A misleading false-negative result using *Neisseria gonorrhoeae opa* MGB multiplex PCR assay in patient's rectal sample due to partial mutations of the *opa* gene

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SUMMARY

A 53-year-old homosexual man presented at his general practitioner (GP) practice with a suspicion of sexually transmitted infection. Initial NAAT screening was performed for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. The patient was positive for *Neisseria gonorrhoeae* both for his urine and rectal sample. The subsequent confirmation test for *Neisseria gonorrhoeae* by a second laboratory was only confirmed for the urine sample and the rectal sample was negative. We report a case of a potential false-negative diagnosis of *Neisseria gonorrhoeae* due to mutations of DNA sequence in the probe region of *opa*-MGB assay of the rectal sample. The patient did not suffer any discomfort as diagnosis of *Neisseria gonorrhoeae* in his urine sample had already led to treatment by prescribing the patient with Ceftriaxone 500 mg IV dissolved in 1 ml lidocaine 2% and 4 mL saline. The patient also received a prescription for Azithromycin (2x500 mg).

KEY WORDS: Neisseria gonorrhea, Sexually transmitted infections, Mutation, Multiplex PCR, GP practice.

INTRODUCTION

Limitations with either sensitivity or specificity of diagnostic test methods have the potential to seriously undermine the ability of diagnostic test systems to detect and identify of certain infectants. Identification of *Neisseria gonorrhoeae* (NG) isolates and its molecular detection in clinical samples can be problematic. NG has the ability to change its phenotypic and genotypic properties (Linz *et al.*, 2000; Whiley *et al.*, 2006).
Such an issue was encountered in our laboratory, which might have resulted in a false neg-

ative diagnosis. Here we describe a case where a low sensitivity of a test method might have had a potentially negative result and diagnosis of *Neisseria gonorrhoeae*.
The urine and rectal sample of a patient were analyzed for the presence of sexually transmitted infections.

CASE REPORT

A 53 year-old homosexual man presented at his general practitioner (GP) practice with complaints of painful urination and discharge from the urinary meatus.
His partner had similar complaints, but had not been tested for sexually transmitted infections (STI). The patient travels extensively around the world and frequently engages in MSM (men

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who have sex with men) activity. The patient has a clean medical history except for previously diagnosed STIs, including syphilis (2009), *Neisseria gonorrhoeae* (2010 and 2012) and *condylomata acuminata* (2012). The patient uses 10 mg of zolpidem daily and 50 mg of sildenafil when necessary.

Based on medical history and the physical examination for discharge from the urethra, which did not yield any abnormalities, the patient was immediately put on Ceftriaxone 500 mg IV dissolved in 1 ml lidocaine 2% and 4 mL saline.

The patient also received a prescription for Azithromycin (2x500 mg). Prior to use of the prescribed antibiotic treatment, clinical samples were taken for laboratory diagnostics: serology for syphilis, hepatitis C and HIV, and urine, rectal and throat swabs for *Chlamydia trachomatis* and NG PCR testing.

After initial screening of rectal and urine, samples tested positive for the presence of NG with a commercial kit targeting the 16s rRNA gene (Vahidnia *et al.*, 2014).

To increase the positive predictive value of the NG screening test and avoiding a potential increase in misdiagnoses (Alexander *et al.*, 2011), a second laboratory performed a confirmatory testing assay on the *opa* gene using a minor groove binding (MGB) probe (Geraats-Peters *et al.*, 2005).

The conformational tests with *opa* MGB assay returned a positive NG result on the urine sample, while the rectal sample was confirmed as negative.

LABORATORY METHODS AND DIAGNOSIS

Due to the discrepant findings between the two samples and the laboratory results, we performed a new confirmatory test to examine the role of the two different testing strategies for confirmation and to determine whether NG strains undergo mutational changes in the intestinal environment, which might lead to the false-negative results with *opa* MGB assay. We developed and validated a new in-house multiplex PCR (m-PCR) based on the primer and probe sequence of Geraarts-Peters *et al.* (2005), replacing the MGB fluorophore by a conventional fluorophore JOE and by elongating the total length of the probe at the 5' in order to achieve optimal annealing temperature for a Taqman-based assay. Furthermore, a *porA* gene for NG was added to the algorithm along with the *PhHv* gene as DNA extraction control for the in-house m-PCR assay (Table 1). We validated our in-house opa and porA assay against 632 patient samples (prospective and retrospective samples) with sensitivity and specificity above 0.95.

The specimens were collected in a lysis buffer eNAT and transport buffer Eswab (Copan, Brescia, Italy) for maximum yield. The samples were subjected to a freeze-thaw cycle of -80°C overnight followed by 95°C for 15 minutes the next day. DNA extraction and detection were performed on Roche Aurora Flow (Vahidnia *et al.*, 2014). The input and output volume for the DNA-extraction on MagnaPure 96 were 100 µl. The in-house m-PCR Taqman-assay for NG was performed on the FLOW under the following

TABLE 1 - *The used primers and probes for the Neisseria gonorrhoeae confirmational tests.*

Name	Sequence 5→3	Fluorophore	Reference
opa-F	GTTGAAACACCGCCCGG		Geraarts-Peters et al. 2005
opa-R	CGGTTTGACCGGTTAAAAAAAGAT		Geraarts-Peters et al. 2005
Probe *opa*-1	CCCTTCAACATCAGTGAAA	MGB	Geraarts-Peters et al. 2005
Probe *opa*-2	CTTTGAACCATCAGTGAAA	MGB	Geraarts-Peters et al. 2005
NG *opa*1 P	CCCGATATAATCCGCCCTTCAACATCAGTG	JOE	This study
NG *opa*2 P	CCCGATATAATCCGCCTTTGAACCATCAGT	JOE	This study
NG *por*A F	GGCTGCAGTTGACCGAG		This study
NG *por*A R	TAAACGAGCCGAAATCACTGAC		This study
NG *por*A P	CCACCCTCAGAAGGTCAAACGGGCAATACA	ROX	This study
PhHv F	CGTTCCAACAACACAACCTACT		This study
PhHv R	CGCCCAAAATTTCTCTCATATCAT		This study
PhHv P	CCACCAATTACTCCTAGTCCACCACCGAAA	Cy5	This study

TABLE 2 - *Multiplex-PCR results of various Neisseria gonorrhoeae genes and the internal control PhHv for urine and rectal samples for the Taqman en the MGB-assays.*

	PhHv	16s rRNA	porA-ROX	opa-JOE	opa-MGB
Urine	26.79	21.07	23.96	21.00	22.33
Rectum	27.48	31.44		30.54	

PCR conditions: 95°C for 15 minutes followed by 5 cycles of 95°C for 5 seconds, 58°C for 20 seconds and 72°C for 15 seconds, followed by 40 cycles of 95°C for 5 seconds, 58°C for 20 seconds and 72°C for 20 seconds (fluorescence detection). Prior to MGB-assay a manual DNA extraction was performed using Qiagen DNeasy minikit (Qiagen, Venlo, the Netherlands) with 200 µl input volume and 50 µl output volume in order to concentrate the DNA-extract.

The manually extracted DNA was used for sequence typing by means of NG-MAST, according to standard operating procedures (Van Looveren *et al.*, 1999). The amplified PCR products with *opa* primers (Table 1) were used for DNA cloning and subsequent sequencing. All plasmids were created by ligation of this specific PCR product into pGEM-Teasy (Promega, Leiden, the Netherlands) and transformation to competent cells (JM109). Plasmid DNA was isolated using the Qiagen Miniprep Kit (Qiagen, Venlo, the Netherlands). Subsequently, plasmids were Sanger-sequenced according to standard procedures (Sanger *et al.*, 1977). The sequenced PCR products for the urine and rectal samples were aligned using the online alignment tool ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). For NG-culture, selective gonococcal agar plates (BD diagnostics, Erembodegem, Belgium) - were inoculated on the same day and subsequently incubated at 36°C in a 5% CO_2-enriched environment. Growth was observed up to 48 hours. Neither urine or rectal sample showed any growth on the selective culture. The newly validated m-PCR conformational test returned positive for *opa* genes of both urine and rectal samples, while the *porA* in the rectal sample was negative (Table 2). The NG sequence typing for the rectal sample was inconclusive due to low DNA load for the rectal sample. Additional Sanger sequencing of both clinical samples showed several mutations by means of insertion of a nucleotide in the annealing area of the MGB probe. This may be the reason why the rectal sample with *opa* MGB assay returned false-negative whereas the *opa*-Taqman assay did not (Figure 1). The mutations have probably resulted in a much lower annealing temperature in MGB than in the Taqman assay and perhaps in combination with a lower NG DNA-load of the rectal sample.

DISCUSSION

In the present study, we have shown that in some cases it is possible for a confirmatory assay to fail. Comparable results have been shown by Luijt and co-workers (Luijt *et al.,* 2014), who showed the emergence of *porA* mutants, and by Herrmann and his group, who first reported a Swedish variant of the CT strain with a 377-bp deletion in the cryptic plasmid, which contained the target sequences for CT molecular assays (Hermann, 2007). It should be noted that the clinical isolates of both laboratories, the laboratory that performed the initial screening as well as the confirmation labora-
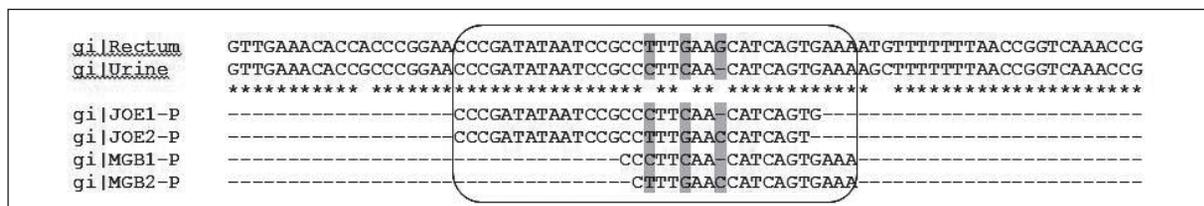


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gi|Rectum   GTTGAAACACCACCCGGAACCCGATATAATCCGCCTTTGAAGCATCAGTGAAATGTTTTTTTAACCGGTCAAACCG
gi|Urine    GTTGAAACACCGCCCGGAACCCGATATAATCCGCCTTCAA-CATCAGTGAAAGCTTTTTTTAACCGGTCAAACCG
            ********** ******** ******************** ** ** *********** ******************
gi|JOE1-P   -----------------CCCGATATAATCCGCCTTCAA-CATCAGTG---------------------------
gi|JOE2-P   -----------------CCCGATATAATCCGCCTTTGAACCATCAGT----------------------------
gi|MGB1-P   -----------------------------CCCTTCAA-CATCAGTGAAA-------------------------
gi|MGB2-P   -----------------------------CTTTGAACCATCAGTGAAA-------------------------
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FIGURE 1 - *The NG sequences of the urine and rectal samples are aligned in this figure and depicted against the JOE and MGB probes (in-boxed). The mutations between the urine and rectal DNA sequences and probes are highlighted in gray.*

tory, were examined with the newly developed in-house m-PCR (Table 2). The Taqman assay tested positive on both the clinical isolates for urine and the rectal swabs, in contrast to *opa* MGB, which only tested positive for the urine samples. The absence of *porA* in the rectal swab is in line with other studies indicating presence of false-negative results with *porA* (Whiley *et al.*, 2011; Ison *et al.*, 2013). Furthermore, concentrating of the DNA-extract for the MGB-assay did not have the desired effect for lower Cp-value (higher DNA load). This could be indicative of a lower sensitivity of the MGB assay than the Taqman-assay (Table 2). In our study, we showed mutational changes in the *opa* gene from the rectal swab (Figure 1). The reason for this mutational change is unclear; phase variation in the *opa* gene as result of mutation could be a possible explanation (Anderson and Seifert, 2013). Whether this mutation will lead to phenotypical changes is subject to further study. One could speculate that mutations in *opa* genes could perhaps lead to differences in the binding capacity of the mannose-binding lectin, a first line defense against microorganisms by complement activation and/or opsonization in the absence of specific antibodies as demonstrated by Eastbrook and coworkers for *N. meningitides* (Eastbrook *et al.*, 2014). Another interesting factor, as proposed by Rank and coworkers (Rank and Yeruva 2014) for *C. trachomatis*, may be the inability of the immune system to clear the bacteria from the lower gastrointestinal tract, thereby creating an ideal environment for recombination and exchange base pairs within the Neisseriaceae itself. However, future studies are needed to verify this hypothesis. But above all, this underscores the need for vigilance in monitoring for abnormalities in the routine diagnostic algorithm.

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