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A comparison of agar dilution with the Calibrated Dichotomous Sensitivity (CDS) and Etest methods for determining the minimum inhibitory concentration of ceftriaxone against *Neisseria gonorrhoeae*

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ABSTRACT

Objectives: The objective of this study was to compare the Calibrated Dichotomous Sensitivity (CDS) based agar dilution (CDS AD) method with the Etest (bioMérieux SA) methods using 2 method protocols for determining the minimum inhibitory concentration (MIC) of ceftriaxone against *Neisseria gonorrhoeae*. The two method protocols were the manufacturer's protocol for which the Clinical and Laboratory Standards Institute (CLSI) interpretative criteria for *Neisseria gonorrhoeae* could be applied, and the CDS-adapted protocol. Comparability of MIC data is critical for situation analysis and monitoring trends in global antimicrobial analysis.
Methods: Two hundred and forty eight clinical isolates of *N. gonorrhoeae* and the World Health Organisation (WHO) *N. gonorrhoeae* reference strains were tested using the three methods.
Results: When compared, CDS AD and CDS Etest gave a regression R^2 value of 94%, the Pearson's correlation coefficient was 97% and a paired comparison within one $\log_2$ dilution was 98%. The CDS AD and the Etest (CLSI) comparison gave a regression R^2 value of 90%, a Pearson's correlation coefficient of 95% and a paired comparison within one $\log_2$ dilution was 98%. The comparison of the CDS Etest and CLSI Etest gave a regression R^2 value of 91%, a Pearson's correlation coefficient of 95% and a paired comparison within one $\log_2$ dilution of 99%. Importantly, there was robust agreement between all three methods for the categorization of susceptibility of Neisseria gonorrhoeae isolates using the WHO nominated breakpoint for decreased susceptibility to ceftriaxone (≥0.125 μg/mL).
Conclusions: The CDS Etest method is comparable to agar dilution and the Etest methods for determining the MIC of ceftriaxone against *N. gonorrhoeae*.

1. Introduction

Neisseria gonorrhoeae has emerged as a challenging pathogen in terms of antimicrobial therapeutic options (Goire et al., 2014). During the past decades, the gonococcus has acquired sequential resistance to every class of antimicrobial agent leaving ceftriaxone as the mainstay of therapy worldwide (Goire et al., 2014). Ceftriaxone-non susceptible strains have been reported recently from Japan, France, Spain and Australia (Cámara et al., 2012; Lahra et al., 2014; Ohnishi et al., 2011; Unemo et al., 2012). International concern about the impact of the spread of ceftriaxone-resistant strains has sharpened the focus on the need for quality antimicrobial resistance (AMR) surveillance (Goire et al., 2014; Lewis, 2010; WHO, 2012).

Agar dilution (AD) is the gold standard for determining the minimum inhibitory concentration (MIC) of antibiotics against *N. gonorrhoeae* (Goire et al., 2014). However, from the clinical perspective the AD method is labor-intensive and time consuming, and the turnaround time may be beyond the timeframe of clinical relevance. In addition, for antimicrobial surveillance, AD is ideal but it is challenging or not possible in resource-limited settings where paradoxically, the burden of gonococcal disease is very high (WHO, 2007).

The comparison of data between gonococcal AMR surveillance programs in different regions internationally, is important for informing local and global public health initiatives, for monitoring trends, and for global AMR analysis, but is problematic, because there is no uniform method internationally. Two widely used methods for determining the MIC values are the United States of America based Clinical Laboratory Standards Institute (CLSI); and the European-developed European Committee on Antimicrobial Susceptibility Testing (EUCAST) and these differ methodologically from each other, and from the

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Table 1
The MIC of ceftriaxone (μg/mL) for the WHO control strains (published data) and those obtained by the CDS AD, the CLSI Etest and CDS Etest methods.

WHO control strains	ATCC49226	WHO F	WHO G	WHO K	WHO L	WHO M	WHO N	WHO P	WHO O
Published MIC(Unemo et al., 2009)	0.004–0.016	<0.002	0.004–0.016	0.032–0.125	0.064–0.25	0.008–0.032	0.002–0.008	0.002–0.008	0.016–0.064
CDS AD	0.016	≤0.001	0.008	0.064	0.125	0.008	0.002	0.004	0.016
CLSI Etest	0.016	<0.002	0.016	0.064	0.125	0.016	0.008	0.008	0.032
CDS Etest	0.008/0.016	<0.002	0.008	0.064	0.125/0.25	0.008	0.004	0.004	0.032

Australian-developed Calibrated Dichotomous Sensitivity (CDS) method. The CDS method is used in Australia, and regionally within the Western Pacific and South East Asia regions to determine the susceptibility of antibiotics against *N. gonorrhoeae*.

Intra-method comparison of AD MIC versus Etest MIC is also of interest given the challenges for performing AD MIC in less resourced settings, and the significant difference in turn-around-times. In a recent study comparing CLSI AD with Etest as performed by the manufacturer's recommendations and using the CLSI interpretive criteria (CLSI Etest), there was good correlation (Liu et al., 2014). A comparison of the CDS AD with CDS Etest and, Etest as performed by the manufacturer's recommendations was not investigated in the recent studies by Singh et al., 2012; Zhu et al., 2014.

In the present study, we evaluated the CDS AD and the CDS-adapted Etest (bioMérieux SA) methods for determining the MIC of ceftriaxone against *N. gonorrhoeae* and we compared these with the Etest method of the manufacturer using the protocol recommended by CLSI for antimicrobial susceptibility testing of *N. gonorrhoeae* by disc diffusion (CLSI, 2012).

2. Materials and methods

Two hundred and forty eight clinical isolates of *N. gonorrhoeae* with a range of ceftriaxone MIC values determined by CDS AD MIC method, as well as the World Health Organization (WHO) *N. gonorrhoeae* reference strains F, G, K, L, M, N, O and P were tested using the three methods. The clinical isolates included the first *N. gonorrhoeae* strain with high level MIC value to ceftriaxone to be isolated in Australia (Lahra et al., 2014). The identity of all strains was determined by MALDI TOF MS (Bruker Daltonics, Bremen, Germany), tested as per the manufacturer's instructions.

Agar dilution MIC testing was performed as per the CDS protocol used by the Australian Gonococcal Surveillance Programme. Briefly, agar plates were prepared using 45 mL of Sensitest Agar (Oxoid, Australia), 1 mL of the required concentration of antibiotic solution and 4 mL of saponin-lysed horse blood (0.5 mL of 10% saponin per 10 mL of horse blood) to give a final volume of 50 mL. The inoculum was prepared by suspending organisms in 2.5 mL of peptone water/saline. The suspension was adjusted to an absorbance of 0.75 at a wavelength of 640 nm using a spectrophotometer (equivalent to 10^9 colony forming units (cfu)/mL). This initial suspension was diluted a hundred-fold to give a further suspension containing 10^7 cfu/mL. A Steer's replicator was used to apply the 10^7 inoculum to the surface of an agar plate which was then incubated at 36 ± 1 °C in 5% CO_2 for 18–24 h. The MIC was determined as the concentration of antibiotic which gave complete inhibition of growth of the 10^4 cfu spot inoculum.

The Etest (bioMérieux SA) was performed using 2 different methods of inoculum preparation and media inoculation. These were the CDS method (Bell, 1975; Bell et al., 2013), and the manufacturer's recommended procedure. For the CDS method of inoculum preparation (Bell, 1975; Bell et al., 2013) the tip of a glass Pasteur pipette was passed through a colony of 1–2 mm diameter which was grown on lysed horse blood agar at 36 ± 1 °C in 5% CO_2 for 18–24 h so that the colony was collected. This material was then suspended in 2.5 mL of 0.9% saline and the surface of a pre-dried (36 ± 1 °C for 1 h) Chocolate Columbia blood agar plate was flooded with the suspension. The inoculum was distributed by rocking the plate to ensure the entire surface of the agar was covered. The excess suspension was removed using a Pasteur pipette and the plate was allowed to dry for 10–15 min. An Etest strip was then placed on the surface of the agar and the MIC was determined after incubation at 36 ± 1 °C in 5% CO_2 for 18–24 h. The MIC value is read where the pointed end of the inhibition ellipse intersects the side of the strip. An Etest MIC value which falls between standard two-fold dilutions must be rounded up to the next upper two-fold value before categorisation (bioMerieux, 2012).

The Etest method was also performed according to the manufacturer's recommendations. This is the protocol recommended by CLSI for antimicrobial susceptibility testing of *N. gonorrhoeae* by disc diffusion (CLSI, 2012). Briefly, 2–5 bacterial colonies from an overnight culture of *N. gonorrhoeae* were suspended in 0.9% saline to give an inoculum equivalent to 0.5 McFarland units. This suspension was then applied to GC agar with 1% defined growth supplement (Vitox byOxoid, Australia) using a cotton swab following a three-way lawn method. An Etest strip was then placed on the surface of the agar and the MIC was determined, as above, after incubation at 36 ± 1^0 C in 5% CO_2 for 18–24 h.

3. Results and discussion

The 248 clinical strains and the WHO control strains F, G, K, L, M, N, O and P of *N. gonorrhoeae* (Unemo et al., 2009) were tested and the MIC values were within the expected published ranges (Table 1). The study compared the CDS AD with Etest and compared the Etest using the CDS, and manufacturer's protocols to evaluate if these methods gave comparable results.

Table 2
The paired comparison of the MIC (μg/mL) of ceftriaxone determined by the CDS agar dilution (AD) and the CDS Etest methods.

	CDS AD									
CDS Etest	≤0.008	0.016	0.032	0.064	0.125	0.25	0.5	1.0	2.0	Total
≤0.008	48	6	1							55
0.016	3	18	11	1						33
0.032		7	55	15	1					78
0.064		1	7	48	4					60
0.125				3	18					21
0.25										0
0.5										0
1.0								1		1
2.0										0
Total	51	32	74	67	23	0	0	1	0	248

Table 3
The paired comparison of the MIC (μg/mL) of ceftriaxone determined by the CDS agar dilution (AD) and the CLSI Etest methods.

	CDS AD									
CLSI Etest	≤0.008	0.016	0.032	0.064	0.125	0.25	0.5	1.0	2.0	Total
≤0.008	45	4								49
0.016	6	12	9	1						28
0.032		15	56	14						85
0.064		1	9	50	8					68
0.125				2	14					16
0.25					1					1
0.5										0
1.0										0
2.0								1		1
Total	51	32	74	67	23	0	0	1	0	248