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Comparative assessment of CDS, CLSI and Etest techniques for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*: A six year study

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ARTICLE SUMMARY

Article focus

- A variety of techniques are available for antimicrobial susceptibility testing (AST) of *Neisseria gonorrhoeae*.
- The aim of this study to find a cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *N. gonorrhoeae* in resource poor countries.

Key messages

- This study highlighted that the CDS technique yielded excellent category agreement with their analogous Etest MICs in comparison to CLSI technique.
- CDS offers the advantage for those laboratories that process small numbers of specimens.
- CDS technique is cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *Neisseria gonorrhoeae* in resource poor countries.
- This technique will facilitate enhanced antimicrobial resistance surveillance and direct and meaningful comparison of resistance data generated within different national and international laboratories.

Strengths and limitations of this study

- This is the first study to determine the comparability of CDS and CLSI disc diffusion method with Etest MICs and to accurately and reproducibly assess *N. gonorrhoeae* susceptibilities for penicillin, tetracycline, ceftriaxone, ciprofloxacin and spectinomycin, commonly used for susceptibility testing.
- Susceptibility testing for cefixime, cefpodoxime, gentamicin and azithromycin was performed only by one method because of non-availability of interpretation criteria for these antimicrobials or limitation of resources. Therefore, comparison of above three techniques could not be carried out for these antibiotics.

ABSTRACT

Background: A variety of techniques are available for antimicrobial susceptibility testing (AST) of *Neisseria gonorrhoeae*.

Objective: The aim of this study was to find a cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *N. gonorrhoeae* in resource poor countries.

Design: Prospective study.

Setting: Male STD clinic and female STD clinic of Regional STD Teaching, Training and Research Centre, New Delhi, India.

Participants: *N. gonorrhoeae* isolates from all male and female patients presenting with acute gonococcal urethritis and cervical discharge.

Material and Methods: A total of consecutive 295 *N. gonorrhoeae* isolates during 2005 to 2010, were used to the compare the CLSI and CDS disc diffusion technique with Etest by performing antimicrobial susceptibility testing in parallel for penicillin, tetracycline, ceftriaxone, ciprofloxacin and spectinomycin. WHO reference strains were used as controls.

Results: CDS disc diffusion zones of inhibition showed that complete percentage agreement for penicillin, ciprofloxacin and tetracycline was high with their analogous Etest MICs in comparison to CLSI technique i.e. 91.5%, 92.9%, 99.3% versus 87.5%, 88.5% and 74.9% respectively. CDS results had less number of major and minor category discrepancies in comparison to CLSI technique and CDS method showed excellent correlation coefficient (r = 1) with Etest for all five antimicrobial agents tested in comparison to CLSI (r = 0.92). It was very poor (r =0.61) by CLSI method for tetracycline.

Conclusions The CDS technique is an attractive alternative for *N. gonorrhoeae* susceptibility testing and is recommended for monitoring the antimicrobial susceptibility in less developed and resource poor settings to facilitate enhanced antimicrobial resistance (AMR) surveillance when the WHO Gonococcal Antimicrobial Surveillance Programme is undergoing expansion to meet the ongoing challenges of surveillance and control of gonococcal AMR.

INTRODUCTION

Gonorrhoea caused by *Neisseria gonorrhoeae* (*N. gonorrhoeae*) is one of the most common sexually transmitted infections and is a global health problem. Uncomplicated urogenital *N. gonorrhoeae* infections can lead to epididymitis in males and salpingitis in females, conditions that are associated with infertility. In some cases localized infections can lead to haematogenic dissemination causing severe complications like sepsis, meningitis and endocarditis. The worldwide distribution of *N. gonorrhoeae* and the emergence of resistance to various antimicrobials emphasize the importance of antimicrobial resistance (AMR) surveillance of clinical gonococcal isolates.

Antimicrobial susceptibility testing (AST) may be carried out by a variety of techniques. The most commonly used methods have been the high-content disc diffusion method first described by Bauer *et al*,[1] and later modified by the National Committee for Clinical Laboratory Standards (NCCLS),[2] the broth microdilution technique as described by the NCCLS,[3] the agar dilution method described by Ericsson and Sherris,[4] and adapted by the NCCLS,[3] more recently Etest (AB Biodisk, Solna, Sweden), a modification of the disc diffusion and the agar dilution methods mentioned previously. The Etest is an impervious carrier with a continuous gradient of antimicrobial agent applied to one side of the strip. The test is performed in the same manner as the disc diffusion test, but determines a minimal inhibitory concentration (MIC) of antimicrobial agent rather than a category result based on a zone size. The Calibrated Dichotomous Sensitivity (CDS) test is a method for determining the antibiotic susceptibility of micro-organisms using agar disc diffusion and was developed in 1969 by Sydney M. Bell assisted by members of the Department of Microbiology, The Prince of Wales Hospital, Sydney, Australia. It was introduced into laboratory practice in

Australia in an effort to correct the unsatisfactory results of antibiotic susceptibility testing in Australia that were obtained from surveys conducted by The Royal College of Pathologists of Australia in the late 60's and early 70's.[5]

The recent emergence of *N. gonorrhoeae* isolates with decreased susceptibility and resistance to the currently recommended treatment guidelines of the Centers for Disease Control and Prevention, including extended-spectrum cephalosporins (ESC), has further established the necessity for a standardized, reliable, economical, less labor intensive and reproducible susceptibility testing method.[6-8] Previous studies with the Etest determined that results correlated well with the reference agar dilution method and that it is a useful guide for determining chemotherapy against many organisms,[9] including gonococci.[9-11] The CDS disc diffusion technique is recommended by WHO Collaborating Centre for STD, Sydney, Australia and was regarded as the only practical and affordable means of phenotypic susceptibility testing.[12] It was found to be cost-effective and more feasible during an external quality assurance scheme in routine diagnostic laboratories in developing countries like India.[12] Earlier this method was evaluated in comparison to Etest for only three antibiotics (ciprofloxacin, penicillin, and ceftriaxone) and that also on a limited number of isolates.[13] Recently, CDS technique has been recommended in less resourced settings for detection of decreased susceptibility to ESCs using cefpodoxime disc.[14] The purpose of this study was to determine the comparability of the Etest, CDS and Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS, to accurately and reproducibly assess *N. gonorrhoeae* susceptibilities for five antibiotics commonly used for susceptibility testing, which included penicillin, tetracycline, ceftriaxone, spectinomycin and ciprofloxacin and to assess the feasibility of recommending CDS technique for its use in developing and resource

poor countries. To our knowledge, this is the first study to compare the two disc diffusion methods with MIC testing for above five antibiotics.

MATERIALS AND METHODS

N. gonorrhoeae isolates

A total of 295 *N. gonorrhoeae* isolates from all male and female patients presenting with acute gonococcal urethritis and cervical discharge respectively to the Regional STD Teaching, Training and Research Centre, Safdarjung Hospital, New Delhi, India from January 2005 to December 2010 were included in the study. The strains were consecutive and nonrepetitive. Methods used for isolation and identification of *N. gonorrheae* have been described previously.[15]

β-lactamase testing

N. gonorrhoeae isolates were tested for β-lactamase production by the chromogenic cephalosporin method using nitrocefin freeze dried powder (Oxoid) or nitrocefin slide (Becton, Dickinson and Company, Maryland).[15]

Antimicrobial susceptibility testing

CDS disc diffusion technique

Antibiotic susceptibility testing of all the 295 *N. gonorrhoeae* isolates was performed by the CDS technique on chocolate agar plates with low concentration antibiotic discs (Oxoid Basingstoke, UK). Six antibiotics with concentrations recommended i.e. penicillin (0.5 IU), tetracycline (10μg), ceftriaxone (0.5μg), ciprofloxacin (1μg), spectinomycin (100μg) and nalidixic acid (30μg) were used as per standard methodology.[16] The strains were

interpreted as susceptible, less susceptible and resistant. Nalidixic acid was used only to identify isolates less susceptible to ciprofloxacin and results of susceptibility to this antibiotic are not included.

CLSI disc diffusion method

Same inoculum was used for this method, which was performed using GC agar base with 1% isovitalex or vitamino growth supplement (Hi Media, India) with higher disc concentration recommended by CLSI i.e. penicillin (10 IU), tetracycline (30µg), ceftriaxone (30µg), ciprofloxacin (5µg), spectinomycin (100µg) and nalidixic acid (30µg) were used.[17] The strains were defined as susceptible, less susceptible and resistant.

Minimum Inhibitory Concentration (MIC) determination

The MICs of all the isolates for penicillin, tetracycline, ciprofloxacin, spectinomycin, and ceftriaxone were determined by the Etest method (AB Biodisk, Solna, Sweden) on GC agar base with 1% isovitalex or vitamino growth supplement. The Etest was performed as specified in the manufacturer's product package insert. The Etest method was selected as the reference method for comparison of results of CDS and CLSI techniques.

Control strains

WHO reference strains C, G, K, L, O and Q were used as controls for disc diffusion and MIC testing. The WHO control strains K and L with decreased susceptibility to extended-spectrum cephalosporins were included in 2010 as these became available to this centre only by the end of 2009. This centre participated in External Quality assurance Scheme (EQAS) of gonococal AST from 2001 to 2010 conducted by the Neisseria Reference Laboratory, WHO Collaborating Centre for STD, Sydney, Australia. EQAS results every year showed

almost 100% agreement with the reference laboratory expected results, except some disagreement of results was observed for one strain for ceftriaxone in 2002, 2007, 2008, 2010 and for one strain for penicillin in 2004 and 2010. On repeat testing, 100% agreement was observed.

Statistical analysis

Discrepancies were differentiated into three categories, minor (involving the less susceptible category), major (false susceptibility) and very major (false resistant) which are defined by U.S. Department of Health and Human Services Food and Drug Administration.[18] Pearson correlation coefficient (r values) was generated for each antimicrobial agent indexed by susceptibility test method. Statistical correlation result was considered perfect for the correlation coefficient (*r* value) of 1.00, desirable for ≥0.90 and acceptable for ≥0.80.

RESULTS

The incidence of susceptible, less susceptible and resistant isolates differed following the performance of both the disc diffusion assays and Etest (Table 1). Table 2 and 3 show the comparison of discrepancies and agreement of CDS and CLSI method with Etest for five different antibiotics. In overall, the rates of discrepancies for different antibiotics differed in both the CDS and CLSI technique on comparison to Etest method. On comparison of CDS method with Etest the highest discrepancy rate was observed for penicillin (8.5%) and lowest for spectinomycin (0) and overall complete agreement was 82.0%. While, for CLSI method highest discrepancy rate was detected for tetracycline (25.1%) and lowest for spectinomycin (0) and overall complete agreement was 49.5%. A total no. of minor and major error was 133 and 16 respectively for CLSI while for CDS they were only 51 and 2 respectively. Complete

percentage agreement for penicillin, ciprofloxacin and tetracycline by CDS test was high in comparison to CLSI technique. It was found to be same for spectinomycin and ceftriaxone. Pearson's correlation coefficient was perfect for all the antibiotics by the CDS method in comparison to CLSI technique i.e. *r* value 1.00 versus 0.92. Moreover, it was very poor (0.61) by CLSI method for tetracycline. Results with individual antibiotics by the three techniques were as follows:

Penicillin

Out of 295 isolates, 34 (11.5%), 156 (52.9%), and 105 (35.6%) isolates were interpreted as susceptible, less susceptible and resistant respectively by the Etest method (Table 1). On comparison of CDS and CLSI with Etest method, minor discrepancies were observed to be 8.5% and 12.5% respectively while no major or very major discrepancies were found. The complete percent agreement of penicillin for the CDS and CLSI method with Etest was 91.5% & 87.5% respectively and the essential agreement for both was found as 100% (Table 2 and 3). Penicillin demonstrated high level of correlation coefficient (0.99) on comparison of both the methods Antibiotic susceptibility by the Etest method revealed that 105 (35.6%) isolates were resistant to penicillin, out of which 95 (32.2%) were β-lactamase positive and 10 (3.4%) were having chromosomally mediated resistance. Out of remaining 190 (64.4%) isolates, 156 (52.9%) had reduced susceptibility to penicillin and 34 (11.5%) were susceptible to penicillin.

Ciprofloxacin

Only 1 (0.3%) isolate was found susceptible for ciprofloxacin with the Etest method. The no. of less susceptible and resistant isolates were 48 (16.3%) and 246 (83.4%) respectively (Table 1). Out of 246 resistant isolates, 102 (34.6%) were observed to have high level

resistance (MIC ≥ 4). On comparison of Etest method with CDS and CLSI methods, minor discrepancies were observed as 7.1% and 11.5% respectively. The complete agreement for both the CDS and CLSI was 92.9% and 88.5% respectively. No major and very major discrepancies occurred. Person's correlation coefficient was excellent (r = 0.99) for both the methods (Table 2 and 3).

Ceftriaxone

Out of 295 isolates, 283 (95.9%) were susceptible and remaining 12 (4.1%) were observed to have decreased susceptibility for ceftriaxone by the Etest method (Table 1). On comparison of CDS and CLSI methods with the Etest method, 1.7% and 1.4% minor discrepancy were observed respectively. The complete percent agreement for ceftriaxone was 98.3% and 98.6% for both the methods. Essential agreement for both the methods was 100% with the Etest method (Table 2 and 3). Person's correlation coefficient was perfect (r = 1) for both the methods.

Spectinomycin

All the isolates were susceptible to spectinomycin, by all the three methods. This resulted in 100% of essential and complete agreement on comparison of the CDS and CLSI method with the Etest method (Table 1-3).

Tetracycline

Out of 295 isolates, 58 (19.7%) were Tetracycline-resistant *N. gonorrhoeae* (TRNG) while 237 (80.3%) were Not-Tetracycline-resistant *N. gonorrhoeae* (N-TRNG) by the Etest method (Table 1). Out of 237 N-TRNG isolates, 116 (39.3%), 91 (30.8%) and 30 (10.2 %) were observed as susceptible, less susceptible and resistant respectively. On comparison of N-TRNG isolates by the CLSI technique with the Etest method, 5.4% and 19.7% major and

minor discrepancies were found respectively. The complete and essential percentage agreement for the CLSI technique was 74.9% and 94.6% respectively. By the CDS method 60 (20.3%) isolates were observed to be TRNG and 235 (79.7%) N-TRNG. Only 2 (0.7%) isolates were found under major discrepancy on comparison of the CDS method with Etest method. Both complete and essential percent agreement between CDS and Etest method was 99.3%. Tetracycline demonstrated perfect correlation coefficient (r = 1) for CDS and Etest method on the basis of TRNG and N-TRNG category comparison. However, for comparison of 80.3% N-TRNG isolates with Etest method, it was observed to be very poor (r = 0.61) by the CLSI technique (Table 2 and 3).

Table 1. Results of susceptibility testing of 295 *N. gonorrhoeae* isolates for five antibiotics by three techniques from 2005-2010.

Method	Penicillin			Ciprofloxacin			Spectinomycin			Ceftriaxone			Tetracycline			Tetracycline	
	S	LS	R	S	LS	R	S	LS	R	S	LS	R	S	LS	R	TRNG	N-TRNG
Etest	34 (11.5)	156 (52.9)	105 (35.6)	1 (0.3)	48 (16.3)	246 (83.4)	295 (100)	-	0	283 (95.9)	12 (4.1)	0	116 (39.3)	91 (30.8)	30 (10.2)	58 (19.7)	237 (80.3)
CDS	30 (10.2)	151 (51.2)	114 (38.6)	1 (0.3)	27 (9.2)	267 (90.5)	295 (100)	-	0	282 (95.6)	13 (4.4)	0	-	-	-	60 (20.3)	235 (79.7)
CLSI	21 (7.1)	157 (53.2)	117 (39.7)	1 (0.3)	64 (21.7)	230 (78.0)	295 (100)	0	0	283 (95.9)	12 (4.1)	0	80 (27.1)	103 (35.9)	64 (21.7)	48 (16.3)	247 (83.7)

S, Susceptible; LS, Less susceptible; R, Resistant; TRNG, Tetracycline Resistant *N. gonorrhoeae*; N-TRNG, Not-TRNG.

Table 2. Comparison of discrepancies and agreement between the CDS and Etest method for 295 *N. gonorrhoeae* isolates.

Antimicrobial Agent	No. of discrepancies			% agreement		Correlation coefficient (r)
	Minor	Major	Very major	Complete	Essential	
Penicillin	25	0	0	91.5	100	0.99
Ciprofloxacin	21	0	0	92.9	100	0.99
Spectinomycin	0	0	0	100	100	1.0
Ceftriaxone	5	0	0	98.3	100	1.0
Tetracycline	0	2	0	99.3	99.3	1.0
All	51	2	0	82.0	99.3	1.0

Table 3. Comparison of discrepancies and agreement between the CLSI and Etest method for 295 *N. gonorrhoeae* isolates.

Antimicrobial Agent	No. of discrepancies			% agreement		Correlation cofficient (r)
	Minor	Major	Very major	Complete	Essential	
Penicillin	37	0	0	87.5	100	0.99
Ciprofloxacin	34	0	0	88.5	100	0.99
Spectinomycin	0	0	0	100	100	1.0
Ceftriaxone	4	0	0	98.6	100	1.0
Tetracycline	58	16	0	74.9	94.6	0.61
All	133	16	0	49.5	94.6	0.92

DISCUSSION

An important and essential measure for monitoring the effectiveness of recommended drugs for the treatment of gonorrhoea is surveillance of the in vitro antimicrobial susceptibilities of clinical isolates of *N. gonorrhoeae*. The recommend procedure for antimicrobial susceptibility testing of gonococci is determination of the MICs by an agar dilution technique.[17] However, this method is laborious and cumbersome, is difficult to standardize, and is performed only in research laboratories, mainly in industrialized countries. Moreover, agar dilution technique requiring a number of isolates at one time is not a feasible technique for determining MIC for this organism because the numbers of isolates even in a central level laboratory are very few and *N. gonorrhoeae* being a fastidious organism is difficult to store for long periods. Etest in a number of studies has already been shown to be an effective alternative and a more appropriate method than the agar dilution technique for MIC determination for *N. gonorrhoeae* by laboratories in developing countries, where resistance of gonococci to antimicrobial agents is a major public health problem.[9-11, 19] Therefore, the Etest method was considered as the reference method in this study for comparison of MIC results with CDS and CLSI disc diffusion techniques, as excellent essential agreement (92-99%) was demonstrated between Etest and standard agar dilution technique for the antibiotics commonly used for susceptibility testing of *N. gonorrhoeae,*[19] and these percentages were above the recommended limitations (≥90%) set by the Food and Drug Administration's *Review Criteria for Assessment of Antimicrobial Susceptibility Devices*, confirming that the Etest satisfactorily approximates the agar reference method.[19] Yeung KH *et al*,[11] also reported 98% overall agreement between Etest and the agar dilution method.

The original purpose of this study was to assess reliability and comparability of the CDS and CLSI method to predict the interpretative categories of susceptibility as compared with standard Etest method. However, because all test procedures were done from a single inoculum and were carefully controlled, we had a unique opportunity to compare the two methods with Etest method which was used as gold standard. Protocols in both the disc diffusion techniques differ in their choices of test medium, antibiotic disc content, and interpretative breakpoint criteria. Zones of inhibition delineating susceptible, less susceptible and resistant isolates from disc diffusion procedures must correlate with corresponding MICs. In the present study, CDS results correlation was excellent with Etest MICs which had less number of major and minor category discrepancies in comparison to CLSI technique i.e. 2 and 52 versus 16 and 133. Complete percent agreement of CDS with Etest was 82.0% while for CLSI method it was 49.5% which was very poor agreement. CDS method showed excellent correlation coefficient (r = 1) with Etest for all five antimicrobial agents tested in comparison to CLSI (r = 0.92). Previously study of our centre had shown 96.9 percent agreement for ceftriaxone for comparision of CDS with Etest technique.[13] Interpretation of disc inhibition zones in CDS method was easier than in the CLSI technique especially when the zone size was near the breakpoint. Double zone of inhibition was observed many times in CLSI technique leading to difficulty in interpretation.

In an another study from WHO Collaborating Centre for STD in Australia and Sweden, the correlation between results of CDS disc diffusion testing (cefpodoxime), and MIC determinations with agar dilution and Etest using cefpodoxime, cefixime and ceftriaxone for the detection of decreased ESC susceptibility in *N. gonorrhoeae* was investigated.[14] CDS technique using cefpodoxime 10 µg disc was shown to provide high sensitivity for detection

of gonococcal isolates with decreased ESC susceptibility and it was suggested that this disc test will make it possible to provide AMR surveillance data also from less-developed and/or less-resourced settings, where disc testing is the only practical and affordable means of AMR testing.[14]

Our findings reveal poor correlation between CLSI disc diffusion results and Etest MICs, most notably for tetracycline, penicillin and ciprofloxacin. Excellent correlation was observed for ceftriaxone and spectinomycin, reason being that resistance was not reported for these antimicrobial agents in the present study. Emergence of resistance to both these antibiotics may lead to poor agreement between CLSI and Etest. Our results compared well with that from China,[20] where poor per cent agreement was reported between the CLSI and agar dilution technique for MIC testing. It was 73.6%, 72.3% and 71.4% for ciprofloxacin, penicillin and ceftriaxone respectively. Given the disparity among susceptibility test results presented here, errors associated with susceptibility testing may result in the unwarranted utilization or elimination of these antibiotics as part of possible treatment regimens.

The results of our study suggest that CDS technique could be reliably used in resistance surveillance programmes for public health purposes and it can be recommended for use by all the focal point laboratories in WHO GASP network in SEAR because of its excellent agreement with Etest results and also it was simple, cost-effective and results are easier to interpret. In many developing countries in SEAR and other regions, most afflicted by gonorrhoea and AMR, MIC determination using agar dilution method or Etest is not readily accessible or affordable. Especially in those countries, the availability of a sensitive, rapid, inexpensive, easily performed and effective disc test can be highly valuable. Use of a

standardized agar diffusion method is practical in these situations and allows fast and reproducible results for clinical microbiology laboratories if standards are observed.[21]

To conclude, this is the first study to compare CDS and CLSI disk diffusion method with Etest and the CDS technique yielded excellent category agreement results when compared with the Etest. The data obtained in the present study suggest that the CDS technique is an accurate alternative method for susceptibility testing of *N. gonorrhoeae* for various antimicrobial agents. It is much less cumbersome than the current reference method because of its simplicity, less consumption of media and glassware and is a more appropriate technique in settings with minimal microbiological resources. CDS offers the advantage for those laboratories that process small numbers of specimens and these laboratories could determine the susceptibilities of gonococcal isolates reasonably accurately. This could facilitate direct and meaningful comparison of resistance data generated within different national and international laboratories.

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There is no additional data available.

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Comparative assessment of CDS, CLSI and Etest techniques for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*: A six year study

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ARTICLE SUMMARY

Article focus

- A variety of techniques are available for antimicrobial susceptibility testing (AST) of *Neisseria gonorrhoeae*.
- The aim of this study to find a cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *N. gonorrhoeae* in resource poor countries.

Key messages

- This study highlighted that the CDS technique yielded excellent category agreement with their analogous Etest MICs in comparison to CLSI technique.
- CDS offers the advantage for those laboratories that process small numbers of specimens.
- CDS technique is cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *Neisseria gonorrhoeae* in resource poor countries.
- This technique will facilitate enhanced antimicrobial resistance surveillance and direct and meaningful comparison of resistance data generated within different national and international laboratories.

Strengths and limitations of this study

- This is the first study to determine the comparability of CDS and CLSI disc diffusion method with Etest MICs and to accurately and reproducibly assess *N. gonorrhoeae* susceptibilities for penicillin, tetracycline, ceftriaxone, ciprofloxacin and spectinomycin, commonly used for susceptibility testing.
- Susceptibility testing for cefixime, cefpodoxime, gentamicin and azithromycin was performed only by one method because of non-availability of interpretation criteria for these antimicrobials or limitation of resources. Therefore, comparison of above three techniques could not be carried out for these antibiotics.

ABSTRACT

Background: A variety of techniques are available for antimicrobial susceptibility testing (AST) of *Neisseria gonorrhoeae*.

Objective: The aim of this study was to find a cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *N. gonorrhoeae* in resource poor countries.

Design: Prospective study.

Setting: Male STD clinic and female STD clinic of Regional STD Teaching, Training and Research Centre, New Delhi, India.

Participants: *N. gonorrhoeae* isolates from all male and female patients presenting with acute gonococcal urethritis and cervical discharge.

Material and Methods: A total of consecutive 295 *N. gonorrhoeae* isolates during 2005 to 2010, were used to the compare the CLSI and CDS disc diffusion technique with Etest by performing antimicrobial susceptibility testing in parallel for penicillin, tetracycline, ceftriaxone, ciprofloxacin and spectinomycin. WHO reference strains were used as controls.

Results: CDS disc diffusion zones of inhibition showed that complete percentage agreement for penicillin, ciprofloxacin and tetracycline was high with their analogous Etest MICs in comparison to CLSI technique i.e. 91.5%, 92.9%, 99.3% versus 87.5%, 88.5% and 74.9% respectively. CDS results had less number of major and minor category discrepancies in comparison to CLSI technique and CDS method showed excellent correlation coefficient (r = 1) with Etest for all five antimicrobial agents tested in comparison to CLSI (r = 0.92). It was very poor (r =0.61) by CLSI method for tetracycline.

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Conclusions The CDS technique is an attractive alternative for *N. gonorrhoeae* susceptibility testing and is recommended for monitoring the antimicrobial susceptibility in less developed and resource poor settings to facilitate enhanced antimicrobial resistance (AMR) surveillance when the WHO Gonococcal Antimicrobial Surveillance Programme is undergoing expansion to meet the ongoing challenges of surveillance and control of gonococcal AMR.

INTRODUCTION

Gonorrhoea caused by *Neisseria gonorrhoeae* (*N. gonorrhoeae*) is one of the most common sexually transmitted infections and is a global health problem. Uncomplicated urogenital *N. gonorrhoeae* infections can lead to epididymitis in males and salpingitis in females, conditions that are associated with infertility. In some cases localized infections can lead to haematogenic dissemination causing severe complications like sepsis, meningitis and endocarditis. The worldwide distribution of *N. gonorrhoeae* and the emergence of resistance to various antimicrobials emphasize the importance of antimicrobial resistance (AMR) surveillance of clinical gonococcal isolates.

Antimicrobial susceptibility testing (AST) may be carried out by a variety of techniques. The most commonly used methods have been the high-content disc diffusion method first described by Bauer *et al*,[1] and later modified by the National Committee for Clinical Laboratory Standards (NCCLS),[2] the broth microdilution technique as described by the NCCLS,[3] the agar dilution method described by Ericsson and Sherris,[4] and adapted by the NCCLS,[3] more recently Etest (AB Biodisk, Solna, Sweden), a modification of the disc diffusion and the agar dilution methods mentioned previously. The Etest is an impervious carrier with a continuous gradient of antimicrobial agent applied to one side of the strip. The test is performed in the same manner as the disc diffusion test, but determines a minimal inhibitory concentration (MIC) of antimicrobial agent rather than a category result based on a zone size. The Calibrated Dichotomous Sensitivity (CDS) test is a method for determining the antibiotic susceptibility of micro-organisms using agar disc diffusion and was developed in 1969 by Sydney M. Bell assisted by members of the Department of Microbiology, The Prince of Wales Hospital, Sydney, Australia. It was introduced into laboratory practice in

Australia in an effort to correct the unsatisfactory results of antibiotic susceptibility testing in Australia that were obtained from surveys conducted by The Royal College of Pathologists of Australia in the late 60's and early 70's.[5]

The recent emergence of *N. gonorrhoeae* isolates with decreased susceptibility and resistance to the currently recommended treatment guidelines of the Centers for Disease Control and Prevention, including extended-spectrum cephalosporins (ESC), has further established the necessity for a standardized, reliable, economical, less labor intensive and reproducible susceptibility testing method.[6-8] Previous studies with the Etest determined that results correlated well with the reference agar dilution method and that it is a useful guide for determining chemotherapy against many organisms,[9] including gonococci.[9-11] The CDS disc diffusion technique is recommended by WHO Collaborating Centre for STD, Sydney, Australia and was regarded as the only practical and affordable means of phenotypic susceptibility testing.[12] It was found to be cost-effective and more feasible during an external quality assurance scheme in routine diagnostic laboratories in developing countries like India.[12] Earlier this method was evaluated in comparison to Etest for only three antibiotics (ciprofloxacin, penicillin, and ceftriaxone) and that also on a limited number of isolates.[13] Moreover, tetracycline and spectinomycin (an alternative drug of choice for treatment of gonorrhea) were not tested in the earlier study of 2005. Recently, CDS technique has been recommended in less resourced settings for detection of decreased susceptibility to ESCs using cefpodoxime disc.[14] The purpose of this study was to determine the comparability of the Etest, CDS and Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS, to accurately and reproducibly assess *N. gonorrhoeae* susceptibilities for five antibiotics commonly used for susceptibility testing, which included penicillin,

tetracycline, ceftriaxone, spectinomycin and ciprofloxacin. The aim was also ~~and~~ to assess the feasibility of recommending CDS technique for its use in developing and resource poor countries. To our knowledge, this is the first study to compare the two disc diffusion methods with Etest MIC testing for above five antibiotics.

MATERIALS AND METHODS

N. gonorrhoeae isolates

A total of 295 *N. gonorrhoeae* isolates from all male and female patients presenting with acute gonococcal urethritis and cervical discharge respectively to the Regional STD Teaching, Training and Research Centre, Safdarjung Hospital, New Delhi, India from January 2005 to December 2010 were included in the study. The strains were consecutive and nonrepetitive. Methods used for isolation and identification of *N. gonorrheae* have been described previously.[15]

β-lactamase testing

N. gonorrhoeae isolates were tested for β-lactamase production by the chromogenic cephalosporin method using nitrocefin freeze dried powder (Oxoid) or nitrocefin slide (Becton, Dickinson and Company, Maryland).[15]

Antimicrobial susceptibility testing

Inoculum Preparation

The inoculum was prepared from 18 to 24 hour pure culture on chocolate agar medium and a homogenized suspension was prepared in 5 ml of sterile saline solution and turbidity was adjusted to an equivalent of 0.5 McFarland standard. Same suspension was used for the

following three methods of antimicrobial susceptibility testing within 15 minutes. All the following three susceptibility tests were run simultaneously on same day.

CDS disc diffusion technique

Antibiotic susceptibility testing of all the 295 *N. gonorrhoeae* isolates was performed by the CDS technique on chocolate agar plates (Columbia agar base, HiMedia Laboratories Pvt. Ltd., Mumbai, India) with low concentration antibiotic discs (Oxoid Basingstoke, UK). Six antibiotics with concentrations recommended i.e. penicillin (0.5 IU), tetracycline (10µg), ceftriaxone (0.5µg), ciprofloxacin (1µg), spectinomycin (100µg) and nalidixic acid (30µg) were used as per standard methodology.[16] The strains were interpreted as susceptible, less susceptible and resistant.[16] Nalidixic acid was used only to identify isolates less susceptible to ciprofloxacin and results of susceptibility to this antibiotic are not included.

CLSI disc diffusion method

Same inoculum was used for this method, which was performed using GC agar base (Oxoid Ltd., Basingstoke, Hampshire, England) with 1% isovitalex or vitamino growth supplement (Hi Media Laboratories Pvt. Ltd., Mumbai, India) with higher disc concentration (HiMedia Laboratories Pvt. Ltd., Mumbai, India) recommended by CLSI i.e. penicillin (10 IU), tetracycline (30µg), ceftriaxone (30µg), ciprofloxacin (5µg), spectinomycin (100µg) and nalidixic acid (30µg) were used.[17] The strains were defined as susceptible, less susceptible and resistant. [17]

Etest Minimum Inhibitory Concentration (MIC) determination

The MICs of all the isolates for penicillin, tetracycline, ciprofloxacin, spectinomycin, and ceftriaxone were determined by the Etest method (AB Biodisk, Solna, Sweden) on GC agar

base with 1% isovitalex or vitamino growth supplement. The Etest was performed as specified in the manufacturer's product package insert. The strains were defined as susceptible, less susceptible and resistant. [17] The Etest method was selected as the reference method for comparison of results of CDS and CLSI techniques.

Control strains

N. gonorrhoeae WHO reference strains C, G, K, L, O and Q were used as controls for disc diffusion and Etest MIC testing. The WHO control strains K and L with decreased susceptibility to extended-spectrum cephalosporins were included in 2010 as these became available to this centre only by the end of 2009.[18] This centre participated in External Quality assurance Scheme (EQAS) of gonococal AST from 2001 to 2010 conducted by the Neisseria Reference Laboratory, WHO Collaborating Centre for STD, Sydney, Australia. EQAS results every year showed almost 100% agreement with the reference laboratory expected results, except some disagreement of results was observed for one strain for ceftriaxone in 2002, 2007, 2008, 2010 and for one strain for penicillin in 2004 and 2010. On repeat testing, 100% agreement was observed.

Statistical analysis

Discrepancies were differentiated into three categories, minor (involving the less susceptible category), major (false susceptibility) and very major (false resistant) which are defined by U.S. Department of Health and Human Services Food and Drug Administration.[198] Complete and essential percent agreement between the reference and test method was evaluated. The complete percent agreement is the percentage of isolates tested by the test method which gave the same category as those tested by the reference method. The essential percent agreement value is the percentage of agreement obtained between the reference and

test method when minor discrepancies are ignored. .[19] Pearson correlation coefficient (r values) was generated for each antimicrobial agent indexed by susceptibility test method. Statistical correlation result was considered perfect for the correlation coefficient (*r* value) of 1.00, desirable for ≥0.90 and acceptable for ≥0.80.

RESULTS

The incidence of susceptible, less susceptible and resistant isolates differed following the performance of both the disc diffusion assays and Etest (Table 1). Table 2 and 3 show the comparison of discrepancies and agreement of CDS and CLSI method with Etest for five different antibiotics. In overall, the rates of discrepancies for different antibiotics differed in both the CDS and CLSI technique on comparison to Etest method. On comparison of CDS method with Etest the highest discrepancy rate was observed for penicillin (8.5%) and lowest for spectinomycin (0) and overall complete agreement was 82.0%. While, for CLSI method highest discrepancy rate was detected for tetracycline (25.1%) and lowest for spectinomycin (0) and overall complete agreement was 49.5%. A total no. of minor and major error was 133 and 16 respectively for CLSI while for CDS they were only 51 and 2 respectively. Complete percentage agreement for penicillin, ciprofloxacin and tetracycline by CDS test was high in comparison to CLSI technique. It was found to be same for spectinomycin and ceftriaxone. Pearson's correlation coefficient was perfect for all the antibiotics by the CDS method in comparison to CLSI technique i.e. *r* value 1.00 versus 0.92. Moreover, it was very poor (0.61) by CLSI method for tetracycline. Results with individual antibiotics by the three techniques were as follows:

Penicillin

Out of 295 isolates, 34 (11.5%), 156 (52.9%), and 105 (35.6%) isolates were interpreted as susceptible, less susceptible and resistant respectively by the Etest method (Table 1). On comparison of CDS and CLSI with Etest method, minor discrepancies were observed to be 8.5% and 12.5% respectively while no major or very major discrepancies were found. The complete percent agreement of penicillin for the CDS and CLSI method with Etest was 91.5% & 87.5% respectively and the essential agreement for both was found as 100% (Table 2 and 3). Penicillin demonstrated high level of correlation coefficient (0.99) on comparison of both the methods Antibiotic susceptibility by the Etest method revealed that 105 (35.6%) isolates were resistant to penicillin, out of which 95 (32.2%) were β-lactamase positive and 10 (3.4%) were having chromosomally mediated resistance. Out of remaining 190 (64.4%) isolates, 156 (52.9%) had reduced susceptibility to penicillin and 34 (11.5%) were susceptible to penicillin.

Ciprofloxacin

Only 1 (0.3%) isolate was found susceptible for ciprofloxacin with the Etest method. The no. of less susceptible and resistant isolates were 48 (16.3%) and 246 (83.4%) respectively (Table 1). Out of 246 resistant isolates, 102 (34.6%) were observed to have high level resistance (MIC ≥ 4). On comparison of Etest method with CDS and CLSI methods, minor discrepancies were observed as 7.1% and 11.5% respectively. The complete agreement for both the CDS and CLSI was 92.9% and 88.5% respectively. No major and very major discrepancies occurred. Person's correlation coefficient was excellent (r = 0.99) for both the methods (Table 2 and 3).

Ceftriaxone

Out of 295 isolates, 283 (95.9%) were susceptible and remaining 12 (4.1%) were observed to have decreased susceptibility for ceftriaxone by the Etest method (Table 1). On comparison of CDS and CLSI methods with the Etest method, 1.7% and 1.4% minor discrepancy were observed respectively. The complete percent agreement for ceftriaxone was 98.3% and 98.6% for both the methods. Essential agreement for both the methods was 100% with the Etest method (Table 2 and 3). Person's correlation coefficient was perfect (r = 1) for both the methods.

Spectinomycin

All the isolates were susceptible to spectinomycin, by all the three methods (Table 1). This resulted in 100% of essential and complete agreement on comparison of the CDS and CLSI method with the Etest method (Table 2~~,1~~3).

Tetracycline

Out of 295 isolates, 58 (19.7%) were Tetracycline-resistant *N. gonorrhoeae* (TRNG) while 237 (80.3%) were Not-Tetracycline-resistant *N. gonorrhoeae* (N-TRNG) by the Etest method (Table 1). Out of 237 N-TRNG isolates, 116 (39.3%), 91 (30.8%) and 30 (10.2 %) were observed as susceptible, less susceptible and resistant respectively. On comparison of N-TRNG isolates by the CLSI technique with the Etest method, 5.4% and 19.7% major and minor discrepancies were found respectively. The complete and essential percentage agreement for the CLSI technique was 74.9% and 94.6% respectively. By the CDS method 60 (20.3%) isolates were observed to be TRNG and 235 (79.7%) N-TRNG. Only 2 (0.7%) isolates were found under major discrepancy on comparison of the CDS method with Etest method. Both complete and essential percent agreement between CDS and Etest method was 99.3%. Tetracycline demonstrated perfect correlation coefficient (r = 1) for CDS and Etest

method on the basis of TRNG and N-TRNG category comparison. However, for comparison of 80.3% N-TRNG isolates with Etest method, it was observed to be very poor (r = 0.61) by the CLSI technique (Table 2 and 3).

Table 1. Results of susceptibility testing of 295 *N. gonorrhoeae* isolates for five antibiotics by three techniques from 2005-2010.

Method	Penicillin			Ciprofloxacin			Spectinomycin			Ceftriaxone			Tetracycline			Tetracycline	
	S	LS	R	S	LS	R	S	LS	R	S	LS	R	S	LS	R	TRNG	N-TRNG
Etest	34	156	105	1	48	246	295	-	0	283	12	0	116	91	30	58	237
	(11.5)	(52.9)	(35.6)	(0.3)	(16.3)	(83.4)	(100)			(95.9)	(4.1)		(39.3)	(30.8)	(10.2)	(19.7)	(80.3)
CDS	30	151	114	1	27	267	295	-	0	282	13	0	-	-	-	60	235
	(10.2)	(51.2)	(38.6)	(0.3)	(9.2)	(90.5)	(100)			(95.6)	(4.4)					(20.3)	(79.7)
CLSI	21	157	117	1	64	230	295	0	0	283	12	0	80	103	64	48	247
	(7.1)	(53.2)	(39.7)	(0.3)	(21.7)	(78.0)	(100)			(95.9)	(4.1)		(27.1)	(35.9)	(21.7)	(16.3)	(83.7)

S, Susceptible; LS, Less susceptible; R, Resistant; TRNG, Tetracycline Resistant *N. gonorrhoeae*; N-TRNG, Not-TRNG.

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Table 2. Comparison of discrepancies and agreement between the CDS and Etest method for 295 *N. gonorrhoeae* isolates.

Antimicrobial Agent	No. of discrepancies			% agreement		Correlation coefficient (r)
	Minor	Major	Very major	Complete	Essential	
Penicillin	25	0	0	91.5	100	0.99
Ciprofloxacin	21	0	0	92.9	100	0.99
Spectinomycin	0	0	0	100	100	1.0
Ceftriaxone	5	0	0	98.3	100	1.0
Tetracycline	0	2	0	99.3	99.3	1.0
All	51	2	0	82.0	99.3	1.0

Table 3. Comparison of discrepancies and agreement between the CLSI and Etest method for 295 *N. gonorrhoeae* isolates.

Antimicrobial Agent	No. of discrepancies			% agreement		Correlation cofficient (r)
	Minor	Major	Very major	Complete	Essential	
Penicillin	37	0	0	87.5	100	0.99
Ciprofloxacin	34	0	0	88.5	100	0.99
Spectinomycin	0	0	0	100	100	1.0
Ceftriaxone	4	0	0	98.6	100	1.0
Tetracycline	58	16	0	74.9	94.6	0.61
All	133	16	0	49.5	94.6	0.92

DISCUSSION

An important and essential measure for monitoring the effectiveness of recommended drugs for the treatment of gonorrhoea is surveillance of the in vitro antimicrobial susceptibilities of clinical isolates of *N. gonorrhoeae*. The recommend procedure for antimicrobial susceptibility testing of gonococci is determination of the MICs by an agar dilution technique.[17] However, this method ~~is laborious and cumbersome, is difficult to standardize, and~~ is performed only in research laboratories, mainly in industrialized countries. Moreover, agar dilution technique requiring a number of isolates at one time is not a feasible technique for determining MIC for this organism because the numbers of isolates even in a central level laboratory are very few and *N. gonorrhoeae* being a fastidious organism is difficult to store for long periods. Etest in a number of studies has already been shown to be an effective alternative and a more appropriate method than the agar dilution technique for MIC determination for *N. gonorrhoeae* by laboratories in developing countries, where resistance of gonococci to antimicrobial agents is a major public health problem.[9-11, 2019] Therefore, the Etest method was considered as the reference method in this study for comparison of MIC results with CDS and CLSI disc diffusion techniques.~~,~~ ~~as~~An excellent essential agreement (92-99%) was demonstrated between Etest and standard agar dilution technique for the antibiotics commonly used for susceptibility testing of *N. gonorrhoeae*,[2019] and these percentages were above the recommended limitations (≥90%) set by the Food and Drug Administration's *Review Criteria for Assessment of Antimicrobial Susceptibility Devices*, confirming that the Etest satisfactorily approximates the agar reference method.[2019] Yeung KH *et al*,[11] also reported 98% overall agreement between Etest and the agar dilution method.

The original purpose of this study was to assess reliability and comparability of the CDS and CLSI method to predict the interpretative categories of susceptibility as compared with standard Etest method. However, because all test procedures were done from ~~a single~~the same inoculum and were carefully controlled, we had a unique opportunity to compare the two methods with Etest method which was used as ~~gold~~ reference standard and the Etest MIC values were supported by satisfactory Quality Control (QC) measures. Protocols in both the disc diffusion techniques differ in their choices of test medium, antibiotic disc content, and interpretative breakpoint criteria. Zones of inhibition delineating susceptible, less susceptible and resistant isolates from disc diffusion procedures must correlate with corresponding MICs. In the present study, CDS results correlation was excellent with Etest MICs which had less number of major and minor category discrepancies in comparison to CLSI technique i.e. 2 and 52 versus 16 and 133. Complete percent agreement of CDS with Etest was 82.0% while for CLSI method it was 49.5% which was very poor agreement. CDS method showed excellent correlation coefficient (r = 1) with Etest for all five antimicrobial agents tested in comparison to CLSI (r = 0.92). Previously study of our centre had shown 96.9 percent agreement for ceftriaxone for comparision of CDS with Etest technique.[13] Interpretation of disc inhibition zones in CDS method was easier than in the CLSI technique especially when the zone size was near the breakpoint. Double zone of inhibition was observed many times in CLSI technique leading to difficulty in interpretation.

In an another study from WHO Collaborating Centre for STD in Australia and Sweden, the correlation between results of CDS disc diffusion testing (cefpodoxime), and MIC determinations with agar dilution and Etest using cefpodoxime, cefixime and ceftriaxone for the detection of decreased ESC susceptibility in *N. gonorrhoeae* was investigated.[14] CDS

technique using cefpodoxime 10 µg disc was shown to provide high sensitivity for detection of gonococcal isolates with decreased ESC susceptibility and it was suggested that this disc test will make it possible to provide AMR surveillance data also from less-developed and/or less-resourced settings, where disc testing is the only practical and affordable means of AMR testing.[14]

Our findings reveal poor correlation between CLSI disc diffusion results and Etest MICs, most notably for tetracycline, penicillin and ciprofloxacin. Further, if tetracycline were removed from the CLSI analysis, then the correlation coefficients between the two methods and the Etest were identical. It is the reported in the literature that there are problems on the reproducibility of results with tetracycline, especially when different media are used.

Excellent correlation was observed for ceftriaxone and spectinomycin, reason being that resistance was not reported for these antimicrobial agents in the present study. Emergence of resistance to both these antibiotics may lead to poor agreement between CLSI and Etest. Our results compared well with that from China,[21~~20~~] where poor per cent agreement was reported between the CLSI and agar dilution technique for MIC testing. It was 73.6%, 72.3% and 71.4% for ciprofloxacin, penicillin and ceftriaxone respectively. Given the disparity among susceptibility test results presented here, errors associated with susceptibility testing may result in the unwarranted utilization or elimination of these antibiotics as part of possible treatment regimens.

The results of our study suggest that CDS technique could be reliably used in resistance surveillance programmes for public health purposes and it can be recommended for use by all the focal point laboratories in WHO GASP network in SEAR because of its excellent agreement with Etest results and also it was simple, cost-effective and results are easier to

interpret. In many developing countries in SEAR and other regions, most afflicted by gonorrhoea and AMR, MIC determination using agar dilution method or Etest is not readily accessible or affordable. Especially in those countries, the availability of a sensitive, rapid, inexpensive, easily performed and effective disc test can be highly valuable. Use of a standardized agar diffusion method is practical in these situations and allows fast and reproducible results for clinical microbiology laboratories if standards are observed.[22~~1~~]

To conclude, this is the first study to compare CDS and CLSI disk diffusion method with Etest and the CDS technique yielded excellent category agreement results when compared with the Etest. The data obtained in the present study suggest that the CDS technique is an accurate alternative method for susceptibility testing of *N. gonorrhoeae* for various antimicrobial agents. It is much less cumbersome than the current reference method because of its simplicity, less consumption of media and glassware and is a more appropriate technique in settings with minimal microbiological resources. CDS offers the advantage for those laboratories that process small numbers of specimens and these laboratories could determine the susceptibilities of gonococcal isolates reasonably accurately. This could facilitate direct and meaningful comparison of resistance data generated within different national and international laboratories.

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There is no additional data available.

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Comparative assessment of CDS, CLSI disc diffusion and Etest techniques for antimicrobial susceptibility testing of Neisseria gonorrhoeae: A six year study

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Comparative assessment of CDS, CLSI disc diffusion and Etest techniques for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*: A six year study

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ARTICLE SUMMARY

Article focus

- A variety of techniques are available for antimicrobial susceptibility testing (AST) of *Neisseria gonorrhoeae*.
- The aim of this study to find a cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *N. gonorrhoeae* in resource poor countries.

Key messages

- This study highlighted that the CDS disc diffusion technique yielded excellent category agreement with their analogous Etest MICs in comparison to CLSI technique.
- CDS offers the advantage for those laboratories that process small numbers of specimens.
- CDS technique is cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *Neisseria gonorrhoeae* in resource poor countries.
- This technique will facilitate enhanced antimicrobial resistance surveillance and direct and meaningful comparison of resistance data generated within different national and international laboratories.

Strengths and limitations of this study

- This is the first study to determine the comparability of CDS and CLSI disc diffusion method with Etest MICs and to accurately and reproducibly assess *N. gonorrhoeae* susceptibilities for penicillin, tetracycline, ceftriaxone, ciprofloxacin and spectinomycin, commonly used for susceptibility testing.
- Susceptibility testing for cefixime, cefpodoxime, gentamicin and azithromycin was performed only by one method because of non-availability of interpretation criteria for these antimicrobials or limitation of resources. Therefore, comparison of above three techniques could not be carried out for these antibiotics.

ABSTRACT

Background: A variety of techniques are available for antimicrobial susceptibility testing (AST) of *Neisseria gonorrhoeae*.

Objective: The aim of this study was to find a cost-effective, reliable and easily applicable microbiological method to detect antimicrobial susceptibilities of *N. gonorrhoeae* in resource poor countries.

Design: Prospective study.

Setting: Male ~~STD clinic~~ and female STD clinic of Regional STD Teaching, Training and Research Centre, New Delhi, India.

Participants: *N. gonorrhoeae* isolates from all male and female patients presenting with acute gonococcal urethritis and cervical discharge.

Material and Methods: A total of ~~consecutive~~ 295 consecutive *N. gonorrhoeae* isolates during 2005 to 2010, was~~ere~~ used to the compare the CLSI and CDS disc diffusion technique with Etest by performing antimicrobial susceptibility testing in parallel for penicillin, tetracycline, ceftriaxone, ciprofloxacin and spectinomycin. WHO reference strains were used as controls.

Results: CDS disc diffusion zones of inhibition showed that complete percentage agreement for penicillin, ciprofloxacin and tetracycline was high with their analogous Etest MICs in comparison to CLSI disc diffusion technique i.e. 91.5%, 92.9%, 99.3% versus 87.5%, 88.5% and 74.9% respectively. CDS results had less number of major and minor category discrepancies in comparison to CLSI ~~technique~~ and CDS method showed excellent correlation coefficient (r = 1) with Etest for all five antimicrobial agents tested in comparison

to CLSI (r = 0.92). It was very poor (r =0.61) by CLSI method for tetracycline. The correlation coefficients between the two methods and the Etest were identical if tetracycline was removed from the CLSI analysis.

Conclusions The CDS technique is an attractive alternative for *N. gonorrhoeae* susceptibility testing and is recommended for monitoring the antimicrobial susceptibility in less developed and resource poor settings to facilitate enhanced antimicrobial resistance (AMR) surveillance when the WHO Gonococcal Antimicrobial Surveillance Programme is undergoing expansion to meet the ongoing challenges of surveillance and control of gonococcal AMR.

INTRODUCTION

Gonorrhoea caused by *Neisseria gonorrhoeae* (*N. gonorrhoeae*) is one of the most common sexually transmitted infections and is a global health problem. Uncomplicated urogenital *N. gonorrhoeae* infections can lead to epididymitis in males and salpingitis in females, conditions that are associated with infertility. In some cases localized infections can lead to haematogenic dissemination causing severe complications like sepsis, meningitis and endocarditis. The worldwide distribution of *N. gonorrhoeae* and the emergence of resistance to various antimicrobials emphasize the importance of antimicrobial resistance (AMR) surveillance of clinical gonococcal isolates.

Antimicrobial susceptibility testing (AST) may be carried out by a variety of techniques. The most commonly used methods have been the high-content disc diffusion method first described by Bauer *et al*,[1] and later modified by the National Committee for Clinical Laboratory Standards (NCCLS),[2] the broth microdilution technique as described by the NCCLS,[3] the agar dilution method described by Ericsson and Sherris,[4] and adapted by the NCCLS,[3] more recently Etest (AB Biodisk, Solna, Sweden), a modification of the disc diffusion and the agar dilution methods mentioned previously. The Etest is an impervious carrier with a continuous gradient of antimicrobial agent applied to one side of the strip. The test is performed in the same manner as the disc diffusion test, but determines a minimal inhibitory concentration (MIC) of antimicrobial agent rather than a category result based on a zone size. The Calibrated Dichotomous Sensitivity (CDS) test is a method for determining

the antibiotic susceptibility of micro-organisms using agar disc diffusion and was developed in 1969 by Sydney M. Bell assisted by members of the Department of Microbiology, The Prince of Wales Hospital, Sydney, Australia. It was introduced into laboratory practice in Australia in an effort to correct the unsatisfactory results of antibiotic susceptibility testing in Australia that were obtained from surveys conducted by The Royal College of Pathologists of Australia in the late 60's and early 70's.[5]

The recent emergence of *N. gonorrhoeae* isolates with decreased susceptibility and resistance to the currently recommended treatment guidelines of the Centers for Disease Control and Prevention, including extended-spectrum cephalosporins (ESC), has further established the necessity for a standardized, reliable, economical, less labor intensive and reproducible susceptibility testing method.[6-8] Previous studies with the Etest determined that results correlated well with the reference agar dilution method and that it is a useful guide for determining chemotherapy against many organisms,[9] including gonococci.[9-11] The CDS disc diffusion technique is recommended by WHO Collaborating Centre for STD, Sydney, Australia and was regarded as the only practical and affordable means of phenotypic susceptibility testing.[12] It was found to be cost-effective and more feasible during an external quality assurance scheme in routine diagnostic laboratories in developing countries like India.[12] Earlier this method was evaluated in comparison to Etest for only three antibiotics (ciprofloxacin, penicillin, and ceftriaxone) and that also on a limited number of isolates.[13] Moreover, tetracycline and spectinomycin (an alternative drug of choice for treatment of gonorrhea) were not tested in the earlier study of 2005. Recently, CDS technique has been recommended in less resourced settings for detection of decreased susceptibility to oral ESCs using cefpodoxime disc,[14] and interpretation criteria for azithromycin by CDS

have been established in September 2011. The purpose of this study was to determine the comparability of the Etest, CDS and Clinical and Laboratory Standards Institute (CLSI) disc diffusion technique, formerly NCCLS, to accurately and reproducibly assess *N. gonorrhoeae* susceptibilities for five antibiotics commonly used for susceptibility testing, which included penicillin, tetracycline, ceftriaxone, spectinomycin and ciprofloxacin. The aim was also to assess the feasibility of recommending CDS technique for its use in developing and resource poor countries. To our knowledge, this is the first study to compare the two disc diffusion methods with Etest MIC testing for above five antibiotics.

MATERIALS AND METHODS

N. gonorrhoeae isolates

A total of 295 *N. gonorrhoeae* isolates from all male and female patients presenting with acute gonococcal urethritis and cervical discharge respectively to the Regional STD Teaching, Training and Research Centre, Safdarjung Hospital, New Delhi, India from January 2005 to December 2010 wasere included in the study. The strains were consecutive and nonrepetitive. Methods used for isolation and identification of *N. gonorrheae* have been described previously.[15]

β-lactamase testing

N. gonorrhoeae isolates were tested for β-lactamase production by the chromogenic cephalosporin method using nitrocefin freeze dried powder (Oxoid) or nitrocefin slide (Becton, Dickinson and Company, Maryland).[15]

Antimicrobial susceptibility testing

Inoculum Preparation

The inoculum was prepared from 18 to 24 hour pure culture on chocolate agar medium and a homogenized suspension was prepared in 5 ml of sterile saline solution and turbidity was adjusted to an equivalent of 0.5 McFarland standard. Same suspension was used for the following three methods of antimicrobial susceptibility testing within 15 minutes. All the following three susceptibility tests were run simultaneously on same day.

CDS disc diffusion technique

Antibiotic susceptibility testing of all the 295 *N. gonorrhoeae* isolates was performed by the CDS technique on chocolate agar plates (Columbia agar base, HiMedia Laboratories Pvt. Ltd., Mumbai, India) with low concentration antibiotic discs (Oxoid Basingstoke, UK). Six antibiotics with concentrations recommended i.e. penicillin (0.5 IU), tetracycline (10µg), ceftriaxone (0.5µg), ciprofloxacin (1µg), spectinomycin (100µg) and nalidixic acid (30µg) were used as per standard methodology.[16] The strains were interpreted as susceptible, less susceptible and resistant.[16] Nalidixic acid was used only to identify isolates less susceptible to ciprofloxacin and results of susceptibility to this antibiotic are not included.

CLSI disc diffusion method

Same inoculum was used for this method, which was performed using GC agar base (Oxoid Ltd., Basingstoke, Hampshire, England) with 1% isovitalex or vitamino growth supplement (Hi Media Laboratories Pvt. Ltd, Mumbai, India) with higher disc concentration (HiMedia Laboratories Pvt. Ltd., Mumbai, India) recommended by CLSI i.e. penicillin (10 IU), tetracycline (30µg), ceftriaxone (30µg), ciprofloxacin (5µg), spectinomycin (100µg) and nalidixic acid (30µg) were used.[17] The strains were defined as susceptible, less susceptible and resistant. [17]

Etest Minimum Inhibitory Concentration (MIC) determination

The MICs of all the isolates for penicillin, tetracycline, ciprofloxacin, spectinomycin, and ceftriaxone were determined by the Etest method (AB Biodisk, Solna, Sweden) on GC agar base with 1% isovitalex or vitamino growth supplement. The Etest was performed as specified in the manufacturer's product package insert. The strains were defined as susceptible, less susceptible and resistant. [17] The Etest method was selected as the reference method for comparison of results of CDS and CLSI disc diffusion techniques.

Control strains

N. gonorrhoeae WHO reference strains C, G, K, L, O and Q were used as controls for disc diffusion and Etest MIC testing. The WHO control strains K and L with decreased susceptibility to extended-spectrum cephalosporins were included in 2010 as these became available to this centre only by the end of 2009.[18] The reproducibility of control strains tested for Etest, CDS and CLSI disc diffusion method was >95%. This centre participated in External Quality assurance Scheme (EQAS) of gonococal AST from 2001 to 2010 conducted by the Neisseria Reference Laboratory, WHO Collaborating Centre for STD, Sydney, Australia. EQAS results every year showed almost 100% agreement with the reference laboratory expected results, except some disagreement of results was observed for one strain for ceftriaxone in 2002, 2007, 2008, 2010 and for one strain for penicillin in 2004 and 2010. On repeat testing, 100% agreement was observed.

Statistical analysis

Discrepancies were differentiated into three categories, minor ((suscetible 'S' interpreted as less sensitive 'LS', 'LS' as 'S' or resistant 'R' and 'R' as 'LS'~~involving the less susceptible category~~), major ('S' interpreted as 'R'~~false susceptibility~~) and very major ('R' interpreted as 'S'~~false resistant~~) which are defined by U.S. Department of Health and Human Services

Food and Drug Administration.[19] Complete and essential percent agreement between the reference and test method was evaluated. The complete percent agreement is the percentage of isolates tested by the test method which gave the same category as those tested by the reference method. The essential percent agreement value is the percentage of agreement obtained between the reference and test method when minor discrepancies are ignored.[19] Pearson's correlation coefficient (r values) was generated for each antimicrobial agent indexed by susceptibility test method. Statistical correlation result was considered perfect for the correlation coefficient (*r* value) of 1.00, desirable for ≥0.90 and acceptable for ≥0.80.

RESULTS

The incidence of susceptible, less susceptible and resistant isolates differed following the performance of both the disc diffusion assays and Etest (Table 1). Table 2 and 3 show the comparison of discrepancies and agreement of CDS and CLSI disc diffusion method with Etest for five different antibiotics. In overall, the rates of discrepancies for different antibiotics differed in both the CDS and CLSI technique on comparison to Etest method. On comparison of CDS method with Etest the highest discrepancy rate was observed for penicillin (8.5%) and lowest for spectinomycin (0) and overall complete agreement was 82.0%.

Highest discrepancy rate ~~While, for~~with the CLSI disc diffusion method ~~highest discrepancy rate~~ was detected for tetracycline (25.1%). ~~and~~ It was lowest for spectinomycin (0) and overall complete agreement was 49.5%. A total no. of minor and major error was 133 and 16 respectively for CLSI disc diffusion metod while for CDS they were only 51 and 2 respectively. Complete percentage agreement for penicillin, ciprofloxacin and tetracycline

by CDS test was high in comparison to CLSI disc diffusion technique. It was found to be same for spectinomycin and ceftriaxone. Pearson's correlation coefficient was perfect for all the antibiotics by the CDS method in comparison to CLSI technique i.e. *r* value 1.00 versus 0.92. Moreover, it was very poor (0.61) by CLSI disc diffusion method for tetracycline. Results with individual antibiotics by the three techniques were as follows:

Penicillin

Out of 295 isolates, 34 (11.5%), 156 (52.9%), and 105 (35.6%) isolates were interpreted as susceptible, less susceptible and resistant respectively by the Etest method (Table 1). On comparison of CDS and CLSI disc diffusion technique with Etest method, minor discrepancies were observed to be 8.5% and 12.5% respectively while no major or very major discrepancies were found (Table 2 and 3). The complete percent agreement of penicillin for the CDS and CLSI method with Etest was 91.5% & 87.5% respectively and the essential agreement for both was found as 100% (Table 2 and 3). Penicillin demonstrated high level of correlation coefficient (0.99) on comparison of both the methods Antibiotic susceptibility by the Etest method revealed that 105 (35.6%) isolates were resistant to penicillin, out of which 95 (32.2%) were β-lactamase positive and 10 (3.4%) were having chromosomally mediated resistance. Out of remaining 190 (64.4%) isolates, 156 (52.9%) had reduced susceptibility to penicillin and 34 (11.5%) were susceptible to penicillin.

Ciprofloxacin

Only 1 (0.3%) isolate was found susceptible for ciprofloxacin with the Etest method. The no. of less susceptible and resistant isolates were 48 (16.3%) and 246 (83.4%) respectively (Table 1). Out of 246 resistant isolates, 102 (34.6%) were observed to have high level resistance (MIC ≥ 4). On comparison of Etest method with CDS and CLSI disc diffusion

methods, minor discrepancies were observed as 7.1% and 11.5% respectively (Table 2 and 3). The complete agreement for both the CDS and CLSI was 92.9% and 88.5% respectively. No major and very major discrepancies occurred. Person's correlation coefficient was excellent (r = 0.99) for both the methods (Table 2 and 3).

Ceftriaxone

Out of 295 isolates, 283 (95.9%) were susceptible and remaining 12 (4.1%) were observed to have decreased susceptibility for ceftriaxone by the Etest method (Table 1). On comparison of CDS and CLSI disc diffusion methods with the Etest method, 1.7% and 1.4% minor discrepancy were observed respectively (Table 2 and 3). The complete percent agreement for ceftriaxone was 98.3% and 98.6% for both the methods. Essential agreement for both the methods was 100% with the Etest method (Table 2 and 3). Pearson's correlation coefficient was perfect (r = 1) for both the methods.

Spectinomycin

All the isolates were susceptible to spectinomycin, by all the three methods (Table 1). This resulted in 100% of essential and complete agreement on comparison of the CDS and CLSI method with the Etest method (Table 2 and 3).

Tetracycline

Out of 295 isolates, 58 (19.7%) were Tetracycline-resistant *N. gonorrhoeae* (TRNG) while 237 (80.3%) were Not-Tetracycline-resistant *N. gonorrhoeae* (N-TRNG) by the Etest method (Table 1). Out of 237 N-TRNG isolates, 116 (39.3%), 91 (30.8%) and 30 (10.2 %) were observed as susceptible, less susceptible and resistant respectively. On comparison of N-TRNG isolates by the CLSI disc diffusion technique with the Etest method, 5.4% and 19.7% major and minor discrepancies were found respectively (Table 3). The complete and essential

percentage agreement for the CLSI technique was 74.9% and 94.6% respectively. By the CDS method 60 (20.3%) isolates were observed to be TRNG and 235 (79.7%) N-TRNG (Table 1). Only 2 (0.7%) isolates were found under major discrepancy on comparison of the CDS method with Etest method. Both complete and essential percent agreement between CDS and Etest method was 99.3% (Table 2). Tetracycline demonstrated perfect correlation coefficient (r = 1) for CDS and Etest method on the basis of TRNG and N-TRNG category comparison (Table 2). However, for comparison of 80.3% N-TRNG isolates with Etest method, it was observed to be very poor (r = 0.61) by the CLSI disc diffusion technique (Table ~~2 and~~ 3).

Table 1. Results of susceptibility testing of 295 *N. gonorrhoeae* isolates for five antibiotics by three techniques from 2005-2010.

Method	Penicillin			Ciprofloxacin			Spectinomycin			Ceftriaxone			Tetracycline			Tetracycline	
	S	LS	R	S	LS	R	S	LS	R	S	LS	R	S	LS	R	TRNG	N-TRNG
Etest	34 (11.5)	156 (52.9)	105 (35.6)	1 (0.3)	48 (16.3)	246 (83.4)	295 (100)	-	0	283 (95.9)	12 (4.1)	0	116 (39.3)	91 (30.8)	30 (10.2)	58 (19.7)	237 (80.3)
CDS	30 (10.2)	151 (51.2)	114 (38.6)	1 (0.3)	27 (9.2)	267 (90.5)	295 (100)	-	0	282 (95.6)	13 (4.4)	0	-	-	-	60 (20.3)	235 (79.7)
CLSI	21 (7.1)	157 (53.2)	117 (39.7)	1 (0.3)	64 (21.7)	230 (78.0)	295 (100)	0	0	283 (95.9)	12 (4.1)	0	80 (27.1)	103 (35.9)	64 (21.7)	48 (16.3)	247 (83.7)

S, Susceptible; LS, Less susceptible; R, Resistant; TRNG, Tetracycline Resistant *N. gonorrhoeae*; N-TRNG, Not-TRNG.

Table 2. Comparison of discrepancies and agreement between the CDS and Etest method for 295 *N. gonorrhoeae* isolates.

Antimicrobial Agent	No. of discrepancies			% agreement		Correlation coefficient (r)
	Minor	Major	Very major	Complete	Essential	
Penicillin	25	0	0	91.5	100	0.99
Ciprofloxacin	21	0	0	92.9	100	0.99
Spectinomycin	0	0	0	100	100	1.0
Ceftriaxone	5	0	0	98.3	100	1.0
Tetracycline	0	2	0	99.3	99.3	1.0
All	51	2	0	82.0	99.3	1.0

Minor, 'S' and 'R' as 'LS'or 'LS' as 'S' or 'R'; Major, 'S' as 'R'; Very major, 'R' as 'S'.

Table 3. Comparison of discrepancies and agreement between the CLSI and Etest method for 295 *N. gonorrhoeae* isolates.

Antimicrobial Agent	No. of discrepancies			% agreement		Correlation cofficient (r)
	Minor	Major	Very major	Complete	Essential	
Penicillin	37	0	0	87.5	100	0.99
Ciprofloxacin	34	0	0	88.5	100	0.99
Spectinomycin	0	0	0	100	100	1.0
Ceftriaxone	4	0	0	98.6	100	1.0
Tetracycline	58	16	0	74.9	94.6	0.61
All	133	16	0	49.5	94.6	0.92

DISCUSSION

An important and essential measure for monitoring the effectiveness of recommended drugs for the treatment of gonorrhoea is surveillance of the in vitro antimicrobial susceptibilities of clinical isolates of *N. gonorrhoeae*. The recommend procedure for antimicrobial susceptibility testing of gonococci is determination of the MICs by an agar dilution technique.[17] However, this method is performed only in research laboratories, mainly in industrialized countries. Moreover, agar dilution technique requiring a number of isolates at one time is not a feasible technique for determining MIC for this organism because the numbers of isolates even in a central level laboratory are very few and *N. gonorrhoeae* being a fastidious organism is difficult to store for long periods. Etest in a number of studies has already been shown to be an effective alternative and a more appropriate method than the agar dilution technique for MIC determination for *N. gonorrhoeae* by laboratories in developing countries, where resistance of gonococci to antimicrobial agents is a major public health problem.[9-11, 20] Therefore, the Etest method was considered as the reference method in this study for comparison of MIC results with CDS and CLSI disc diffusion techniques. An excellent essential agreement (92-99%) was demonstrated between Etest and standard agar dilution technique for the antibiotics commonly used for susceptibility testing of *N. gonorrhoeae,*[20] and these percentages were above the recommended limitations (≥90%) set by the Food and Drug Administration's *Review Criteria for Assessment of Antimicrobial Susceptibility Devices*, confirming that the Etest satisfactorily approximates the agar reference method.[20] Yeung KH *et al*,[11] also reported 98% overall agreement between Etest and the agar dilution method.

The original purpose of this study was to assess reliability and comparability of the CDS and CLSI disc diffusion method to predict the interpretative categories of susceptibility as

compared with standard Etest method. However, because all test procedures were done from the same inoculum and were carefully controlled, we had a unique opportunity to compare the two methods with Etest method which was used as reference standard and the Etest MIC values were supported by satisfactory Quality Control (QC) measures. Protocols in both the disc diffusion techniques differ in their choices of test medium, antibiotic disc content, and interpretative breakpoint criteria. Zones of inhibition delineating susceptible, less susceptible and resistant isolates from disc diffusion procedures must correlate with corresponding MICs. In the present study, CDS results correlation was excellent with Etest MICs which had less number of major and minor category discrepancies in comparison to CLSI disc diffusion technique i.e. 2 and 52 versus 16 and 133. Complete percent agreement of CDS with Etest was 82.0% while for CLSI disc diffusion method it was 49.5% which was very poor agreement. CDS method showed excellent correlation coefficient (r = 1) with Etest for all five antimicrobial agents tested in comparison to CLSI (r = 0.92). Previously study of our centre had shown 96.9 percent agreement for ceftriaxone for comparision of CDS with Etest technique.[13] Interpretation of disc inhibition zones in CDS method was easier than in the CLSI technique especially when the zone size was near the breakpoint. Double zone of inhibition was observed many times in CLSI technique leading to difficulty in interpretation. In an another study from WHO Collaborating Centre for STD in Australia and Sweden, the correlation between results of CDS disc diffusion testing (cefpodoxime), and MIC determinations with agar dilution and Etest using cefpodoxime, cefixime and ceftriaxone for the detection of decreased ESC susceptibility in *N. gonorrhoeae* was investigated.[14] CDS technique using cefpodoxime 10 µg disc was shown to provide high sensitivity for detection of gonococcal isolates with decreased ESC susceptibility and it was suggested that this disc

test will make it possible to provide AMR surveillance data also from less-developed and/or less-resourced settings, where disc testing is the only practical and affordable means of AMR testing.[14]

Our findings reveal poor correlation between CLSI disc diffusion results and Etest MICs, most notably for tetracycline, penicillin and ciprofloxacin. Further, if tetracycline were removed from the CLSI analysis, then the correlation coefficients between the two methods and the Etest were identical. It is the reported in the literature that there are problems on the reproducibility of results with tetracycline, especially when different media are used. Excellent correlation was observed for ceftriaxone and spectinomycin, reason being that resistance was not reported for these antimicrobial agents in the present study. ~~Emergence of resistance to both these antibiotics may lead to poor agreement between CLSI and Etest.~~ Our results compared well with that from China,[21] where poor per cent agreement was reported between the CLSI and agar dilution technique for MIC testing. It was 73.6%, 72.3% and 71.4% for ciprofloxacin, penicillin and ceftriaxone respectively. Given the disparity among susceptibility test results presented here, errors associated with susceptibility testing may result in the unwarranted utilization or elimination of these antibiotics as part of possible treatment regimens.

The results of our study suggest that CDS disc diffusion technique could be reliably used in resistance surveillance programmes for public health purposes and it can be recommended for use by all the focal point laboratories in WHO GASP network in SEAR because of its excellent agreement with Etest results and also it was simple, cost-effective and results are easier to interpret. In many developing countries in SEAR and other regions, most afflicted by gonorrhoea and AMR, MIC determination using agar dilution method or Etest is not

readily accessible or affordable. Especially in those countries, the availability of a sensitive, rapid, inexpensive, easily performed and effective disc test can be highly valuable. Use of a standardized agar diffusion method is practical in these situations and allows fast and reproducible results for clinical microbiology laboratories if standards are observed.[22]

To conclude, this is the first study to compare CDS and CLSI disk diffusion method with Etest and the CDS technique yielded excellent category agreement results when compared with the Etest. The data obtained in the present study suggest that the CDS technique is an accurate alternative method for susceptibility testing of *N. gonorrhoeae* for various antimicrobial agents. It is much less cumbersome than the current reference method because of its simplicity, less consumption of media and glassware and is a more appropriate technique in settings with minimal microbiological resources. CDS offers the advantage for those laboratories that process small numbers of specimens and these laboratories could determine the susceptibilities of gonococcal isolates reasonably accurately. This could facilitate direct and meaningful comparison of resistance data generated within different national and international laboratories.

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Contributors: MB designed the study and coordinated the work. VS and MB carried out analysis of data and preparation of manuscript. MK participated in data analysis and VR collaborated in the writing of the manuscript.

There is no additional data available.

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