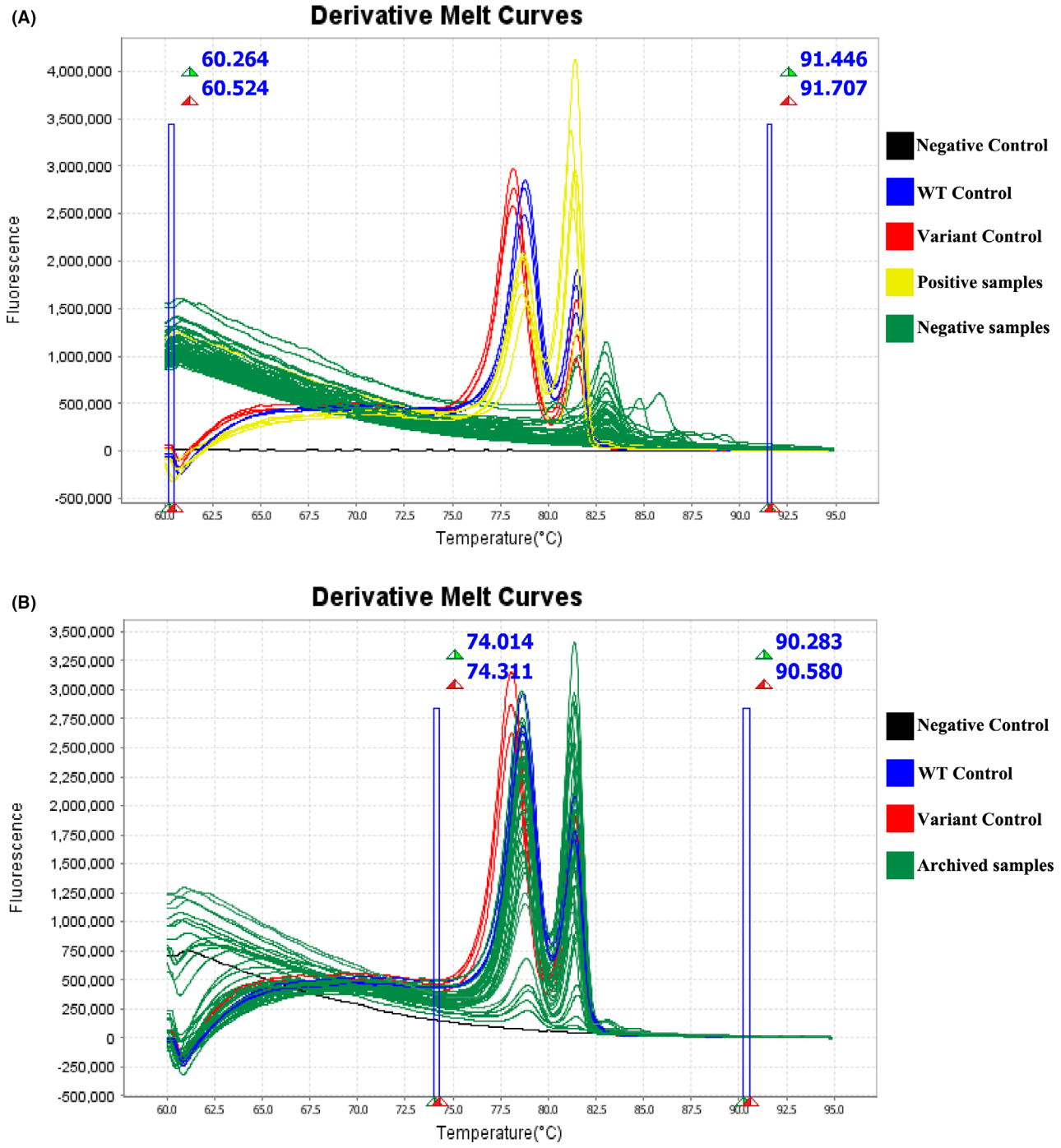

Fig. 2. The HRM scanning assay for detecting the nvCT in tested samples. (A) The results of clinical samples. (B) The results of DNA specimens with *C. trachomatis* positive.

possible limitation in the development of this assay, it is important to note that the application of this method for nvCT detection would not be affected. A further limitation of this molecular screening assay, which is common to other HRM-based molecular methods, was that targets with the same GC content were difficult to distinguish. Indeed, we did not correctly distinguish between the C1515T and G1523A mutations. However, the HRM assay correctly differentiated the C1514T, C1515T and G1523A mutations from the wide-type. As high

Table 3. Clinical performance comparison of HRM and STI-MS assay for the detection of *Chlamydia trachomatis*.

	STI-MS assay			% agreement (95% CI) between assays		
HRM assay	Positive	Negative	Total	Positive	Negative	Overall
Positive	34	2	36	87.2 (71.8–95.2)	99.6 (98.5–99.9)	98.8 (97.4–99.5)
Negative	5	529	534			
Total	39	531	570			

Table 4. Previous studies and the current study on screening for the nvCT escaping detection in the AC2assay.

Country	Year[a]	Type of tested samples	No. retested samples	No. the th nvCT	Mutations in the 23S rRNA gene	Reference
Finland	2019	AC2-negative or equivocal ACT-positive samples	180	10	C1515T (*n* = 10)	Rantakokko-Jalava *et al.* (2019)
Sweden	2019	AC2-negative or equivocal samples	70	2	C1515T (*n* = 2)	Unemo *et al.* (2019)
England	2019	AC2/ACT discrepant specimens	266	2	C1514T (*n* = 1) G1523A (*n* = 1)	Roberts *et al.* (2019)
Finland	2018–2019	AC2-negative or equivocal ACT-positive samples	>200	39	C1515T (*n* = 39)	Hokynar *et al.* (2019)
Norway	2019	AC2/ACT discordant samples	97	81	C1514T (*n* = 1) C1515T (*n* = 79) G1523A (*n* = 1)	Johansen *et al.* (2019)
Denmark	2019	AC2-negative or equivocal ACT-positive samples	150	80	C1514T (*n* = 2) C1515T (*n* = 2) G1523A (*n* = 76)	Hadad *et al.* (2020)
China	2018	CT positive samples by Roche Cobas® 4800 CT/NG	404	0	ND	This study

AC2, Aptima Combo 2; ACT, Aptima *Chlamydia trachomatis*; nvCT, AC2 new variant of *Chlamydia trachomatis*; ND, not detected.
a. Collection date of retest samples.

sequence homology among the 23S rRNA genes is found in all Chlamydia species, a similar melting profile for target 23S rRNA was observed for the *C. pneumoniae* and *C. trachomatis* in tested strains. This further illustrates the importance of including dual-target detection methods for *C. trachomatis* in the investigation of Chlamydia cases with false-positive results in PCR targeted exclusively at the *C. trachomatis*. Even so, the emergence of variants lacking the entire 7.5 kb chlamydial cryptic plasmid (Magbanua *et al.*, 2007; Sweeney *et al.*, 2019) that eludes assay detection may occur. However, the two human species of Chlamydia have a different mode of transmission and that *C. pneumoniae* is not sexually transmitted. Therefore, the overall utility of this assay for *C. trachomatis* and nvCT detection is not affected.

Although the nvCT was not been found outside the Europe countries, its potentially wide geographical distribution cannot be excluded (Johansen *et al.*, 2019; Rantakokko-Jalava *et al.*, 2019; Unemo *et al.*, 2019a). To confirm this, the retrospective analysis of AC2 negative samples all over the world needs to be conducted (Chi Wai Wong *et al.*, 2019; Johansen *et al.*, 2019; Xiu *et al.*, 2019). In addition, data from this small-scale screening program have shown that this nvCT was not present in our samples. To further investigate whether this nvCT was present and circulating in China, a large-scale laboratory-based surveillance program must be incepted. By means of the herein described protocol, it is possible to obtain the results within two hours in less than $ 0.50 for each sample. Therefore, our HRM approach may represent a useful laboratory tool for the identification of *C. trachomatis* in clinical specimens, with the potential ability to provide clues for distinguishing nvCT from wild-type. Implementation of such a screening strategy could reduce the time and cost to effectively detect *C. trachomatis,* thus facilitating a greater understanding of nvCT spread in populations.

Conclusion

The HRM scanning assay is not only able to directly identify *C. trachomatis* in clinical specimens, but also suitable for distinguishing nvCTs (three different mutations in the 23S rRNA gene) from the wild-type. Our small-scale screening study showed that nvCTs were not identified in our sample collection. Thus, further large-scale investigations must determine whether nvCTs are present and circulating in China.

Experimental procedures

Overview

This molecular screening assay was developed using high-resolution melting (HRM) analysis technology. HRM

is a simple, reliable and cost-effective method that distinguishes wild-type and mutant variants by the presence of specific DNA targets (Montgomery *et al*., 2010; Tong and Giffard, 2012). To avoid false-negative results, the HRM assay targets two genes (23S rRNA and cryptic plasmid) from *C. trachomatis* to increase the sensitivity and specificity. The 23S rRNA target was used to identify *C. trachomatis* and distinguish the nvCT from the wild-type. The presence of *C. trachomatis* was then further confirmed by the other target, the cryptic plasmid.

Study samples

The performance of this assay was initially assessed using *C. trachomatis* samples ($n = 28$) confirmed using the STI-MS assay (Xiu *et al*., 2019; STI-MS is a detection method combining multiplex-PCR with MALDI-TOF analysis). For these 28 samples, the 23S rRNA gene was previously characterized with respect to the wild-type, using Sanger sequencing. Three control plasmids, bearing individual mutations within the 23S rRNA target region for AC2, were used to develop and evaluate the HRM assay. These recombinant plasmids were directly constructed by Tsingke Biotechnology (Beijing, China). The plasmids bearing the 23S rRNA C1514T, C1515T and G1523A mutations were confirmed by sequencing. Our assay was also validated using 570 urethral/genital swabs, from which DNA was extracted using the MagNA Pure LC DNA Isolation Kit 1 (Roche Diagnostics, Mannheim, Germany) and tested for sexually transmitted infections (STI) agents using the STI-MS method (Xiu *et al*., 2019). Moreover, a total of 404 archived DNA specimens extracted from first void urine (FVU) and previously confirmed as positive for *C. trachomatis* using the Roche Cobas® 4800 CT/NG test (Roche Diagnostics, Indianapolis, IN, USA) were also screened using the HRM assay. These clinical samples were collected from clinical laboratories of different hospitals in the Shenzhen area between 2015 and 2018, obtained as part of the Shenzhen Gonococcal and Chlamydial Intervention Programme (SGCIP). A majority of the patients recruited to the study presented with urinary symptoms or for an asymptomatic sexual health screen and had a higher risk of sexually transmitted infections. No information regarding the sexual orientation of the patient or sexual contacts were available. All study participants older than 18 years who were normally resident in the Shenzhen.

Assay design and reaction conditions

Primers for the two targets (23S rRNA and cryptic plasmid) used in this study were designed by the software package Beacon Designer 7.0 (PremierBiosoft, Palo Alto, USA). All primers were synthesized by Tsingke Biotechnology (Beijing, China). Six specific primer sets were designed on a relatively conserved region of the cryptic plasmid. Six candidate primer pairs were selected to amplify the 23S rRNA region, where the nvCT hotspot mutations were located. The optimum primer pairs were selected using the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The single HRM assay was initially developed and performed using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosciences, Foster City, CA, USA). HRM assay reactions were carried out in a final volume of 20 μl, consisting of 10 μl EvaGreen Master Mix (Biotium, Hayward, CA, USA), 1 μl of each primer and 2 μl of the DNA template. Based on this established single HRM assay, a series of experiments were performed to optimize a multiplex HRM assay, including primer combinations, reagent concentrations and cycling parameters. For multiplex HRM analyses, reaction mixtures containing 10 μl EvaGreen Master, 0.5 μl each corresponding primer to 23S rRNA (HRM-CT-23S_F: 5'-AGTTAAGCACGCGGACGATT-3' and HRM-CT-23S_R: 5'- GCGGATTTGCCTACTAACCG-3') and cryptic plasmid (HRM-CT-cryP_F: 5'-CCGGCGGCGGGCCAGCACTCCAATTTCTGAC-3' and HRM-CT-cryP_R: 5'- CGGCGGCCGCCCTCGATGATTTGAGCGTGT-3'), and 2 μl of bacterial genomic DNA, or extracted DNA from clinical sample. The final reaction volume was adjusted to 20 μl with sterile water. The bases, forming the extra GC-rich tail were added to adjust and distinguish the melting temperature on the 5' end of cryptic plasmid primers, are underlined. Cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles at 95 °C for 15 s and 60 °C for 1 min. The melting analysis for the HRM assay was the same as described previously (Xiu *et al*., 2020): an initial holding step for 1 min at 60°C, followed by a slow temperature increase at a rate of 0.025°C/s to 95°C with continuous fluorescence signal collection. Melting curves were automatically generated by the QuantStudio 6 and 7 Flex Real-Time PCR software v1.0 (Applied Biosciences, Foster City, CA, USA) and melting temperature (Tm) values were obtained. Finally, using the Melt Curves (Derivative and/or Aligned Melt Curves), differences in melting curve shapes were analysed by comparing each amplicon curve with the control. This further enabled the clustering of samples into different groups.

Sanger sequencing

The V2 region of the 23S rRNA gene was amplified using conventional PCR methods, as described previously (Hokynar *et al*., 2019). Sanger sequencing was accomplished at Tsingke Biotechnology (Beijing, China). To confirm the nvCT, the obtained sequences were compared with the 23S rRNA sequences of *C. trachomatis* E/Bour strain (GenBank accession no. HE601870) and

three previously described AC2 diagnostic-escape nvCTs using the ClustalW algorithms implemented in BioEdit software version 7.0.9 (Ibis Bioscience, Inc., USA).

Statistical analysis

Statistical analyses of the HRM assay's limit of detection (LOD) were performed using Probit analysis, 95 % confidence interval (CI) was calculated using logistic regression model in SPSS statistical software package version 17.0 (SPSS Inc., Chicago, IL, USA). Overall per cent agreement, positive per cent agreement, negative per cent agreement and associated 95% CI were also calculated.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

JP, FW and LX were involved in the concept of the study. JP and FW contributed to the study design and formulating the research question. LX performed the experiments, analysed the results and wrote the manuscript. YL conducted the experiments and assisted in writing the manuscript. CZ, YL and YZ conducted the experiments and analysed the results. All authors reviewed and revised the final version of manuscript. All authors approve the work for publication and agree to be accountable for the work.

Ethical approval

All experiments were performed according to the ethical standards of the national research committee and approved by the Institutional Review Boards of the Institute of Pathogen Biology. All samples were obtained under approved ethical protocols and with informed consent from each patient.

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