INVESTIGATIVE REPORT

Appropriate Time for Test-of-Cure when Diagnosing Gonorrhoea with a Nucleic Acid Amplification Test

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Short title: Appropriate time for test-of-cure of *N. gonorrhoeae* using NAAT

Culture is commonly regarded as the gold standard for diagnosis of *Neisseria gonorrhoeae*. However, nucleic acid amplification tests (NAATs) have rapidly replaced culture for diagnostics in many settings. The aim of the present study was to investigate the appropriate time for test-of-cure (TOC) when NAATs are used for diagnosis of gonorrhoea. In total, 30 patients (28 men and 2 women) provided urethral, cervical, rectal or pharyngeal specimens for TOC. All included patients, except one who did not return for second TOC before day 19, tested negative within two weeks after treatment with cefixime 400 mg×1. Antimicrobial susceptibility testing showed that 68% of the culture positive strains were resistant to ciprofloxacin. Thus the recommended empirical treatment with ciprofloxacin in Norway should promptly be changed. TOC can be performed 2 weeks after treatment when NAATs are used for diagnosis of gonorrhoea.

Keywords: TOC, test-of-cure, *N. gonorrhoeae*, PCR, NAAT.

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Neisseria gonorrhoeae is the etiological agent of gonorrhoea, which is the second most prevalent bacterial sexually transmitted infection globally. Microscopy of urethral smears (Gram or methylene blue stained) has a high sensitivity (≥95%) for symptomatic men. However, the sensitivity of microscopy in asymptomatic men or cervical samples is too low for reliable diagnostics (≤55%) (1). Culture has been regarded as the gold standard for diagnosis of gonorrhoea, but during recent decade nucleic acid amplification tests (NAATs) have rapidly replaced culture due to, among other advantages, the improved sensitivity (2).

However, NAATs do not allow antimicrobial susceptibility testing. This is a major disadvantage, especially when the level of antimicrobial resistance in *N. gonorrhoeae* is high to all antimicrobials previously recommended as first-line treatment options (3-6). Furthermore, the susceptibility to the currently recommended first-line treatment, extended-spectrum cephalosporins (ESCs) is declining globally (3;4;6). These ESCs, i.e. ceftriaxone (injectable) and cefixime (oral), are the last remaining treatment options in several settings. Using ceftriaxone, no treatment failure of urogenital gonorrhoea has yet been reported. However, verified treatment failures with cefixime, which is the standard treatment in many countries, have been reported in Japan since 2007 (7). Worryingly, the first two cases of failure outside Japan were recently reported in Norway (8). Test-of-cure (TOC) after provision of gonorrhoea therapy may soon be crucial in many settings.

Appropriate evidence-based recommendations for the appropriate time for TOC using different NAATs for diagnosis of gonorrhoea is lacking. In contrast to some international guidelines (1;9), which do not recommend TOC for uncomplicated gonorrhoea if a recommended treatment has been given, in Norway TOC is recommended for all gonorrhoea cases. This recommendation has been fortunate considering the reluctance to abandon ciprofloxacin as the recommended empirical treatment for gonorrhoea in Norway (10;11). Currently a strictly validated in-house *porA* pseudogene PCR (12;13) is extensively used for diagnosis of gonorrhoea in Norway, nevertheless, the national recommendations for TOC are based upon the time for TOC using culture, i.e. seven days.

The aim of the present study was to investigate the appropriate time for test-of-cure when detection of *N. gonorrhoeae* is performed using an in house *porA* pseudogene PCR.

MATERIALS AND METHODS

Study population

Two hundred and fifty-seven consecutive patients with suspected genital and/or extra-genital gonorrhoea attending an STI outpatient clinic (Olafiaklinikken) in Oslo, Norway were recruited

from June 2006 through January 2007. The 257 patients comprised 23 women (mean age: 31 years, range: 15 to 46 years) and 234 men (mean age: 33.7 years, range: 15 to 73 years), where the majority (66.7%) was men having sex with men (MSM).

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK NORD) and each included participant gave a written consent.

Clinical samples

Samples for PCR were collected using either a urethral flocked swab (Copan, Brescia, Italy) or an endocervical flocked swab (Copan, Brescia, Italy). The urethral swab was used for sampling the urethra. The endocervical swab was used for sampling the cervix, rectum and pharynx. Each sample was collected with individual swabs that were placed and transported in UTM-RT (universal transport media – room temperature) transport media (Copan, Brescia, Italy) for PCR analysis. The samples for culture were consistently taken before the samples for PCR to avoid reducing the quality of the routine culture diagnostics. The sampling sites were chosen based on the medical history of each patient. Patients were asked to return after one week for a follow-up examination and TOC, and subsequently every week until two negative samples were deposited. In total 669 clinical samples were collected from the 257 patients. Patients with positive samples who did not return for any TOC within 2 weeks were excluded from the study.

Culture diagnostics

The culture diagnostics was performed at Oslo University Hospital Ullevål, Oslo, Norway as part of their routine diagnostics by identification of characteristic colonies on selective culture medium according to standard laboratory procedures. For thorough species verification, oxidase test, identification of Gram-negative diplococci in microscopy, sugar utilisation test, and Phadebact GC Monoclonal test (Bactus AB, Huddinge, Sweden) were used.

Antimicrobial susceptibility testing

Determination of the minimum inhibitory concentrations (MICs, mg/L) of ceftriaxone, ciprofloxacin and spectinomycin was performed using the Etest method according to the instructions of the manufacturer (bioMérieux, Solna, Sweden). Interpretative criteria from the European Committee on Antimicrobial Susceptibility testing (EUCAST, www.eucast.org) were used.

DNA preparation

All the UTM-RT samples were vortexed for 10 seconds and 200 µl sample were subsequently used for DNA preparation with the infectious disease protocol on the Biorobot M48 workstation (Qiagen, Hilden, Germany), with an elution volume of 100 µl.

Real-time PCR

A real-time TaqMan FAST *porA* pseudogene PCR was performed as previously described (12;13), using 11.5 µl DNA template and 13.5 µl mastermix. All positive specimens were confirmed by repeated testing from a new DNA isolation.

Antimicrobial treatment

All patients except one were treated with cefixime 400 mg×1 (oral dose), despite the Norwegian recommendations of using ciprofloxacin for empirical treatment. The remaining patient was administered spectinomycin 2 g×1 intramuscularly.

RESULTS

In total 50 gonorrhoea patients were identified. Eight of these patients were excluded because they did not return for any TOC, and twelve were excluded for returning later than two weeks after treatment for the first TOC. Accordingly, 30 patients that were *N. gonorrhoeae* positive, diagnosed by culture (n = 27) and/or NAAT (n = 30), in at least one clinical specimen were further examined. These 30 positive patients comprised 2 women (mean age: 24.5 years, range: 21 to 28 years) and 28 men (mean age: 37.4 years, range: 22 to 58 years), of which 50% (n=14) were MSM. Seven clinical specimens (representing different anatomical sites) from seven patients were positive using NAAT, but negative with culture. Three of these patients (10% of all included patients) did not have any positive culture sample and, accordingly, would have been reported falsely negative if not also NAAT was used for diagnostics. No patients were positive by culture only. Twenty-five patients were diagnosed with gonorrhoea at a single urogenital site, two patients had only extra-genital gonorrhoea (pharyngeal and rectal), and three patients had multiple infected sites (Supplemental Table). All patients diagnosed with urogenital gonorrhoea reported symptoms such as discharge and dysuria, while all the extra-genital infections were asymptomatic.

Nineteen patients (63%) returned for TOC within seven days (day four to day seven) after treatment, and 16 (84%) of these were negative using NAAT. Two of the patients that remained positive (positive TOC on day four and day six) provided a negative sample within 14 days (day 11

for both patients). The remaining patient (positive TOC on day seven) did not return before day 19, but then had a negative TOC (Supplemental Table; Fig. 1A).

There were eleven (37%) patients who did not return for their initial TOC before day eight to day 14 after treatment, however they were then all negative (Supplemental Table; Fig. 1B).

Antimicrobial resistance testing was performed on *N. gonorrhoeae* isolates from 25 of the 30 included patients. Isolates from 17 (68%) of these patients were ciprofloxacin resistant; however, no isolate was resistant to ceftriaxone (Supplemental Table), or spectinomycin.

DISCUSSION

High level of antimicrobial resistance in *N. gonorrhoeae* is a public health problem worldwide. The internationally recommended first-line ESCs are the only remaining options for effective treatment of gonorrhoea in several settings (8;14). However, the susceptibility to all the ESCs is decreasing and treatment failures of urogenital gonorrhoea have been identified using the oral ESC cefixime (6-8). Furthermore, Ohnishi et al. (15) recently described the first strain with high-level resistance to ceftriaxone, which most likely was related to a treatment failure of pharyngeal gonorrhoea. Accordingly, gonorrhoea may become untreatable in certain circumstances and especially in some settings. Appropriate verification/falsification of presumed clinical treatment failures need to be emphasized worldwide.

A more extensive use of TOC to assess treatment outcome is accordingly warranted. However, the use of TOC for *N. gonorrhoeae* is debated and the recommendations vary for uncomplicated gonorrhoea. Centers for Disease Control and Prevention (CDC) (16) recommend re-testing within 3 months rather than TOC for uncomplicated gonococcal infection, as a high number of positive cases after treatment may be re-infections (17;18). The British Association of Sexual Health and HIV (BASHH; www.bashh.org/documents/3611) and The International Union against Sexually Transmitted Infections (IUSTI) (1) does not recommend TOC routinely for anogenital infections if a recommended treatment has been given. Indications for TOC are persistence of symptoms, re-exposure to infection, possible antimicrobial resistance, stipulated by national practice or in case of pharyngeal infections. Manavi et al. (19) also recommends TOC for pharyngeal infections because of higher rate of treatment failure. Accordingly, especially pharyngeal gonococcal infections pose an additional problem because these can be difficult to treat (20-22), and are often asymptomatic resulting in potential reservoirs for further transmission.

NAATs are rapidly replacing culture for detection of *N. gonorrhoeae*, and for non-culture based diagnostics, such as NAATs, adequate evidence-based recommendations for appropriate time for TOC are lacking. One study has previously been published regarding appropriate time for TOC

using NAAT for detection of *N. gonorrhoeae*. This study by Bachmann et al. (23) examined urine and patient-obtained vaginal swab specimens using ligase chain reaction (LCR). They found that all gonococcal DNA was absent from urine samples by day 6 (regardless of sex) and vaginal swabs by day 9, and concluded that TOC could be taken within 14 days after appropriate treatment regardless of specimen. In the present study all individuals, except one, were negative within two weeks after treatment using an in house *porA* pseudogene PCR. The remaining patient did not return before day 19 for his second TOC, but then had a negative TOC. Furthermore, 84% of the patients returning for their first TOC (within one week) were already negative. The present study using an in house *porA* pseudogene PCR (12;13) fully supports the findings by Bachmann et al. (23). Accordingly, an appropriate time for TOC using NAATs for diagnosis of gonorrhoea seems to be 14 days after treatment. It is advantageous to avoid having a longer time before TOC, i.e. to reduce the risk of a positive TOC due to re-infection instead of treatment failure.

This study also showed that 24% of the initial 50 gonorrhoea patients did not return within 2 weeks for TOC despite strict instructions to return already after 1 week, and 16% never returned for TOC. Failure to return for TOC is a greater concern in cases where only molecular results are available and treatment outcome cannot be assessed due to lack of antimicrobial resistance testing. Partner notification and close follow-up of the index patient was vigilantly performed in this study.

The main limitations of the present study included that it was a low number of examined gonorrhoea patients, several patients did not return for TOC at requested time points, and additional TOCs were not performed with short intervals (not possible in the routine diagnostics), as performed in the study by Bachman et al. (23).

In Norway, the national recommendations for empirical first-line treatment of gonorrhoea remains ciprofloxacin (10). In the present study, 68% of the culture positive patients were infected by a ciprofloxacin resistant *N. gonorrhoeae* strain. Thus the guidelines for empirical treatment should be changed promptly, which is also further supported by a few previous Norwegian publications (13;24) as well as numerous international reports (25-29).

In conclusion, an appropriate time for TOC seems to be 14 days after appropriate treatment when an in house *porA* pseudogene PCR is used for detection of *N. gonorrhoeae*. Despite differences in sensitivity, this time for TOC is most probably similar using most NAATs (at least the DNA-based NAATs). TOC and re-testing should be more frequently performed, and part of a vigilant follow-up of partners and index patients. Facing the threat of untreatable gonorrhoea, selection and use of antimicrobials should be evidence-based and warrants timely surveillance programs nationally and internationally. Accordingly, the Norwegian gonorrhoea treatment guidelines should be modified promptly based upon the high prevalence of ciprofloxacin resistant isolates.

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The authors declare no conflicts of interest.

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Supplemental Table. *Gonorrhoea patient characteristics: sex, age, sample site, test of cure (TOC), initial method of diagnosis, and result of antimicrobial susceptibility testing*

Sex	Age	Sample site	Days to 1st TOC	Days to cure (1st or 2nd TOC)	Initial method of diagnosis		MIC[a], mg/L	
					PCR	Culture	ciprofloxacin	ceftriaxone
M	47	Rectum			Neg			
		Pharynx			Neg			
		Urethra	6	6	Pos	Pos	32	0.047
M	22	Rectum	6	6	Pos	Neg		
		Pharynx			Neg			
		Urethra	6	11	Pos	Pos	3	0.023
M	40	Urethra	7	7	Pos	Pos	2	0.002
M	22	Rectum	7	7	Pos	Pos	0.004	0.003
		Pharynx	7	7	Pos	Neg		
		Urethra	7	7	Pos	Pos	0.004	0.003
M	29	Urethra	7	7	Pos	Pos	0.003	0.004
M	43	Urethra	7	7	Pos	Pos	32	0.004
M	43	Urethra	7	7	Pos	Pos	1	0.004
M	39	Urethra	7	7	Pos	Pos	32	0.047
M	53	Rectum			Neg			
		Pharynx			Neg			
		Urethra	7	7	Pos	Pos	0.004	0.003
M	28	Urethra	7	7	Pos	Pos	8	0.047
M	51	Urethra	7	7	Pos	Pos	3	0.032
M	34	Urethra	7	7	Pos	Pos	3	0.008
F	28	Cervix	7	7	Pos	Pos	0.38	0.003
		Urethra	7	7	Pos	Pos	not performed	not performed
M	54	Urethra	7	7	Pos	Pos	not performed	not performed
M	22	Rectum			Neg			
		Pharynx			Neg			
		Urethra	7	7	Pos	Pos	0.002	0.002
M	29	Urethra	7	7	Pos	Pos	0.002	0.003
M	58	Urethra	7	7	Pos	Pos	not performed	not performed
M	49	Urethra	8	8	Pos	Neg		
M	35	Urethra	8	8	Pos	Pos	32	0.016
M	34	Urethra	8	8	Pos	Pos	0.002	0.002
M	37	Urethra	8	8	Pos	Pos	6	0.006
F	21	Cervix	8	8	Pos	Pos	3	0.004
		Urethra	8	8	Pos	Neg		
M	46	Rectum			Neg	Pos		
		Pharynx			Neg			

		Urethra	11	11	Pos	Pos	0.002	0.002
M	25	Urethra	11	11	Pos	Pos	1.5	0.002
M	29	Rectum			Neg			
		Pharynx			Neg			
		Urethra	4	11	Pos	Pos	0.006	0.25
M	44	Urethra	12	12	Pos	Pos	0.38	0.003
M	27	Rectum			Neg			
		Pharynx	12	12	Pos	Neg		
M	42	Rectum	13	13	Pos	Neg		
		Pharynx			Neg			
		Urethra	13	13	Pos	Pos	32	0.004
M	39	Rectum	14	14	Pos	Neg		
M	27	Rectum			Neg			
		Pharynx			Neg			
		Urethra	7	19	Pos	Pos	32	0.047

[a] minimal inhibitory concentration
EUCAST (www.eucast.org) breakpoints were applied. Neg, negative; Pos, positive.

Figure 1. A. Number of positive patients, of the ones that returned within seven days for their first test-of-cure (TOC), following treatment of gonorrhoea. *Did not return before day 19 for the second TOC. B. Number of positive patients, of the ones that returned after eight or more days for their first TOC, following treatment of gonorrhoea.