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Utility of MALDI-TOF MS for differentiation of *Neisseria gonorrhoeae* isolates with dissimilar azithromycin susceptibility profiles

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Abstract

Background: Antibiotic-resistant gonorrhoea has been a chronic public health burden since the mid-1930s. Recent emergence of isolates resistant to the current recommended antibiotics for gonorrhoea further magnifies the threat of untreatable gonorrhoea. The lack of new, effective antibiotics highlights the need for better understanding of the population structure of *Neisseria gonorrhoeae* in order to provide greater insight on how to curtail the spread of antimicrobial-resistant *N. gonorrhoeae*.

Objectives: To explore a potential application of MALDI-TOF MS to differentiate *N. gonorrhoeae* displaying different levels of susceptibility to the antibiotic azithromycin.

Methods: We conducted MALDI-TOF MS using the Bruker Biotyper on 392 *N. gonorrhoeae* isolates collected through the Gonococcal Isolate Surveillance Project (GISP) and/or the Strengthening the United States Response to Resistant Gonorrhea (SURRG) project. The MALDI-TOF MS spectra were visually analysed to assess the presence of distinctive peak(s). Statistical

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None to declare.

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Supplementary data
Table S1 is available as Supplementary data at *JAC* Online

analysis was performed to assess the relationship between gonococcal isolates with the distinct protein peak and antibiotic susceptibility.

Results: In this study, we were able to differentiate *N. gonorrhoeae* isolates into two distinct subpopulations using MALDI-TOF MS. Isolates were distinguished by the presence or absence of a spectral peak at 11 300 Da. Notably, these two groups exhibited different levels of susceptibility to azithromycin.

Conclusions: We have shown that in addition to its ability to identify *N. gonorrhoeae*, MALDI-TOF MS could also be used to differentiate gonococcal isolates with different levels of susceptibility to azithromycin.

Introduction

Neisseria gonorrhoeae is the aetiological agent of the sexually transmitted disease gonorrhoea. Serious health complications, including disseminated infection, pelvic inflammatory disease, infertility and HIV acquisition, can result from untreated gonorrhoea.[1–3] Managing this disease remains a major public health concern as the treatment recommendations for gonorrhoea have constantly evolved with the development of antibiotic resistance to each successive recommended antibiotic treatment regimen. Since the mid-1940s, more than five different antimicrobial agents have been employed to treat gonorrhoea.[4–6] In 2015, the CDC recommended a combination-therapy strategy consisting of ceftriaxone and azithromycin in an effort to slow the emergence and spread of antibiotic-resistant *N. gonorrhoeae*.[7] In spite of this effort, *N. gonorrhoeae* isolates with dual resistance to ceftriaxone and azithromycin have been reported around the world.[8]

In some countries, epidemiological surveillance of antimicrobial-resistant *N. gonorrhoeae* is an essential tool in curtailing the spread of untreatable gonorrhoea. Data from these gonococcal surveillance programmes, particularly antimicrobial susceptibility data, help guide the development of treatment guidelines for gonorrhoea.[5,9] In addition to monitoring the antimicrobial susceptibility patterns, molecular-based assays have also been used to explain the emergence and/or transmission of antimicrobial-resistant *N. gonorrhoeae*. MLST, *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) and, recently, WGS have been employed to explain the molecular epidemiology of various gonococcal populations.[10–12] Understanding the antimicrobial susceptibility pattern(s) and the underlying molecular epidemiology of gonococcal isolates may provide greater insight into the molecular identification of antimicrobial-resistant gonorrhoea.

In recent years, MALDI-TOF MS has been explored for use in detecting other antibiotic-resistant bacteria.[13] MALDI-TOF MS has been successfully integrated into the clinical microbiology toolbox, specifically for the identification of microorganisms.[14–17] This technology can be used to identify microbes through the profiles of their ribosomal proteins.[14–17] In this study, we explored and report the utilization of MALDI-TOF MS to differentiate *N. gonorrhoeae* isolates based on a spectral peak at the 11 300 Da molecular weight (MW) mark.

Materials and methods

N. gonorrhoeae isolates and growth conditions

N. gonorrhoeae isolates were obtained through the US CDC's Gonococcal Isolate Surveillance Project (GISP) and/or the Strengthening the United States Response to Resistant Gonorrhea (SURRG) project.[18] The isolates selected for this study were based solely on azithromycin MIC. A total of 392 isolates were included in this study. All isolates were previously identified as *N. gonorrhoeae* by laboratories participating in the GISP and the SURRG project. Moreover, the MALDI-TOF MS assay that we used to assess the peak also identified these isolates as *N. gonorrhoeae* by MALDI-TOF MS. For species identification, the instrument's FlexAnalysis software (Bruker Daltonics) compared the whole spectrum (all peaks from 2000 to 20 000 Da) generated from protein lysates of each isolate with the reference spectra in its database.

All 392 *N. gonorrhoeae* isolates analysed in this study were collected from the USA between 2010 and 2018, with most of the isolates (83%) collected from 2016 to 2018. All isolates were collected from nine US Department of Health & Human Services (HHS) regions—HHS regions 2 to 10 (Table 1).[19] The regions with the highest representation included HHS regions 9 (Arizona, California, Hawaii and Nevada) and 5 (Illinois, Indiana, Ohio, Michigan, Minnesota and Wisconsin), with approximately 33% and 18% of the isolates, respectively.

All isolates were propagated on gonococcal (GC) medium base agar supplemented with 1% haemoglobin and/or 1% IsoVitaleX (BD, Franklin Lakes, NJ, USA) and were incubated at 36±1°C/5% CO_2.

MALDI-TOF MS assay and spectrum analysis

MALDI-TOF MS assay was performed using the Microflex Biotyper (Bruker Daltonics, Billerica, MA, USA). A crude protein lysate of *N. gonorrhoeae* was prepared using the ethanol/formic acid extraction method as previously described.[20] Briefly, a suspension of an overnight gonococcal culture was prepared in 300 µl of deionized water followed by the addition of 900 µl of pure ethanol. After 5–10 min of incubation at room temperature, the gonococcal suspension was pelleted by centrifugation at 15.7×10^3 **g** for 2 min followed by the decantation of the supernatant and air-drying of the pellet. The air-dried gonococcal pellet was first resuspended in 25–50 µl of 70% formic acid [70:30 (v/v)] and an equal volume of acetonitrile. The lysate was centrifuged at 15.7×10^3 **g** for 2 min to remove the cell debris. One microlitre of this crude protein lysate was spotted, in duplicate, on a MALDI-TOF MS sample preparation (MSP) target plate (MSP 96). The spots were allowed to air-dry at room temperature and then overlaid with 1 µl of a-cyano-4-hydroxycinnamic acid ('HCCA'; Bruker Daltonics) and analysed with the Microflex Biotyper. The bacterial test standard ('BTS'; Bruker Daltonics) was also included in all experiments for quality control purposes for the MALDI-TOF MS assay.

The spectrum generated by the MALDI-TOF MS assay was visually examined using the FlexAnalysis software (Bruker Daltonics). The spectra of all isolates were examined to

determine the presence [intensity >500 arbitrary units (a.u.)] or absence of a peak at the 11 300 Da MW mark.

Antimicrobial susceptibility testing (AST)

The MICs of the antibiotics azithromycin, cefixime, ceftriaxone, ciprofloxacin, penicillin and tetracycline for *N. gonorrhoeae* were determined using an agar dilution AST method, as described by the CLSI.[21] The Etest® AST method was used to further characterize the azithromycin susceptibility of isolates that displayed an azithromycin MIC >16 mg/L using agar dilution (highest concentration for agar dilution).[22] The highest azithromycin concentration on an azithromycin Etest® strip is 256 mg/L. All antibiotic powders were purchased from Sigma–Aldrich (St Louis, MO, USA), while all Etest® strips were purchased from bioMérieux (Durham, NC, USA). Categorical interpretation of the MICs as susceptible, non-susceptible, intermediate susceptible and resistant was based on CLSI's guidelines.

Statistical analysis

The OR was calculated to determine the relationship between antibiotic susceptibility and gonococcal subpopulations with or without the 11 300 Da protein peak (also referred to as 'peak' elsewhere in the text). These analyses were conducted in Rstudio version 1.0.44 (Boston, MA, USA; http://www.rstudio.com/). The ORs were calculated to test the following hypotheses (H_0 and H_1): H_0, Y and X are not associated; and H_1, H_0 is not true [where Y (isolate groupings based on MICs) and X (presence or absence of the 11 300 Da protein peak) were converted into binary variables]. The grouping of isolates based on MIC was achieved as follows. The susceptible and low-range MIC (SLM) group consisted of isolates with an MIC of azithromycin of <1 mg/L, MICs of ceftriaxone and cefixime of <0.125 mg/L and MICs in the susceptible category for ciprofloxacin (<0.125 mg/L), penicillin (<0.125 mg/L) and tetracycline (<0.5 mg/L). The intermediate, resistant and high-range MIC (IRHM) group consisted of isolates with an MIC of azithromycin of ≥1 mg/L, MICs of ceftriaxone and cefixime of ≥0.125 mg/L and MICs in the intermediate susceptible and resistant categories for ciprofloxacin (≥0.125 mg/L), penicillin (≥0.125 mg/L) and tetracycline (≥0.5 mg/L). A constant value of 0.5 was added to the variables that contained zero because the OR equals zero or infinity if any of the cell counts is zero.[23] ORs and the 95% CIs were calculated using the adjusted frequency table.

Results

A subset of N. gonorrhoeae isolates displayed a unique peak at 11 300 Da

The spectra generated from the gonococcal protein lysates consisted of distinct protein peaks with MW ranging from 2000 to 12 000 Da (Figure 1). The majority (>92%) of the peaks on the spectra were below 11 000 Da. A subset of the isolates, approximately 34.5%, displayed a unique peak at 11 300 Da, while the remaining isolates displayed less dominant peaks at 11 200 and 11 400 Da. The 11 300 Da protein peak was present in the spectrum of all years and in all HHS regions examined in this study, except for HHS region 7 (Table 2). No other unique peaks could be identified based on visual inspection of all the spectra. We also assessed whether growth media had any effects on the appearance of this protein peak

because we did observe some growth differences associated with different growth media (C. D. Pham, unpublished data). To this end, we analysed the peak in isolates grown on two of the most commonly used media for *N. gonorrhoeae*: GC medium base agar supplemented with 1% IsoVitaleX plus 1% haemoglobin and GC medium base agar supplemented with 1% IsoVitaleX only. We did not observe any effects of growth media on the manifestation of the 11 300 Da peak (data not shown).

N. gonorrhoeae isolates displaying the 11 300 Da protein peak appeared to be less susceptible to azithromycin

The gonococcal isolates selected for this study had an azithromycin MIC range of 0.008 to ≥256 mg/L (Figure 2). In this study, approximately 60.3% (129/214) of the isolates with an MIC of azithromycin of ≥1 mg/L had the detectable 11 300 Da protein peak [Table 3 and Table S1 (available as Supplementary data at *JAC* Online)]. Of the 136 isolates displaying this spectral peak, 94.9% (129/136) had an MIC of azithromycin of ≥1 mg/L. In contrast, 96.1% (171/178) of the isolates with an azithromycin MIC of <1 mg/L lacked this spectral peak (Figure 2). Overall, the OR for an isolate having the detectable 11 300 Da spectral peak and an MIC of azithromycin of ≥1 mg/L was 36.79 (95% CI = 16.46–82.20).

In contrast to azithromycin, the distribution of gonococcal isolates was not evenly distributed across the different susceptibility categories (e.g. susceptible, non-susceptible, intermediate susceptible and resistant) for non-macrolide antibiotics (Tables 3 and S1). More than 90% of the isolates belonged to either the susceptible subpopulation for ceftriaxone and cefixime or to the intermediate susceptible/resistant subpopulation for penicillin and tetracycline. The distributions of susceptible and intermediate susceptible/resistant subpopulations for ciprofloxacin were 62.4% and 37.6%, respectively. Association analyses performed on cephalosporin antibiotics showed ORs of 0.05 (95% CI = 0.01–0.34) and 0.03 (95% CI = 0.002–0.45) for isolates with the 11 300 Da protein peak and MICs ≥0.125 mg/L for ceftriaxone and cefixime, respectively (Table 3). The ORs for isolates having this protein peak and belonging in the intermediate susceptible/resistant categories for ciprofloxacin, tetracycline and penicillin were 0.12 (95% CI = 0.07–0.21), 37.86 (95% CI = 2.30–623.70) and 8.15 (95% CI = 0.46–143.73), respectively (Table 3).

Discussion

AST has been an integral component of many countries' gonococcal surveillance programmes in the effort to curtail the spread of antimicrobial-resistant gonorrhoea. In the USA, an AST-based surveillance approach has been highly successful in monitoring and maintaining therapeutic efficacy for the antibiotic(s) used in treating gonorrhoea. However, concerns around untreatable gonococcal infection and the spread of antimicrobial-resistant gonococcal isolates have highlighted the need for better understanding of the population structure of *N. gonorrhoeae*. Using WGS, Sánchez-Busó *et al.*[24] (2019) demonstrated that the current gonococcal population could be subdivided into two lineages—an MDR lineage and a multidrug-susceptible lineage. Particularly for azithromycin susceptibility, Thomas *et al.*[25] (2019) reported that 67% of isolates with an azithromycin MIC of ≥2 mg/L belong to a single clade. The quest for better insight into the dynamic relationship between AST

pattern(s) and molecular epidemiology led us to explore an additional molecular tool to study population structure in *N. gonorrhoeae.*

In recent years, MALDI-TOF MS has been explored for uses other than species identification, such as antimicrobial susceptibility assessment, strain typing and detection of virulence.[14,17] MALDI-TOF MS was shown to be able to differentiate ESBL-producing and non-ESBL-producing *Klebsiella pneumoniae* and *Escherichia coli*.[13] Carannante *et al*.[26] demonstrated that MALDI-TOF MS was able to segregate *N. gonorrhoeae* into different clusters. Unlike Carannante *et al*.,[26] who analysed the whole spectrum, our study showed that MALDI-TOF MS may be able to segregate *N. gonorrhoeae* isolates into two subgroups based on a distinct peak in the MALDI-TOF MS spectrum. The two gonococcal subgroups were discernible by a spectral peak at 11 300 Da. This protein peak appears to be intrinsic to *N. gonorrhoeae* and is not influenced by extrinsic factors such as growth media and geographical regions (HHS). However, the data seem to suggest that the gonococcal subpopulations discernible by this specific MALDI-TOF MS peak displayed different levels of susceptibility to azithromycin.

Approximately 35% (136/392) of the isolates examined in this study displayed this spectral peak, while the rest of the isolates did not have the peak (Table 3). Interestingly, nearly 95% (129/136) of the isolates with the peak had an azithromycin MIC of ≥1 mg/L. In contrast, >96% (171/178) of the isolates with an azithromycin MIC of <1 mg/L did not have the protein peak. Based on these observations, we decided to set the azithromycin MIC threshold at 1 mg/L for the association analysis. Our analysis suggests a strong association between an isolate with the peak and an azithromycin MIC of ≥1 mg/L. The OR for an isolate with both the peak and an azithromycin MIC of ≥1 mg/L is 36.79 (95% CI = 16.46–82.20). This indicates that having the peak corresponds to a high likelihood that the isolate has an azithromycin MIC of ≥1 mg/L. Although most of the isolates with the peak have a higher azithromycin MIC than those without the peak, it is worthwhile to point out that roughly 40% of the isolates with an azithromycin MIC ≥1 mg/L did not display this specific protein peak. One possible explanation for this observation could be attributed to the mechanisms of azithromycin resistance in conjunction with the detection capability of the Microflex Biotyper instrument.

Azithromycin exerts its antimicrobial effects by binding to the 50S ribosomal subunit at the 23S rRNA–ribosomal protein interface and abrogates protein synthesis.[27–29] Modifications to the gonococcus's ribosomal proteins (e.g. RplV and RplD), the protein–drug interaction interface (e.g. 23S rRNA mutations) and the multidrug efflux pump (*mtr* locus) have been reported to influence azithromycin's efficacy.[30–32] Not all these mechanisms of azithromycin resistance are detectable by MALDI-TOF MS assay. The Microflex Biotyper instrument primarily detects ribosomal proteins, which are the basis for microorganism identification and have a significant presence within the measured MW range.[14–17] Therefore, ribosomal protein modifications (e.g. changes to the ribosomal protein's MW or expression level) would more likely be detected by this assay. In contrast, non-ribosomal protein modifications (e.g. 23S rRNA mutation and *mtr* locus) may not be detectable by the MALDI-TOF MS assay. Interestingly, Thomas *et al*.[25] (2019) reported that the majority of gonococcal isolates that displayed an MIC of azithromycin of ≥4 mg/L have

mutations in the 23S rRNA. In our study, approximately 66% (56/85) of the isolates with an azithromycin MIC of ≥1 mg/L and without the 11 300 Da peak had an MIC of azithromycin of ≥4 mg/L. This suggests that these isolates might harbour the previously described 23S rRNA mutations. The detection capability of MALDI-TOF MS and the mechanisms of azithromycin resistance may also explain the observed stronger association between the 11 300 Da protein peak and azithromycin's susceptibility in comparison with other antibiotics. To this end, we did not investigate ribosomal protein modifications in the studied isolates because of the inability of the Microflex Biotyper to identify the proteins it detected. More studies are required to determine the protein(s) that contributed to the 11 300 Da peak in the MALDI-TOF MS spectrum.

In addition to the constraint in protein identification, association analyses for the peak and non-macrolide antibiotics were also limited because the gonococcal isolates selected for this study were based solely on azithromycin MIC. This narrow selection criterion inadvertently skewed the distribution of isolates, based on MIC, for the non-macrolide antibiotics (e.g. ceftriaxone, cefixime, ciprofloxacin, penicillin and tetracycline). This limits our inferencing power on the associations between the peak and antimicrobial susceptibilities for ceftriaxone, cefixime, ciprofloxacin, penicillin and tetracycline. However, our data suggest associations between the peak and the susceptibility patterns for tetracycline, ciprofloxacin, ceftriaxone and cefixime, while no association was found for penicillin. For tetracycline, analysis showed a positive association (OR = 37.86; 95% CI = 2.30–623.70) for isolates having the 11 300 Da protein peak and belonging in the intermediate susceptible/resistant categories (IRHM). This indicates that gonococcal isolates with this protein peak might display reduced susceptibility to tetracycline. In contrast, a negative association (OR = 0.12; 95% CI = 0.07–0.21) was found for those isolates having this protein peak and belonging in the intermediate susceptible/resistant categories for ciprofloxacin. Likewise, analyses performed on the cephalosporin antibiotics and the protein peak showed negative associations for isolates with ceftriaxone (OR = 0.05; 95% CI = 0.01–0.34) and cefixime (OR = 0.03; 95% CI = 0.002–0.45) MICs of ≥0.125 mg/L. This MIC is one doubling dilution below the susceptible breakpoints (≤0.25 mg/L) for these two antibiotics. This implies that isolates harbouring this protein peak might be susceptible to ciprofloxacin, ceftriaxone and cefixime. Additional assessments, with more gonococcal isolates, are required in order to determine the true associations between the protein peak and ceftriaxone, cefixime, ciprofloxacin, penicillin and tetracycline susceptibilities. Finally, the antimicrobial susceptibility profiles of the isolates employed in this study may not represent the circulating gonococcal population because of the non-random isolate selection process.

In conclusion, effective and efficient techniques for the detection of antimicrobial resistance are essential for better tracking of resistant gonorrhoea. MALDI-TOF MS has been widely employed as an alternative to phenotypic identification of microorganisms in the last decade.[14–17] Our study suggests that MALDI-TOF MS can be utilized for identification, and possibly differentiation, of subpopulations of *N. gonorrhoeae* that may respond differently to azithromycin. Although more research is needed, our findings may mean a promising new application for MALDI-TOF MS and the study of population structure in *N. gonorrhoeae*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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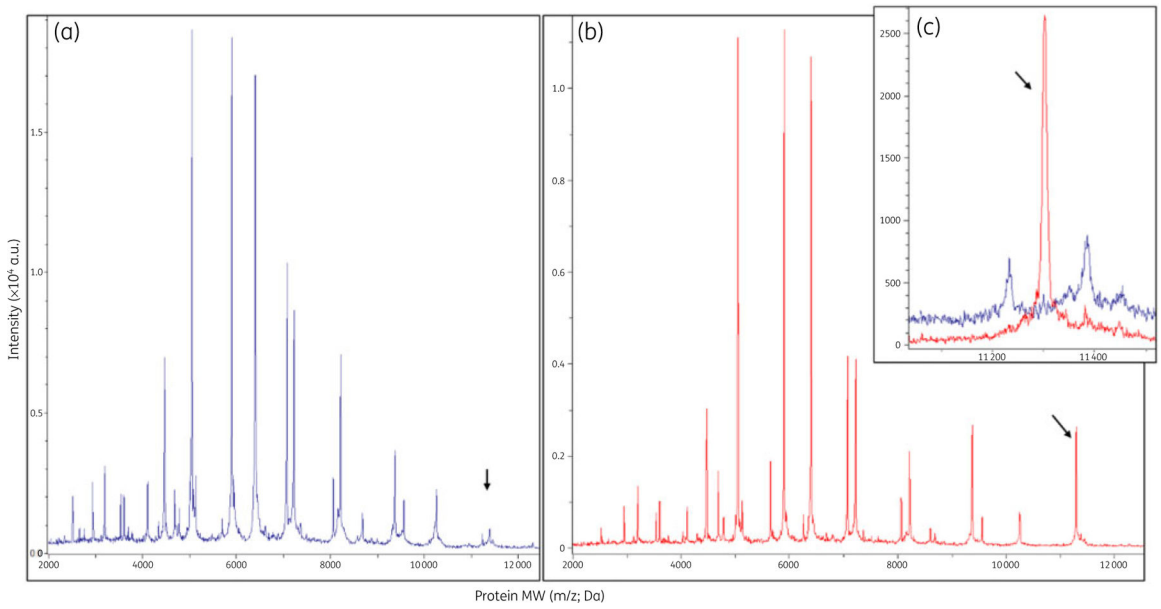

Figure 1.

N. gonorrhoeae MALDI-TOF MS spectra. Spectra of an isolate without a peak at 11300 Da (a; arrow) and an isolate with a peak at 11 300 Da (b; arrow). The inset (c) is a close-up, merged image of the two spectra around the protein peak of interest (arrow; approximate intensity = 2.5×10^3 a.u.).

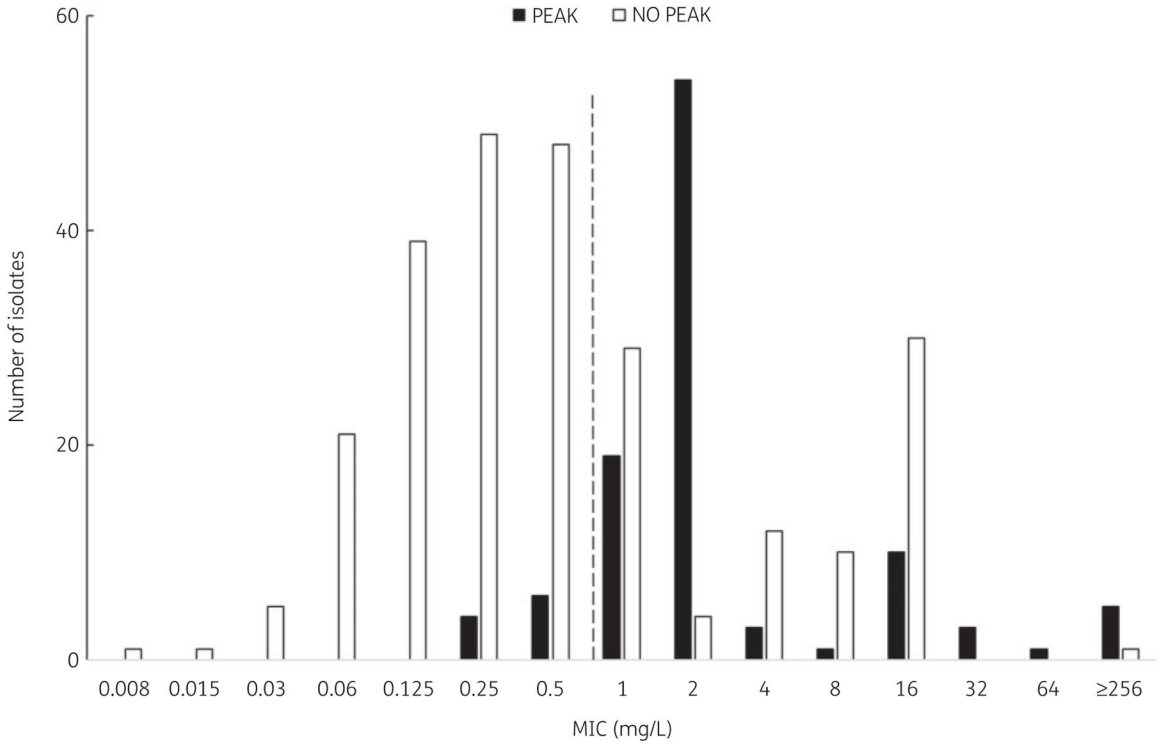

Figure 2.

Azithromycin MIC distribution for *N. gonorrhoeae* isolates used for peak analysis. Isolates with and without the peak are plotted in relation to azithromycin MICs. The line of demarcation at 1 mg/L indicates the azithromycin MIC differentiating the two populations of isolates.

Table 1.

Numbers and percentages of *N. gonorrhoeae* isolates by HHS region and year

	HHS region									
Year	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**total (%)**
2010	0	0	0	0	0	0	0	1	0	1 (0.3)
2012	0	0	0	0	0	0	0	2	1	3 (0.8)
2013	0	1	0	0	0	0	0	1	1	3 (0.8)
2014	1	1	0	1	0	0	0	13	6	22 (5.6)
2015	2	4	0	0	2	0	0	21	7	36 (9.2)
2016	5	12	0	6	1	0	1	45	13	83 (21.2)
2017	12	0	24	51	33	10	31	28	0	189 (48.2)
2018	0	13	0	11	0	0	0	17	14	55 (14)
Total (%)	20 (5.1)	31 (7.9)	24 (6.1)	69 (17.6)	36 (9.2)	10 (2.6)	32 (8.2)	128 (32.7)	42 (10.7)	392 (100)

Table 2.

Numbers and percentages of *N. gonorrhoeae* isolates displaying the 11 300 Da peak by HHS region and year

	HHS region									
Year	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**total (%)**
2010	0	0	0	0	0	0	0	1	0	1 (100)
2012	0	0	0	0	0	0	0	2	1	3 (100)
2013	0	0	0	0	0	0	0	1	1	2 (66.7)
2014	0	0	0	1	0	0	0	5	0	6 (27.3)
2015	0	1	0	0	2	0	0	5	1	9 (25)
2016	0	3	0	0	1	0	1	12	3	20 (24.1)
2017	6	0	3	12	6	0	20	6	0	53 (28)
2018	0	9	0	10	0	0	0	9	14	42 (76.4)
Total (%)	6 (30)	13 (41.9)	3 (12.5)	23 (33.3)	9 (25)	0 (0)	21 (65.6)	41 (32)	20 (47.6)	136 (34.7)

Table 3.

Data and association analysis

	Antibiotic					
	ceftriaxone	**cefixime**	**penicillin**	**tetracycline**	**ciprofloxacin**	**azithromycin**
No. of samples	391	391	391	391	391	392
Peak, presence/absence (%)	34.5/65.5	34.5/65.5	34.5/65.5	34.5/65.5	34.5/65.5	34.7/65.3
Susceptibility, IRHM/SLM (%)	9.5/90.5	7.7/92.3	98.2/1.8	92.1/7.9	37.6/62.4	54.6/45.4
$IRHM^{peak}/SLM^{peak}$ (%)	2.7/37.9	0/37.4	35.2/0	37.5/0	10.2/49.2	60.3/3.9
$Peak^{IRHM}/peak^{SLM}$ (%)	0.7/99.3	0/100	100/0	100/0	11.1/88.9	94.9/5.1
Peak:IRHM ORs (95% CI)	0.05 (0.01–0.34)	0.03[a] (0.002–0.45)	8.15[a] (0.46–143.73)	37.86[a] (2.30–623.70)	0.12 (0.07–0.21)	36.79 (16.46–82.20)

OR interpretation[33] is as follows: OR = 1 means the 11 300 Da protein peak (peak) does not affect odds of IRHM outcome, OR >1 means the peak is associated with higher odds of IRHM outcome and OR <1 means the peak is associated with lower odds of IRHM outcome.

[a]A constant value of 0.5 was added to each cell that contained zero because the OR equals zero or infinity if any of the cell counts are zero.