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Evaluation of the Siemens VERSANT® CT/GC DNA 1.0 Assay (kPCR) for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

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ABSTRACT

The Siemens VERSANT kPCR system is an automated system which combines extraction of nucleic acids from 96 samples with subsequent real-time PCR. The VERSANT CT/GC DNA 1.0 (kPCR) assay detects *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (GC) in a multiplex real-time PCR on this system. We compared this assay with the BD ProbeTe™ ET System (PT) and the Roche Cobas Amplicor (CA). Three different sets of samples were tested in the kPCR: PT pre-treated samples, prospectively collected urine samples during routine CT/GC testing and urine samples obtained in a blinded fashion by an external lab facility. Agreement of kPCR with the comparator tests was >0.99 for sample set I and complete agreement was observed for sample set II and III. The kPCR assay demonstrated to be an easy to use robust diagnostic platform. A few modifications to the manufacturer's instructions are recommended to intercept false positivity. We advise to retest samples with Cq values above 35 cycles at least one time and we suggest checking the amplification curves.

1. Introduction

Chlamydia trachomatis (CT) and *Neisseria gonorrhoeae* (GC) are among the most prevalent sexual transmitted bacterial diseases. A nationwide study in the Netherlands showed an overall CT prevalence rate of 2% (Van Bergen et al., 2005). The prevalence of GC was much lower (Van Bergen et al., 2006). In Limburg, the region where most samples in this study were obtained, the prevalence of CT among young sexually active people was 5% (Van Bergen et al., 2010).

Already more than a decade diagnosis of these pathogens has relied on nucleic acid amplification tests like polymerase chain reaction (PCR), strand displacement amplification or similar molecular techniques (Olshen and Shrier, 2005).

In recent years real-time PCR systems were developed which combine high sensitivity and specificity in a closed tube format. This reduces contamination problems substantially.

Streamlining of the complete process from sample preparation to PCR result is highly desirable to reduce hands-on time. An automated nucleic acid purification procedure with automated pipetting steps and complete integration of the PCR setup is needed for high throughput within diagnostic laboratories.

The Siemens VERSANT kPCR system is such an automated system which combines extraction of nucleic acids from 96 samples with subsequent real-time PCR. The VERSANT CT/GC DNA 1.0 Assay (kPCR) assay detects CT and GC in a multiplex real-time PCR on this automated system including the recently described new variant of CT (nvCT) which was discovered in Sweden in 2006 (Ripa and Nilsson, 2006). We compared this assay with the BD ProbeTe™ ET System (PT). In addition we blindly tested urine samples obtained at a different lab site, which were tested with the Roche Cobas Amplicor (CA). This test has become available recently and only one evaluation has been done (Kerndt et al., 2011).

2. Materials and methods

2.1. Samples

Three different sets of samples were tested in the kPCR assay. Sample set I) urine from male and female ($n=111$), male urethral and female endocervical swab samples ($n=188$) pre-treated for use in the PT system (so called lysates) were collected and stored at −80 °C. From November 2008 until February 2010 all CT or GC positive lysates with approximately one negative control lysate for each positive per testrun were stored. These lysates contain DMSO and glycerol and were 1:1 diluted with M4RT medium (Remel, Lenexa, USA) (M4RT) as the kPCR system could not pipette and handle the pure lysate. Sample set II) From November 2009 until February

Abbreviations: CT, *Chlamydia trachomatis*; GC, *Neisseria gonorrhoeae*; kPCR, VERSANT CT/GC DNA 1.0 Assay; PT, BD ProbeTec™ ET System; CA, Roche Cobas Amplicor.

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2010 243 urine samples from male and female patients for CT/GC testing (by PT) were also collected in Siemens Urine transport medium (UTK), stored at 4 °C and tested by kPCR in February 2010. Sample set III) Finally, 292 urine samples were collected in UTK at a remote lab, stored at 4 °C and tested blindly by kPCR in August 2010. Information about gender was not available for this sample set.

The Quality Control for Molecular Diagnostics (QCMD; www.QCMD.org) panels for CT (2010–1) and GC (2010–1) were also tested during the evaluation period.

2.2. Tests

The kPCR assay was performed according to the manufacturer's instructions. The kPCR assay targets the cryptic plasmid of CT and the *piv*NG gene of GC in an internally controlled multiplex real-time PCR format. The assay is validated by the manufacturer for female endocervical swabs, male urethral swabs and female and male urines. Swabs should be collected in a collection and transport medium such as M4RT, urines should be collected with UTK. Urines collected in UTK are stable for three months at 2° to 30 °C. Two millilitres of first voided urine is added to the UTK, which contains a preservative (5 M guanidine thiocyanate). The tube is coded with a barcode and placed on the sample preparation (SP) module of the system. The machine is completely automated and takes 250 µl of the urine and finally elutes in 100 µl. An internal control which is derived from *Methanobacterium thermoautrophicum* is added to each sample by the system. For the subsequent PCR in the amplification and detection (AD) module 25 µl eluate is used as input. The pipetting device sets up the complete PCR by adding mastermix (including Uracil N-glycosylase (UNG) to prevent carryover) and primer/probe mix to all the samples. The PCR program contains a ten-minute UNG activation step (50 °C), 15 min Taq polymerase activation step (95 °C) followed by 40 amplification cycles of 15 s 95 °C and 1 min 62 °C.

Remaining eluate can be used for additional PCR tests. In our lab we currently use the remaining eluate for *Trichomonas vaginalis* real-time PCR (data not shown but details obtainable from the corresponding author). For 94 samples a kPCR workflow needs 6 h. This includes one hour hands-on time for sample handling and system setup (loading disposables, reagents and samples), 3 h processing time for extraction and automated PCR setup (2 h for 47 samples) followed by 10 min hands-on time to seal the PCR plate and transfer to PCR machine and 1 h and 45 min for real-time PCR. Processing two full runs in a 9 h shift is possible.

The PT and CA are well known assays (Olshen and Shrier, 2005) and were performed according to the manufacturer's instructions. PT and CA are well known for false positive GC results. Therefore GC confirmation is routinely performed for GC positive samples. In the Netherlands frequently a home-brew GC *opa* gene real-time PCR is used (Geraats-Peters et al., 2005; Palmer et al., 2003). The *opa* gene codes for an outer membrane protein that contributes to colony opacity (*opa*) during growth on agar plates. This test is known to be non-reactive with 84 non-GC strains and is performed by accredited medical microbiology labs which participate in external quality control programs.

2.3. Test algorithm

The first and second set of samples were tested by kPCR and PT. These samples were not blinded and were intended to observe discrepant results. All discrepant results were re-tested by kPCR. The results of the first and second set of samples were used to refine the interpretation of the CT/GC test and a final operating procedure was implemented. Subsequently the third set was tested by kPCR and compared with CA. kPCR testing of this third set of samples was performed without knowledge of the results at the remote testing site. After comparison of the results with the remote test site CT discrepant samples in this set were analysed by the Dutch reference centre for CT infections (VU University Medical Centre, Amsterdam) with a commercially available real-time PCR (Presto CT/NG assay kit, Goffin Technologies, Beek, NL), which targets the cryptic plasmid. Samples were considered true positive when at least two tests were positive. For all sample sets a positive GC result by PT, CA or kPCR had to be confirmed by the *opa* gene PCR before the final result was definitely judged as positive.

2.4. Analysis

For sample sets I and II a sample was considered true positive when both tests (kPCR and PT) were positive. Similarly, a sample was considered true negative if both tests were negative.

In case of a discordant result kPCR was only considered positive or negative when this result was repeated by retesting.

The final result of sample set I was analysed and a κ-agreement was calculated (Graphpad Quick calcs).

For sample set III the samples were considered true positive when both tests (kPCR and CA) were positive. Samples were considered true negative if both tests were negative. Samples were repeated when Cq values exceeded 35 cycles. Only repeatedly positive samples were judged as true positive.

3. Results

3.1. Sample set I

The set contained 299 samples which were all tested for CT and GC. The results are shown in Table 1. Although the sample type in this set was not validated by the manufacturer, the internal control was amplified in all samples. This makes it unlikely that inhibition occurred and excludes false negative results. For GC no discrepancies were observed. One endocervical sample was positive for GC by PT and negative in kPCR, however the confirmation PCR was also negative, thus agreement with kPCR was observed. K-agreement was 1.0. The range of Cq (quantification cycle, cycle at which the target is quantified) values of all kPCR positives was 16.5–32.6.

One discrepant CT sample was found. This female urine sample was positive in PT and negative in kPCR (sample repeatedly negative in kPCR). K-agreement was 0.993. The range of Cq values was 15.1–33.6.

Three samples were retested by kPCR because of an initial discrepant result between PT and kPCR. These three samples had high Cq

Table 1
Results of sample set I obtained with kPCR and PT.

$n=299$			PT			
			CT		GC	
			Positive (m/f)[a]	Negative (m/f)[a]	Positive (m/f)[a]	Negative (m/f)[a]
kPCR	Urine ($n=111$)	Positive	55/7	0/0	10/0	0/0
		Negative	0/1	42/6	0/0	87/14
	Male urethral swab/female	Positive	3/63	0/0	5/7	0/0
	Endocervical swab ($n=188$)	Negative	0/0	5/117	0/0	3/173

[a] Male versus female counts.

Table 2
Results for sample set II obtained with kPCR and PT and sample set III obtained with kPCR and CA.

		PT ($n=243$)				CA			
		CT		GC		CT ($n=292$)		GC ($n=267$)	
		Positive (m/f)[a]	Negative (m/f)[a]	Positive (m/f)[a]	Negative (m/f)[a]	Positive	Negative	Positive	Negative
kPCR	Positive	14/2	0/0	5/0	0/0	27	1	2	0
	Negative	0/0	189/38	0/0	198/40	4	260	0	265

[a] Male versus female counts.

values above 35 cycles. After retesting all three samples were negative and in agreement with PT.

3.2. Sample set II

203 male and 40 female urines were tested (Table 2). In this set two samples were repeated as Cq values were above 35 cycles (above the upper limit of Cq values we had seen in the evaluation of sample set I). Both samples were negative on retesting and definitely judged negative. With this small adjustment, results of both tests were complete in agreement.

3.3. Sample set III

292 urine samples were tested for CT and 267 urine samples were tested for GC, as testing at the remote lab site was only performed when CT or GC was specifically requested by the physician. Data were shown in Table 2. Two samples were GC positive by CA but the confirmation PCR was negative. Both samples were also negative by kPCR. Surprising discrepancies were observed in the CT series. Five discrepant results were obtained. These five samples were tested by the Dutch reference centre for CT infections (VU University Medical Centre, Amsterdam). All five samples were in agreement with kPCR. CA therefore generated four out of 292 (1.4%) false-positive results.

When sample sets II and III are combined, agreement for CT ($n=535$) and GC ($n=510$) after discrepancy analysis was 100% with the notification that Cq values>35 cycles should be repeated.

3.4. QCMD panels

QCMD panels for CT and GC were also tested during the evaluation period. For CT there were no discrepancies with the expected result. The panel included the Swedish variant strain. For GC all samples with one exception were in agreement with final results. The exception was a sample (QCMD NG10-02) containing a *N. cinerea* strain which was isolated from a clinical specimen. The sample was positive as judged by Cq value. We observed an unexpected amplification curve which was positive at a Cq value of 27 cycles but fluorescence value did not exceed the curve of the positive control (Fig. 1). The positive control is a low copy positive control and positive around 34 cycles. The *N. cinerea* strain was tested three times and each time fluorescence was approximately three quarters of the fluorescence level (dRn value) as compared with the positive control.

4. Discussion

This study shows that the kPCR assay is a useful and accurate test for detection of CT and GC.

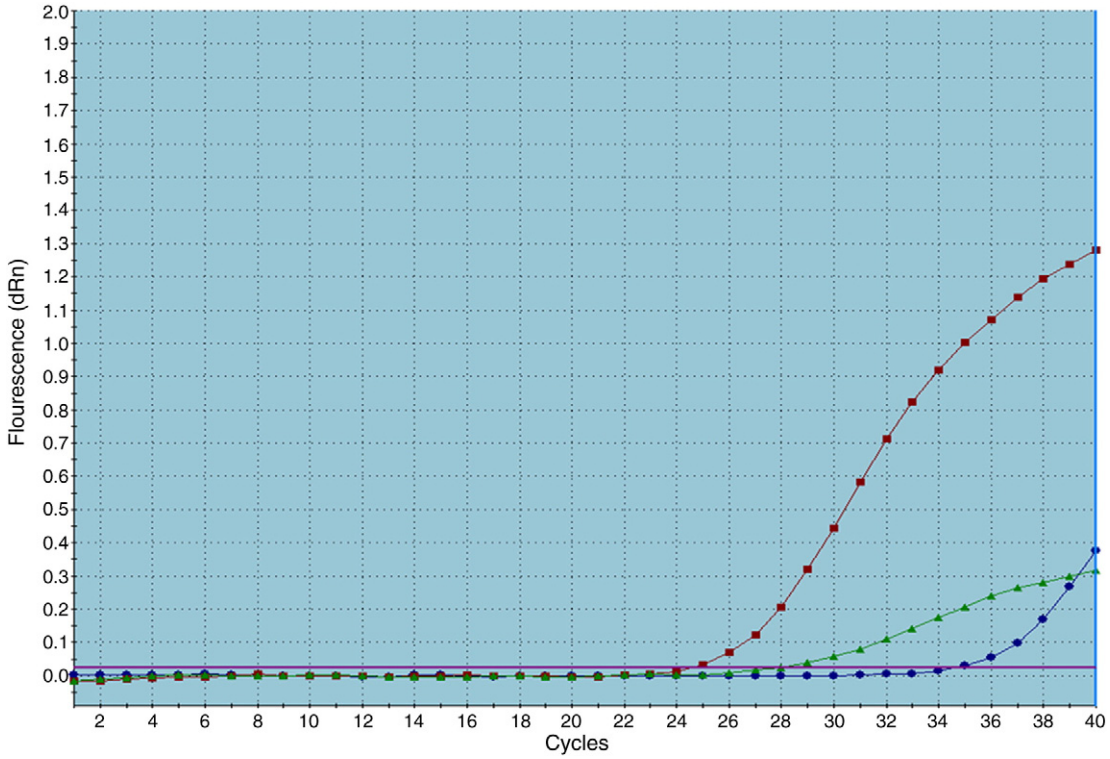

Fig. 1. Amplification plot of the GC QCMD samples. ■ Shows a positive sample with a Cq 24,63. ● Shows the positive control with Cq 34,65. ▲ Shows QCMD sample NG10-02. This sample with Cq 27,84, gives a non-logarithmic curve.

In addition the system (VERSANT kPCR system) underlying this assay is convenient and needs less hands-on time compared to our previously used PT system. As the amplification curves can be viewed in the results screen it is possible to see the underlying information concerning shape and relative fluorescence of the curves. Concerning the different sample sets there is overall very good agreement of kPCR with the different comparator tests. In sample set I discrepancy analysis was not performed on the only CT positive sample in PT that was repeatedly negative in kPCR as the result will hardly influence the overall image. Furthermore these lysates are not official sample types for kPCR. This set is especially important since it has compressed all positive samples obtained in a one and a half year period in one relatively small sample set.

Sample set two mimics the normal diagnostic situation without blinding of the results of the comparator test. Two samples with Cq values transcending 35 cycles were repeated and became negative on retesting. We judged these samples as negative. With this adjustment, agreement with PT was complete.

The third sample set was intended to proof the final test protocol. Unexpected, we observed five CT discordant. After discrepancy analysis all samples were in agreement with kPCR. CA produced four false-positive results in this sample set. This unexpected high proportion of false-positives is worrisome. It is not sure if this proportion of false-positives is a general or just a local problem. Although, this high percentage false-positives by CA were also shown in other studies (Chan et al., 2000).

CT QCMD samples were all correct including the nvCT Swedish variant strain. One GC QCMD sample containing *N. cinerea* showed a positive Cq value but a strange, non-logarithmic amplification curve. A low final fluorescence value (dRn) was observed. Fluorescence did not exceed the fluorescence value of the low positive control. In the contrary, fluorescence was approximately three quarters the value of the positive control. This phenomenon is probably caused by a probe that can partly bind to this *N. cinerea* strain. We reported this experience to Siemens Healthcare Diagnostics (SHD). With this knowledge this strain can easily be recognised as a false-positive signal. We did not observe such unusual curves in all the positive samples tested in this evaluation. These results were reported to the manufacturer and have meanwhile contributed to the fact that the package insert of the assay will state the false-positivity observed with this QCMD strain. More *N. cinerea* strains will be tested and sequenced in the near future by SHD.

Previous validation for specificity of the assay by the manufacturer for *N. cinerea* strains did not reveal positive signals (Meng et al., 2007) although this specific QCMD provided strain was not included. In another study this strain was also found positive by kPCR (Kerndt et al., 2011). The clinical relevance of this observation has to be determined in the near future. Until then we advise to perform a confirmation test if these kinds of curves are observed. From a clinical perspective, it is reassuring that all positive GC kPCR results were confirmed by a second PCR directed at the *opa* gene.

In addition to the manufacturer's instructions we suggest a few modifications to the reporting of the results. We observed a few positive samples with Cq values above 35 cycles that could not be reproduced by retesting and were also negative in the comparator tests. Therefore we advise to retest these kinds of samples at least one time (including a new extraction) on the kPCR system. This observation was perhaps also seen by the study of Kerndt et al. (Kerndt et al., 2011). They reported 13 positive results ($n=1125$) by kPCR in comparison with the Aptima Combo 2 assay (Gen-Probe) which became all negative after retesting. Unfortunately, Cq values of these samples were not shown.

In all tested clinical samples so far no false positive GC results were obtained. Although it should be kept in mind that we only tested urine and endocervical swab samples. Evaluation of extragenital samples is still needed and these samples should be subject to a confirmatory test (Palmer et al., 2003).

5. Conclusion

Overall excellent agreement with PT and CA was observed for urine and endocervical samples. The kPCR assay is a real-time PCR assay with less hands-on time due to complete integration of DNA extraction and PCR setup in one single system.

A few modifications to the manufacturer's instructions are recommended to intercept false positivity. We advise to retest samples with Cq values above 35 cycles two times and we suggest checking the amplification curves.

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