



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

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4 **Comparative assessment of CDS, CLSI and Etest techniques for**
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6 **antimicrobial susceptibility testing of *Neisseria gonorrhoeae*: A six year**
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8 **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 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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3 **Conclusions** The CDS technique is an attractive alternative for *N. gonorrhoeae* susceptibility
4 testing and is recommended for monitoring the antimicrobial susceptibility in less developed
5 and resource poor settings to facilitate enhanced antimicrobial resistance (AMR) surveillance
6 when the WHO Gonococcal Antimicrobial Surveillance Programme is undergoing expansion
7 to meet the ongoing challenges of surveillance and control of gonococcal AMR.
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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

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3 Australia in an effort to correct the unsatisfactory results of antibiotic susceptibility testing in
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6 Australia that were obtained from surveys conducted by The Royal College of Pathologists
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8 of Australia in the late 60's and early 70's.[5]
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11 The recent emergence of *N. gonorrhoeae* isolates with decreased susceptibility and resistance
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13 to the currently recommended treatment guidelines of the Centers for Disease Control and
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15 Prevention, including extended-spectrum cephalosporins (ESC), has further established the
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17 necessity for a standardized, reliable, economical, less labor intensive and reproducible
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19 susceptibility testing method.[6-8] Previous studies with the Etest determined that results
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21 correlated well with the reference agar dilution method and that it is a useful guide for
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23 determining chemotherapy against many organisms,[9] including gonococci.[9-11] The CDS
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25 disc diffusion technique is recommended by WHO Collaborating Centre for STD, Sydney,
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27 Australia and was regarded as the only practical and affordable means of phenotypic
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29 susceptibility testing.[12] It was found to be cost-effective and more feasible during an
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31 external quality assurance scheme in routine diagnostic laboratories in developing countries
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33 like India.[12] Earlier this method was evaluated in comparison to Etest for only three
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35 antibiotics (ciprofloxacin, penicillin, and ceftriaxone) and that also on a limited number of
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37 isolates.[13] Recently, CDS technique has been recommended in less resourced settings for
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39 detection of decreased susceptibility to ESCs using cefpodoxime disc.[14] The purpose of
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41 this study was to determine the comparability of the Etest, CDS and Clinical and Laboratory
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43 Standards Institute (CLSI), formerly NCCLS, to accurately and reproducibly assess *N.*
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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

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3 poor countries. To our knowledge, this is the first study to compare the two disc diffusion
4 methods with MIC testing for above five antibiotics.
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10 11 **MATERIALS AND METHODS**

12 13 14 ***N. gonorrhoeae* isolates**

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16 A total of 295 *N. gonorrhoeae* isolates from all male and female patients presenting with
17 acute gonococcal urethritis and cervical discharge respectively to the Regional STD
18 Teaching, Training and Research Centre, Safdarjung Hospital, New Delhi, India from
19 January 2005 to December 2010 were included in the study. The strains were consecutive
20 and nonrepetitive. Methods used for isolation and identification of *N. gonorrhoeae* have been
21 described previously.[15]
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30 31 **β -lactamase testing**

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33 *N. gonorrhoeae* isolates were tested for β -lactamase production by the chromogenic
34 cephalosporin method using nitrocefin freeze dried powder (Oxoid) or nitrocefin slide
35 (Becton, Dickinson and Company, Maryland).[15]
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40 41 **Antimicrobial susceptibility testing**

42 43 *CDS disc diffusion technique*

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45 Antibiotic susceptibility testing of all the 295 *N. gonorrhoeae* isolates was performed by the
46 CDS technique on chocolate agar plates with low concentration antibiotic discs (Oxoid
47 Basingstoke, UK). Six antibiotics with concentrations recommended i.e. penicillin (0.5 IU),
48 tetracycline (10 μ g), ceftriaxone (0.5 μ g), ciprofloxacin (1 μ g), spectinomycin (100 μ g) and
49 nalidixic acid (30 μ g) were used as per standard methodology.[16] The strains were
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3 interpreted as susceptible, less susceptible and resistant. Nalidixic acid was used only to
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5 identify isolates less susceptible to ciprofloxacin and results of susceptibility to this antibiotic
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7 are not included.
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10 11 *CLSI disc diffusion method*

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13 Same inoculum was used for this method, which was performed using GC agar base with 1%
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15 isovitalex or vitamino growth supplement (Hi Media, India) with higher disc concentration
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17 recommended by CLSI i.e. penicillin (10 IU), tetracycline (30µg), ceftriaxone (30µg),
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19 ciprofloxacin (5µg), spectinomycin (100µg) and nalidixic acid (30µg) were used.[17] The
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21 strains were defined as susceptible, less susceptible and resistant.
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25 26 *Minimum Inhibitory Concentration (MIC) determination*

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28 The MICs of all the isolates for penicillin, tetracycline, ciprofloxacin, spectinomycin, and
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30 ceftriaxone were determined by the Etest method (AB Biodisk, Solna, Sweden) on GC agar
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32 base with 1% isovitalex or vitamino growth supplement. The Etest was performed as
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34 specified in the manufacturer's product package insert. The Etest method was selected as the
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36 reference method for comparison of results of CDS and CLSI techniques.
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40 41 **Control strains**

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

11 12 13 **Statistical analysis**

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

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

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

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15 Out of 295 isolates, 283 (95.9%) were susceptible and remaining 12 (4.1%) were observed to
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17 have decreased susceptibility for ceftriaxone by the Etest method (Table 1). On comparison
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19 of CDS and CLSI methods with the Etest method, 1.7% and 1.4% minor discrepancy were
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21 observed respectively. The complete percent agreement for ceftriaxone was 98.3% and
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23 98.6% for both the methods. Essential agreement for both the methods was 100% with the
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25 Etest method (Table 2 and 3). Person's correlation coefficient was perfect ($r = 1$) for both the
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27 methods.
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33 **Spectinomycin**

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35 All the isolates were susceptible to spectinomycin, by all the three methods. This resulted in
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37 100% of essential and complete agreement on comparison of the CDS and CLSI method with
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39 the Etest method (Table 1-3).
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43 **Tetracycline**

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45 Out of 295 isolates, 58 (19.7%) were Tetracycline-resistant *N. gonorrhoeae* (TRNG) while
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47 237 (80.3%) were Not-Tetracycline-resistant *N. gonorrhoeae* (N-TRNG) by the Etest method
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49 (Table 1). Out of 237 N-TRNG isolates, 116 (39.3%), 91 (30.8%) and 30 (10.2 %) were
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51 observed as susceptible, less susceptible and resistant respectively. On comparison of N-
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53 TRNG isolates by the CLSI technique with the Etest method, 5.4% and 19.7% major and
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3 minor discrepancies were found respectively. The complete and essential percentage
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5 agreement for the CLSI technique was 74.9% and 94.6% respectively. By the CDS method
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7 60 (20.3%) isolates were observed to be TRNG and 235 (79.7%) N-TRNG. Only 2 (0.7%)
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9 isolates were found under major discrepancy on comparison of the CDS method with Etest
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11 method. Both complete and essential percent agreement between CDS and Etest method was
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13 99.3%. Tetracycline demonstrated perfect correlation coefficient ($r = 1$) for CDS and Etest
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15 method on the basis of TRNG and N-TRNG category comparison. However, for comparison
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17 of 80.3% N-TRNG isolates with Etest method, it was observed to be very poor ($r = 0.61$) by
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19 the CLSI technique (Table 2 and 3).
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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 coefficient (r)
	Minor	Major	Very major	Complete	Essential	
	Penicillin	37	0	0	87.5	
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 ($\geq 90\%$) 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.

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

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22 In an another study from WHO Collaborating Centre for STD in Australia and Sweden, the
23 correlation between results of CDS disc diffusion testing (cefpodoxime), and MIC
24 determinations with agar dilution and Etest using cefpodoxime, cefixime and ceftriaxone for
25 the detection of decreased ESC susceptibility in *N. gonorrhoeae* was investigated.[14] CDS
26 technique using cefpodoxime 10 µg disc was shown to provide high sensitivity for detection
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3 of gonococcal isolates with decreased ESC susceptibility and it was suggested that this disc
4 test will make it possible to provide AMR surveillance data also from less-developed and/or
5 less-resourced settings, where disc testing is the only practical and affordable means of AMR
6 testing.[14]
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12 Our findings reveal poor correlation between CLSI disc diffusion results and Etest MICs,
13 most notably for tetracycline, penicillin and ciprofloxacin. Excellent correlation was
14 observed for ceftriaxone and spectinomycin, reason being that resistance was not reported for
15 these antimicrobial agents in the present study. Emergence of resistance to both these
16 antibiotics may lead to poor agreement between CLSI and Etest. Our results compared well
17 with that from China,[20] where poor per cent agreement was reported between the CLSI and
18 agar dilution technique for MIC testing. It was 73.6%, 72.3% and 71.4% for ciprofloxacin,
19 penicillin and ceftriaxone respectively. Given the disparity among susceptibility test results
20 presented here, errors associated with susceptibility testing may result in the unwarranted
21 utilization or elimination of these antibiotics as part of possible treatment regimens.
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36 The results of our study suggest that CDS technique could be reliably used in resistance
37 surveillance programmes for public health purposes and it can be recommended for use by all
38 the focal point laboratories in WHO GASP network in SEAR because of its excellent
39 agreement with Etest results and also it was simple, cost-effective and results are easier to
40 interpret. In many developing countries in SEAR and other regions, most afflicted by
41 gonorrhoea and AMR, MIC determination using agar dilution method or Etest is not readily
42 accessible or affordable. Especially in those countries, the availability of a sensitive, rapid,
43 inexpensive, easily performed and effective disc test can be highly valuable. Use of a
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3 standardized agar diffusion method is practical in these situations and allows fast and
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5 reproducible results for clinical microbiology laboratories if standards are observed.[21]
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8 To conclude, this is the first study to compare CDS and CLSI disk diffusion method with
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10 Etest and the CDS technique yielded excellent category agreement results when compared
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12 with the Etest. The data obtained in the present study suggest that the CDS technique is an
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14 accurate alternative method for susceptibility testing of *N. gonorrhoeae* for various
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16 antimicrobial agents. It is much less cumbersome than the current reference method because
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18 of its simplicity, less consumption of media and glassware and is a more appropriate
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20 technique in settings with minimal microbiological resources. CDS offers the advantage for
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22 those laboratories that process small numbers of specimens and these laboratories could
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24 determine the susceptibilities of gonococcal isolates reasonably accurately. This could
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26 facilitate direct and meaningful comparison of resistance data generated within different
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28 national and international laboratories.
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6 collaborated in the writing of the manuscript.
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13 There is no additional data available.
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16 17 18 **Copyright** 19

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34 35 **REFERENCES** 36

- 37 1 Bauer AW, Kirby WM, Sherris JC, *et al.* Antibiotic susceptibility testing by a
38 standardized single disk method. *Am J Clin Pathol* 1966;**45**:493-6.
39
40
- 41 2 National Committee for Clinical Laboratory Standards. Performance standards for
42 antimicrobial disk susceptibility test. *Tentative standard, M2-T4*, 1988;vol 8. National
43 Committee for Clinical Laboratory Standards, Villanova, Pa.
44
45
46
47
48
- 49 3 National Committee for Clinical Laboratory Standards. Methods for dilution
50 antimicrobial susceptibility test for bacteria that grow aerobically. *Tentative standard,*
51 *M7-T2*, 1988; vol. 8. National Committee for Clinical Laboratory Standards,
52 Villanova, Pa.
53
54
55
56
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60

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4 Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Report of an international
5 collaborative study. *Acta Pathol Microbiol Scand* 1971;**217** Sect. B Suppl:1-90.
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55
56
57
58
59
60
- 4 Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Report of an international collaborative study. *Acta Pathol Microbiol Scand* 1971;**217** Sect. B Suppl:1-90.
- 5 Bell SM. The Calibrated Dichotomous Sensitivity (CDS) disc method of antibiotic sensitivity testing. *Pathology* 1975;**7** Suppl:1-48.
- 6 Bala M, Ray K, Gupta SM, *et al.* Changing trends of antimicrobial susceptibility pattern of *Neisseria gonorrhoeae* in India and the emergence of ceftriaxone less susceptible *N. gonorrhoeae* strains. *J Antimicrob Chemother* 2007;**60**:582-6.
- 7 Unemo M, Golparian D, Syversen G, *et al.* Two cases of verified clinical failures using internationally recommended first-line cefixime for gonorrhoea treatment, Norway, 2010. *Euro Surveill* 2010;**15**(47):pii=19721.
- 8 Ohnishi M, Golparian D, Shimuta K, *et al.* Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea? Detailed characterization of the first strain with high-level resistance to ceftriaxone. *Antimicrob Agents Chemother* 2011;**55**:3538-45.
- 9 Sanchez ML, Barrett MS, Jones RN. The Etest applied to susceptibility test of gonococci, multiple-resistant enterococci and Entobacteriaceae producing potent beta-lactamases. *Diagn Microbiol Infect Dis* 1992;**15**:459-63.
- 10 Dyck EV, Smet H, Piot P. Comparison of E test with agar dilution for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*. *J Clin Microbiol* 1994;**32**:1586-8.
- 11 Yeung KH, Ng LK, Dillon JA. Evaluation of Etest for testing antimicrobial susceptibilities of *Neisseria gonorrhoeae* isolates with different growth media. *J Clin Microbiol* 1993;**31**:3053-5.

- 1
2
3
4 12 Bala M, Tapsall JW, Limnios A, *et al.* Experience with an external quality assurance
5 scheme for antimicrobial susceptibility testing of *Neisseria gonorrhoeae* in India,
6 2001–2007. *Epidemiol Infect* 2010;**138**:69–75.
7
8
9
10
11 13 Bala M, Ray K, Gupta S. Comparison of disc diffusion results with minimum
12 inhibitory concentration (MIC) values for antimicrobial susceptibility testing of
13 *Neisseria gonorrhoeae*. *Indian J Med Res* 2005;**122**:48-51.
14
15
16
17
18 14 Limnios A, Tapsall J, Kahlmeter J, *et al.* Cefpodoxime 10 µg disc screening test for
19 detection of *Neisseria gonorrhoeae* with mosaic PBP2 and decreased susceptibility to
20 extended-spectrum cephalosporins for public health purposes. *APMIS* 2011;**119**:356-
21 63.
22
23
24
25
26
27
28 15 Laboratory diagnosis of gonorrhoea. *WHO Regional Publication, South East Asia*
29 *series no 33*: World Health Organisation, Geneva, 1999.
30
31 <http://w3.whosea.org/book33/> [accessed 22 December, 2011]
32
33
34
35
36 16 Bell SM, Pham JN, Fisher GT. Antibiotic susceptibility testing by the CDS method. *A*
37 *manual for medical and veterinary laboratories*. Fifth edition. 2009; P42-58.
38
39 <http://web.med.unsw.edu.au/cdstest> [accessed 22 December, 2011]
40
41
42
43 17 Clinical and Laboratory Standards Institute (CLSI). Performance standards for
44 antimicrobial susceptibility testing. Seventeenth Informational Supplement. *CLSI*
45 *document M100-S17*. Wayne, USA: Clinical and Laboratory Standards Institute 2007.
46
47
48
49
50
51 18 U.S. Food and Drug Administration. Class II Special Controls Guidance Document:
52 Antimicrobial Susceptibility Test (AST) Systems. U.S. Department of Health and
53 Human Services Food and Drug Administration, Center for Devices and Radiological
54
55
56
57
58
59
60

Health. Issued March 5, 2007.

<http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080187.htm> [accessed 28 December, 2011]

- 19 Biedenbach DJ, Jones RN. Comparative Assessment of Etest for Testing Susceptibilities of *Neisseria gonorrhoeae* to Penicillin, Tetracycline, Ceftriaxone, Cefotaxime, and Ciprofloxacin: Investigation Using 510(k) Review Criteria, recommended by the Food and Drug Administration. *J Clin Microbiol* 1996;**32**:14-7.
- 20 Guoming L, Qun C, Shengchun W. Resistance of *Neisseria gonorrhoeae* epidemic strains to antibiotics: report of resistant isolates and surveillance in Zhanjiang, China: 1998 to 1999. *Sex Transm Dis* 2000;**27**:115-8.
- 21 Jones RN, Gerlach EH, Koontz FP, *et al.* Development of *Neisseria gonorrhoeae* in vitro susceptibility test methods for cefixime including quality control guidelines. *Diagn Microbiol Infect Dis* 1990;**14**:383-8.

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

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Running title: Comparison of CDS, CLSI, Etest for susceptibility testing of *N. gonorrhoeae*

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 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

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9 Australia in an effort to correct the unsatisfactory results of antibiotic susceptibility testing in
10 Australia that were obtained from surveys conducted by The Royal College of Pathologists
11 of Australia in the late 60's and early 70's.[5]
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14 The recent emergence of *N. gonorrhoeae* isolates with decreased susceptibility and resistance
15 to the currently recommended treatment guidelines of the Centers for Disease Control and
16 Prevention, including extended-spectrum cephalosporins (ESC), has further established the
17 necessity for a standardized, reliable, economical, less labor intensive and reproducible
18 susceptibility testing method.[6-8] Previous studies with the Etest determined that results
19 correlated well with the reference agar dilution method and that it is a useful guide for
20 determining chemotherapy against many organisms,[9] including gonococci.[9-11] The CDS
21 disc diffusion technique is recommended by WHO Collaborating Centre for STD, Sydney,
22 Australia and was regarded as the only practical and affordable means of phenotypic
23 susceptibility testing.[12] It was found to be cost-effective and more feasible during an
24 external quality assurance scheme in routine diagnostic laboratories in developing countries
25 like India.[12] Earlier this method was evaluated in comparison to Etest for only three
26 antibiotics (ciprofloxacin, penicillin, and ceftriaxone) and that also on a limited number of
27 isolates.[13] Moreover, tetracycline and spectinomycin (an alternative drug of choice for
28 treatment of gonorrhea) were not tested in the earlier study of 2005. Recently, CDS technique
29 has been recommended in less resourced settings for detection of decreased susceptibility to
30 ESCs using cefpodoxime disc.[14] The purpose of this study was to determine the
31 comparability of the Etest, CDS and Clinical and Laboratory Standards Institute (CLSI),
32 formerly NCCLS, to accurately and reproducibly assess *N. gonorrhoeae* susceptibilities for
33 five antibiotics commonly used for susceptibility testing, which included penicillin,
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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. gonorrhoeae* 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% isovitalax 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 gonococcal 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. [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 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

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9 Out of 295 isolates, 34 (11.5%), 156 (52.9%), and 105 (35.6%) isolates were interpreted as
10 susceptible, less susceptible and resistant respectively by the Etest method (Table 1). On
11 comparison of CDS and CLSI with Etest method, minor discrepancies were observed to be
12 8.5% and 12.5% respectively while no major or very major discrepancies were found. The
13 complete percent agreement of penicillin for the CDS and CLSI method with Etest was
14 91.5% & 87.5% respectively and the essential agreement for both was found as 100% (Table
15 2 and 3). Penicillin demonstrated high level of correlation coefficient (0.99) on comparison
16 of both the methods. Antibiotic susceptibility by the Etest method revealed that 105 (35.6%)
17 isolates were resistant to penicillin, out of which 95 (32.2%) were β -lactamase positive and
18 10 (3.4%) were having chromosomally mediated resistance. Out of remaining 190 (64.4%)
19 isolates, 156 (52.9%) had reduced susceptibility to penicillin and 34 (11.5%) were
20 susceptible to penicillin.
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23 **Ciprofloxacin**

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

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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 2,3).

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).

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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.

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
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 coefficient (r)
	Minor	Major	Very major	Complete	Essential	
	Penicillin	37	0	0	87.5	
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 ($\geq 90\%$) 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.

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9 The original purpose of this study was to assess reliability and comparability of the CDS and
10 CLSI method to predict the interpretative categories of susceptibility as compared with
11 standard Etest method. However, because all test procedures were done from ~~a single~~the
12 same inoculum and were carefully controlled, we had a unique opportunity to compare the
13 two methods with Etest method which was used as ~~gold-reference~~ standard and the Etest
14 MIC values were supported by satisfactory Quality Control (QC) measures. Protocols in both
15 the disc diffusion techniques differ in their choices of test medium, antibiotic disc content,
16 and interpretative breakpoint criteria. Zones of inhibition delineating susceptible, less
17 susceptible and resistant isolates from disc diffusion procedures must correlate with
18 corresponding MICs. In the present study, CDS results correlation was excellent with Etest
19 MICs which had less number of major and minor category discrepancies in comparison to
20 CLSI technique i.e. 2 and 52 versus 16 and 133. Complete percent agreement of CDS with
21 Etest was 82.0% while for CLSI method it was 49.5% which was very poor agreement. CDS
22 method showed excellent correlation coefficient ($r = 1$) with Etest for all five antimicrobial
23 agents tested in comparison to CLSI ($r = 0.92$). Previously study of our centre had shown
24 96.9 percent agreement for ceftriaxone for comparison of CDS with Etest technique.[13]
25 Interpretation of disc inhibition zones in CDS method was easier than in the CLSI technique
26 especially when the zone size was near the breakpoint. Double zone of inhibition was
27 observed many times in CLSI technique leading to difficulty in interpretation.
28 In an another study from WHO Collaborating Centre for STD in Australia and Sweden, the
29 correlation between results of CDS disc diffusion testing (cefpodoxime), and MIC
30 determinations with agar dilution and Etest using cefpodoxime, cefixime and ceftriaxone for
31 the detection of decreased ESC susceptibility in *N. gonorrhoeae* was investigated.[14] CDS
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9 technique using cefpodoxime 10 µg disc was shown to provide high sensitivity for detection
10 of gonococcal isolates with decreased ESC susceptibility and it was suggested that this disc
11 test will make it possible to provide AMR surveillance data also from less-developed and/or
12 less-resourced settings, where disc testing is the only practical and affordable means of AMR
13 testing.[14]

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18 Our findings reveal poor correlation between CLSI disc diffusion results and Etest MICs,
19 most notably for tetracycline, penicillin and ciprofloxacin. Further, if tetracycline were
20 removed from the CLSI analysis, then the correlation coefficients between the two methods
21 and the Etest were identical. It is the reported in the literature that there are problems on the
22 reproducibility of results with tetracycline, especially when different media are used.
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28 Excellent correlation was observed for ceftriaxone and spectinomycin, reason being that
29 resistance was not reported for these antimicrobial agents in the present study. Emergence of
30 resistance to both these antibiotics may lead to poor agreement between CLSI and Etest. Our
31 results compared well with that from China,[~~21~~20] where poor per cent agreement was
32 reported between the CLSI and agar dilution technique for MIC testing. It was 73.6%, 72.3%
33 and 71.4% for ciprofloxacin, penicillin and ceftriaxone respectively. Given the disparity
34 among susceptibility test results presented here, errors associated with susceptibility testing
35 may result in the unwarranted utilization or elimination of these antibiotics as part of possible
36 treatment regimens.

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45 The results of our study suggest that CDS technique could be reliably used in resistance
46 surveillance programmes for public health purposes and it can be recommended for use by all
47 the focal point laboratories in WHO GASP network in SEAR because of its excellent
48 agreement with Etest results and also it was simple, cost-effective and results are easier to
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9 interpret. In many developing countries in SEAR and other regions, most afflicted by
10 gonorrhoea and AMR, MIC determination using agar dilution method or Etest is not readily
11 accessible or affordable. Especially in those countries, the availability of a sensitive, rapid,
12 inexpensive, easily performed and effective disc test can be highly valuable. Use of a
13 standardized agar diffusion method is practical in these situations and allows fast and
14 reproducible results for clinical microbiology laboratories if standards are observed.[22+]
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16 To conclude, this is the first study to compare CDS and CLSI disk diffusion method with
17 Etest and the CDS technique yielded excellent category agreement results when compared
18 with the Etest. The data obtained in the present study suggest that the CDS technique is an
19 accurate alternative method for susceptibility testing of *N. gonorrhoeae* for various
20 antimicrobial agents. It is much less cumbersome than the current reference method because
21 of its simplicity, less consumption of media and glassware and is a more appropriate
22 technique in settings with minimal microbiological resources. CDS offers the advantage for
23 those laboratories that process small numbers of specimens and these laboratories could
24 determine the susceptibilities of gonococcal isolates reasonably accurately. This could
25 facilitate direct and meaningful comparison of resistance data generated within different
26 national and international laboratories.
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REFERENCES

- 1 Bauer AW, Kirby WM, Sherris JC, *et al.* Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966;**45**:493-6.

- 1
2
3
4
5
6
7
8
9 2 National Committee for Clinical Laboratory Standards. Performance standards for
10 antimicrobial disk susceptibility test. *Tentative standard, M2-T4*, 1988;vol 8. National
11 Committee for Clinical Laboratory Standards, Villanova, Pa.
12
- 13
14 3 National Committee for Clinical Laboratory Standards. Methods for dilution
15 antimicrobial susceptibility test for bacteria that grow aerobically. *Tentative standard,*
16 *M7-T2*, 1988; vol. 8. National Committee for Clinical Laboratory Standards,
17 Villanova, Pa.
18
19
- 20
21 4 Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Report of an international
22 collaborative study. *Acta Pathol Microbiol Scand* 1971;**217** Sect. B Suppl:1-90.
23
24
- 25
26 5 Bell SM. The Calibrated Dichotomous Sensitivity (CDS) disc method of antibiotic
27 sensitivity testing. *Pathology* 1975;**7** Suppl:1-48.
28
29
- 30
31 6 Bala M, Ray K, Gupta SM, *et al.* Changing trends of antimicrobial susceptibility
32 pattern of *Neisseria gonorrhoeae* in India and the emergence of ceftriaxone less
33 susceptible *N. gonorrhoeae* strains. *J Antimicrob Chemother* 2007;**60**:582-6.
34
35
- 36
37 7 Unemo M, Golparian D, Syversen G, *et al.* Two cases of verified clinical failures
38 using internationally recommended first-line cefixime for gonorrhoea treatment,
39 Norway, 2010. *Euro Surveill* 2010;**15**(47):pii=19721.
40
41
- 42
43 8 Ohnishi M, Golparian D, Shimuta K, *et al.* Is *Neisseria gonorrhoeae* initiating a
44 future era of untreatable gonorrhea? Detailed characterization of the first strain with
45 high-level resistance to ceftriaxone. *Antimicrob Agents Chemother* 2011;**55**:3538-45.
46
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2
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8
9 9 Sanchez ML, Barrett MS, Jones RN. The Etest applied to susceptibility test of
10 gonococci, multiple-resistant enterococci and Entobacteriaceae producing potent
11 beta-lactamases. *Diagn Microbiol Infect Dis* 1992;**15**:459–63.
12
13
14
15 10 Dyck EV, Smet H, Piot P. Comparison of E test with agar dilution for antimicrobial
16 susceptibility testing of *Neisseria gonorrhoeae*. *J Clin Microbiol* 1994;**32**:1586–8.
17
18
19 11 Yeung KH, Ng LK, Dillon JA. Evaluation of Etest for testing antimicrobial
20 susceptibilities of *Neisseria gonorrhoeae* isolates with different growth media. *J Clin*
21 *Microbiol* 1993;**31**:3053–5.
22
23
24
25 12 Bala M, Tapsall JW, Limnios A, *et al*. Experience with an external quality assurance
26 scheme for antimicrobial susceptibility testing of *Neisseria gonorrhoeae* in India,
27 2001–2007. *Epidemiol Infect* 2010;**138**:69–75.
28
29
30
31 13 Bala M, Ray K, Gupta S. Comparison of disc diffusion results with minimum
32 inhibitory concentration (MIC) values for antimicrobial susceptibility testing of
33 *Neisseria gonorrhoeae*. *Indian J Med Res* 2005;**122**:48–51.
34
35
36
37 14 Limnios A, Tapsall J, Kahlmeter J, *et al*. Cefpodoxime 10 µg disc screening test for
38 detection of *Neisseria gonorrhoeae* with mosaic PBP2 and decreased susceptibility to
39 extended-spectrum cephalosporins for public health purposes. *APMIS* 2011;**119**:356-
40 63.
41
42
43
44
45 15 Laboratory diagnosis of gonorrhoea. *WHO Regional Publication, South East Asia*
46 *series no 33*: World Health Organisation, Geneva, 1999.
47 <http://w3.whosea.org/book33/> [accessed 22 December, 2011]
48
49
50
51
52
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4
5
6
7
8
9 16 Bell SM, Pham JN, Fisher GT. Antibiotic susceptibility testing by the CDS method. *A*
10 *manual for medical and veterinary laboratories*. Fifth edition. 2009; P42-58.
11 <http://web.med.unsw.edu.au/cdstest> [accessed 22 December, 2011]
12
13
14
15 17 Clinical and Laboratory Standards Institute (CLSI). Performance standards for
16 antimicrobial susceptibility testing. Seventeenth Informational Supplement. *CLSI*
17 *document M100-S17*. Wayne, USA: Clinical and Laboratory Standards Institute 2007.
18
19
20
21 18 [Unemo M, Fasth O, Fredlund H, et al. Phenotypic and genetic characterization of the](#)
22 [2008 WHO Neisseria gonorrhoeae reference strain panel intended for global quality](#)
23 [assurance and quality control of gonococcal antimicrobial resistance surveillance for](#)
24 [public health purposes. *J Antimicrob Chemother* 2009;**63**:1142–51.](#)
25
26
27
28
29 19. U.S. Food and Drug Administration. Class II Special Controls Guidance Document:
30 Antimicrobial Susceptibility Test (AST) Systems. U.S. Department of Health and
31 Human Services Food and Drug Administration, Center for Devices and Radiological
32 Health. Issued March 5, 2007.
33 [http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocum](http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080187.htm)
34 [ents/ucm080187.htm](http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080187.htm) [accessed 28 December, 2011]
35
36
37
38
39
40 ~~20~~¹⁹ Biedenbach DJ, Jones RN. Comparative Assessment of Etest for Testing
41 Susceptibilities of *Neisseria gonorrhoeae* to Penicillin, Tetracycline, Ceftriaxone,
42 Cefotaxime, and Ciprofloxacin: Investigation Using 510(k) Review Criteria,
43 recommended by the Food and Drug Administration. *J Clin Microbiol* 1996;**32**14-7.
44
45
46
47
48 21⁰ Guoming L, Qun C, Shengchun W. Resistance of *Neisseria gonorrhoeae* epidemic
49 strains to antibiotics: report of resistant isolates and surveillance in Zhanjiang, China:
50 1998 to1999. *Sex Transm Dis* 2000;**27**:115-8.
51
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8
9 | **22+** Jones RN, Gerlach EH, Koontz FP, *et al.* Development of *Neisseria gonorrhoeae* in
10 vitro susceptibility test methods for cefixime including quality control guidelines.
11
12 *Diagn Microbiol Infect Dis* 1990;**14**:383-8.
13
14
15
16
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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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Keywords:	Microbiology < BASIC SCIENCES, Diagnostic microbiology < INFECTIOUS DISEASES, PUBLIC HEALTH

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15 **Vikram Singh, Manju Bala, Monika Kakran and V. Ramesh**
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45 Antimicrobial Surveillance Programme.
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48 **Running title:** Comparison of CDS, CLSI, Etest for susceptibility testing of *N. gonorrhoeae*
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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~~ used to 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

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3 to CLSI ($r = 0.92$). It was very poor ($r = 0.61$) by CLSI method for tetracycline. The
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5 correlation coefficients between the two methods and the Etest were identical if tetracycline
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7 was removed from the CLSI analysis.
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10 **Conclusions** The CDS technique is an attractive alternative for *N. gonorrhoeae* susceptibility
11 testing and is recommended for monitoring the antimicrobial susceptibility in less developed
12 and resource poor settings to facilitate enhanced antimicrobial resistance (AMR) surveillance
13 when the WHO Gonococcal Antimicrobial Surveillance Programme is undergoing expansion
14 to meet the ongoing challenges of surveillance and control of gonococcal AMR.
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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

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3 the antibiotic susceptibility of micro-organisms using agar disc diffusion and was developed
4 in 1969 by Sydney M. Bell assisted by members of the Department of Microbiology, The
5 Prince of Wales Hospital, Sydney, Australia. It was introduced into laboratory practice in
6 Australia in an effort to correct the unsatisfactory results of antibiotic susceptibility testing in
7 Australia that were obtained from surveys conducted by The Royal College of Pathologists
8 of Australia in the late 60's and early 70's.[5]
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18 The recent emergence of *N. gonorrhoeae* isolates with decreased susceptibility and resistance
19 to the currently recommended treatment guidelines of the Centers for Disease Control and
20 Prevention, including extended-spectrum cephalosporins (ESC), has further established the
21 necessity for a standardized, reliable, economical, less labor intensive and reproducible
22 susceptibility testing method.[6-8] Previous studies with the Etest determined that results
23 correlated well with the reference agar dilution method and that it is a useful guide for
24 determining chemotherapy against many organisms,[9] including gonococci.[9-11] The CDS
25 disc diffusion technique is recommended by WHO Collaborating Centre for STD, Sydney,
26 Australia and was regarded as the only practical and affordable means of phenotypic
27 susceptibility testing.[12] It was found to be cost-effective and more feasible during an
28 external quality assurance scheme in routine diagnostic laboratories in developing countries
29 like India.[12] Earlier this method was evaluated in comparison to Etest for only three
30 antibiotics (ciprofloxacin, penicillin, and ceftriaxone) and that also on a limited number of
31 isolates.[13] Moreover, tetracycline and spectinomycin (an alternative drug of choice for
32 treatment of gonorrhea) were not tested in the earlier study of 2005. Recently, CDS technique
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56 oral ESCs using cefpodoxime disc;[14] and interpretation criteria for azithromycin by CDS
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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 were included in the study. The strains were consecutive and nonrepetitive. Methods used for isolation and identification of *N. gonorrhoeae* 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

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3 The inoculum was prepared from 18 to 24 hour pure culture on chocolate agar medium and a
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5 homogenized suspension was prepared in 5 ml of sterile saline solution and turbidity was
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7 adjusted to an equivalent of 0.5 McFarland standard. Same suspension was used for the
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9 following three methods of antimicrobial susceptibility testing within 15 minutes. All the
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11 following three susceptibility tests were run simultaneously on same day.
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14 15 *CDS disc diffusion technique*

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17 Antibiotic susceptibility testing of all the 295 *N. gonorrhoeae* isolates was performed by the
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19 CDS technique on chocolate agar plates (Columbia agar base, HiMedia Laboratories Pvt.
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21 Ltd., Mumbai, India) with low concentration antibiotic discs (Oxoid Basingstoke, UK). Six
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23 antibiotics with concentrations recommended i.e. penicillin (0.5 IU), tetracycline (10µg),
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25 ceftriaxone (0.5µg), ciprofloxacin (1µg), spectinomycin (100µg) and nalidixic acid (30µg)
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27 were used as per standard methodology.[16] The strains were interpreted as susceptible, less
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29 susceptible and resistant.[16] Nalidixic acid was used only to identify isolates less
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31 susceptible to ciprofloxacin and results of susceptibility to this antibiotic are not included.
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37 38 *CLSI disc diffusion method*

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40 Same inoculum was used for this method, which was performed using GC agar base (Oxoid
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42 Ltd., Basingstoke, Hampshire, England) with 1% isovitalax or vitamino growth supplement
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44 (Hi Media Laboratories Pvt. Ltd, Mumbai, India) with higher disc concentration (HiMedia
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46 Laboratories Pvt. Ltd., Mumbai, India) recommended by CLSI i.e. penicillin (10 IU),
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48 tetracycline (30µg), ceftriaxone (30µg), ciprofloxacin (5µg), spectinomycin (100µg) and
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50 nalidixic acid (30µg) were used.[17] The strains were defined as susceptible, less susceptible
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52 and resistant. [17]
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57 58 *Etest Minimum Inhibitory Concentration (MIC) determination*

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3 The MICs of all the isolates for penicillin, tetracycline, ciprofloxacin, spectinomycin, and
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5 ceftriaxone were determined by the Etest method (AB Biodisk, Solna, Sweden) on GC agar
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8 base with 1% isovitalex or vitamino growth supplement. The Etest was performed as
9
10 specified in the manufacturer's product package insert. The strains were defined as
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12 susceptible, less susceptible and resistant. [17] The Etest method was selected as the
13
14
15 reference method for comparison of results of CDS and CLSI [disc diffusion](#) techniques.
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18 **Control strains**

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

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49 Discrepancies were differentiated into three categories, minor ([susceptible 'S' interpreted as
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51 less sensitive 'LS', 'LS' as 'S' or resistant 'R' and 'R' as 'LS' involving the less susceptible
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53 category](#)), major (['S' interpreted as 'R' false susceptibility](#)) and very major (['R' interpreted as
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55 'S' false resistant](#)) which are defined by U.S. Department of Health and Human Services
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3 Food and Drug Administration.[19] Complete and essential percent agreement between the
4 reference and test method was evaluated. The complete percent agreement is the percentage
5 of isolates tested by the test method which gave the same category as those tested by the
6 reference method. The essential percent agreement value is the percentage of agreement
7 obtained between the reference and test method when minor discrepancies are ignored.[19]

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15 Pearson's correlation coefficient (r values) was generated for each antimicrobial agent
16 indexed by susceptibility test method. Statistical correlation result was considered perfect for
17 the correlation coefficient (r value) of 1.00, desirable for ≥ 0.90 and acceptable for ≥ 0.80 .
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25 RESULTS

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28 The incidence of susceptible, less susceptible and resistant isolates differed following the
29 performance of both the disc diffusion assays and Etest (Table 1). Table 2 and 3 show the
30 comparison of discrepancies and agreement of CDS and CLSI disc diffusion method with
31 Etest for five different antibiotics. In overall, the rates of discrepancies for different
32 antibiotics differed in both the CDS and CLSI technique on comparison to Etest method. On
33 comparison of CDS method with Etest the highest discrepancy rate was observed for
34 penicillin (8.5%) and lowest for spectinomycin (0) and overall complete agreement was
35 82.0%.
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47 ~~Highest discrepancy rate~~ While, ~~for with the~~ CLSI disc diffusion method ~~highest discrepancey~~
48 ~~rate~~ was detected for tetracycline (25.1%), ~~and It was~~ lowest for spectinomycin (0) and
49 overall complete agreement was 49.5%. A total no. of minor and major error was 133 and 16
50 respectively for CLSI disc diffusion metod while for CDS they were only 51 and 2
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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 ($MIC \geq 4$). On comparison of Etest method with CDS and CLSI [disc diffusion](#)

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3 methods, minor discrepancies were observed as 7.1% and 11.5% respectively ([Table 2 and](#)
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6 [3](#)). The complete agreement for both the CDS and CLSI was 92.9% and 88.5% respectively.

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8 No major and very major discrepancies occurred. Person's correlation coefficient was
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10 excellent ($r = 0.99$) for both the methods (Table 2 and 3).

11 12 **Ceftriaxone**

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14 Out of 295 isolates, 283 (95.9%) were susceptible and remaining 12 (4.1%) were observed to
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16 have decreased susceptibility for ceftriaxone by the Etest method (Table 1). On comparison
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18 of CDS and CLSI [disc diffusion](#) methods with the Etest method, 1.7% and 1.4% minor
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20 discrepancy were observed respectively ([Table 2 and 3](#)). The complete percent agreement for
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22 ceftriaxone was 98.3% and 98.6% for both the methods. Essential agreement for both the
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24 methods was 100% with the Etest method (Table 2 and 3). Pearson's correlation coefficient
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26 was perfect ($r = 1$) for both the methods.
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31 32 **Spectinomycin**

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34 All the isolates were susceptible to spectinomycin, by all the three methods (Table 1). This
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36 resulted in 100% of essential and complete agreement on comparison of the CDS and CLSI
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38 method with the Etest method (Table 2 [and 3](#)).
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41 42 **Tetracycline**

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44 Out of 295 isolates, 58 (19.7%) were Tetracycline-resistant *N. gonorrhoeae* (TRNG) while
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46 237 (80.3%) were Not-Tetracycline-resistant *N. gonorrhoeae* (N-TRNG) by the Etest method
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48 (Table 1). Out of 237 N-TRNG isolates, 116 (39.3%), 91 (30.8%) and 30 (10.2 %) were
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50 observed as susceptible, less susceptible and resistant respectively. On comparison of N-
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52 TRNG isolates by the CLSI [disc diffusion](#) technique with the Etest method, 5.4% and 19.7%
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54 major and minor discrepancies were found respectively ([Table 3](#)). The complete and essential
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3 percentage agreement for the CLSI technique was 74.9% and 94.6% respectively. By the
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5 CDS method 60 (20.3%) isolates were observed to be TRNG and 235 (79.7%) N-TRNG
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7 [\(Table 1\)](#). Only 2 (0.7%) isolates were found under major discrepancy on comparison of the
8
9 CDS method with Etest method. Both complete and essential percent agreement between
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11 CDS and Etest method was 99.3% [\(Table 2\)](#). Tetracycline demonstrated perfect correlation
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13 coefficient ($r = 1$) for CDS and Etest method on the basis of TRNG and N-TRNG category
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15 comparison [\(Table 2\)](#). However, for comparison of 80.3% N-TRNG isolates with Etest
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17 method, it was observed to be very poor ($r = 0.61$) by the CLSI [disc diffusion](#) technique
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19 [\(Table 2 and 3\)](#).
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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.

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'.

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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 coefficient (r)
	Minor	Major	Very major	Complete	Essential	
	Penicillin	37	0	0	87.5	
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 ($\geq 90\%$) 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

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3 compared with standard Etest method. However, because all test procedures were done from
4 the same inoculum and were carefully controlled, we had a unique opportunity to compare
5 the two methods with Etest method which was used as reference standard and the Etest MIC
6 values were supported by satisfactory Quality Control (QC) measures. Protocols in both the
7 disc diffusion techniques differ in their choices of test medium, antibiotic disc content, and
8 interpretative breakpoint criteria. Zones of inhibition delineating susceptible, less susceptible
9 and resistant isolates from disc diffusion procedures must correlate with corresponding
10 MICs. In the present study, CDS results correlation was excellent with Etest MICs which had
11 less number of major and minor category discrepancies in comparison to CLSI disc diffusion
12 technique i.e. 2 and 52 versus 16 and 133. Complete percent agreement of CDS with Etest
13 was 82.0% while for CLSI disc diffusion method it was 49.5% which was very poor
14 agreement. CDS method showed excellent correlation coefficient ($r = 1$) with Etest for all
15 five antimicrobial agents tested in comparison to CLSI ($r = 0.92$). Previously study of our
16 centre had shown 96.9 percent agreement for ceftriaxone for comparison of CDS with Etest
17 technique.[13] Interpretation of disc inhibition zones in CDS method was easier than in the
18 CLSI technique especially when the zone size was near the breakpoint. Double zone of
19 inhibition was observed many times in CLSI technique leading to difficulty in interpretation.
20 In an another study from WHO Collaborating Centre for STD in Australia and Sweden, the
21 correlation between results of CDS disc diffusion testing (cefpodoxime), and MIC
22 determinations with agar dilution and Etest using cefpodoxime, cefixime and ceftriaxone for
23 the detection of decreased ESC susceptibility in *N. gonorrhoeae* was investigated.[14] CDS
24 technique using cefpodoxime 10 μ g disc was shown to provide high sensitivity for detection
25 of gonococcal isolates with decreased ESC susceptibility and it was suggested that this disc
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3 test will make it possible to provide AMR surveillance data also from less-developed and/or
4 less-resourced settings, where disc testing is the only practical and affordable means of AMR
5 testing.[14]
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10 Our findings reveal poor correlation between CLSI disc diffusion results and Etest MICs,
11 most notably for tetracycline, penicillin and ciprofloxacin. Further, if tetracycline were
12 removed from the CLSI analysis, then the correlation coefficients between the two methods
13 and the Etest were identical. It is reported in the literature that there are problems on the
14 reproducibility of results with tetracycline, especially when different media are used.
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20 Excellent correlation was observed for ceftriaxone and spectinomycin, reason being that
21 resistance was not reported for these antimicrobial agents in the present study. ~~Emergence of
22 resistance to both these antibiotics may lead to poor agreement between CLSI and Etest.~~ Our
23 results compared well with that from China,[21] where poor per cent agreement was reported
24 between the CLSI and agar dilution technique for MIC testing. It was 73.6%, 72.3% and
25 71.4% for ciprofloxacin, penicillin and ceftriaxone respectively. Given the disparity among
26 susceptibility test results presented here, errors associated with susceptibility testing may
27 result in the unwarranted utilization or elimination of these antibiotics as part of possible
28 treatment regimens.
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43 The results of our study suggest that CDS disc diffusion technique could be reliably used in
44 resistance surveillance programmes for public health purposes and it can be recommended
45 for use by all the focal point laboratories in WHO GASP network in SEAR because of its
46 excellent agreement with Etest results and also it was simple, cost-effective and results are
47 easier to interpret. In many developing countries in SEAR and other regions, most afflicted
48 by gonorrhoea and AMR, MIC determination using agar dilution method or Etest is not
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3 readily accessible or affordable. Especially in those countries, the availability of a sensitive,
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5 rapid, inexpensive, easily performed and effective disc test can be highly valuable. Use of a
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7 standardized agar diffusion method is practical in these situations and allows fast and
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9 reproducible results for clinical microbiology laboratories if standards are observed.[22]

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11 To conclude, this is the first study to compare CDS and CLSI disk diffusion method with
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13 Etest and the CDS technique yielded excellent category agreement results when compared
14
15 with the Etest. The data obtained in the present study suggest that the CDS technique is an
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17 accurate alternative method for susceptibility testing of *N. gonorrhoeae* for various
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19 antimicrobial agents. It is much less cumbersome than the current reference method because
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21 of its simplicity, less consumption of media and glassware and is a more appropriate
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23 technique in settings with minimal microbiological resources. CDS offers the advantage for
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25 those laboratories that process small numbers of specimens and these laboratories could
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27 determine the susceptibilities of gonococcal isolates reasonably accurately. This could
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29 facilitate direct and meaningful comparison of resistance data generated within different
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31 national and international laboratories.
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9 analysis of data and preparation of manuscript. MK participated in data analysis and VR
10 collaborated in the writing of the manuscript.
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18 There is no additional data available.
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40 **REFERENCES**

- 41
42
43 1 Bauer AW, Kirby WM, Sherris JC, *et al.* Antibiotic susceptibility testing by a
44 standardized single disk method. *Am J Clin Pathol* 1966;**45**:493-6.
45
46
47
48 2 National Committee for Clinical Laboratory Standards. Performance standards for
49 antimicrobial disk susceptibility test. *Tentative standard, M2-T4*, 1988;vol 8. National
50 Committee for Clinical Laboratory Standards, Villanova, Pa.
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- 3 National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. *Tentative standard, M7-T2*, 1988; vol. 8. National Committee for Clinical Laboratory Standards, Villanova, Pa.
- 4 Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Report of an international collaborative study. *Acta Pathol Microbiol Scand* 1971;**217** Sect. B Suppl:1-90.
- 5 Bell SM. The Calibrated Dichotomous Sensitivity (CDS) disc method of antibiotic sensitivity testing. *Pathology* 1975;**7** Suppl:1-48.
- 6 Bala M, Ray K, Gupta SM, *et al.* Changing trends of antimicrobial susceptibility pattern of *Neisseria gonorrhoeae* in India and the emergence of ceftriaxone less susceptible *N. gonorrhoeae* strains. *J Antimicrob Chemother* 2007;**60**:582-6.
- 7 Unemo M, Golparian D, Syversen G, *et al.* Two cases of verified clinical failures using internationally recommended first-line cefixime for gonorrhoea treatment, Norway, 2010. *Euro Surveill* 2010;**15**(47):pii=19721.
- 8 Ohnishi M, Golparian D, Shimuta K, *et al.* Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea? Detailed characterization of the first strain with high-level resistance to ceftriaxone. *Antimicrob Agents Chemother* 2011;**55**:3538-45.
- 9 Sanchez ML, Barrett MS, Jones RN. The Etest applied to susceptibility test of gonococci, multiple-resistant enterococci and Entobacteriaceae producing potent beta-lactamases. *Diagn Microbiol Infect Dis* 1992;**15**:459-63.
- 10 Dyck EV, Smet H, Piot P. Comparison of E test with agar dilution for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*. *J Clin Microbiol* 1994;**32**:1586-8.

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59
60
- 11 Yeung KH, Ng LK, Dillon JA. Evaluation of Etest for testing antimicrobial susceptibilities of *Neisseria gonorrhoeae* isolates with different growth media. *J Clin Microbiol* 1993;**31**:3053–5.
- 12 Bala M, Tapsall JW, Limnios A, *et al.* Experience with an external quality assurance scheme for antimicrobial susceptibility testing of *Neisseria gonorrhoeae* in India, 2001–2007. *Epidemiol Infect* 2010;**138**:69–75.
- 13 Bala M, Ray K, Gupta S. Comparison of disc diffusion results with minimum inhibitory concentration (MIC) values for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*. *Indian J Med Res* 2005;**122**:48-51.
- 14 Limnios A, Tapsall J, Kahlmeter J, *et al.* Cefpodoxime 10 µg disc screening test for detection of *Neisseria gonorrhoeae* with mosaic PBP2 and decreased susceptibility to extended-spectrum cephalosporins for public health purposes. *APMIS* 2011;**119**:356-63.
- 15 Laboratory diagnosis of gonorrhoea. *WHO Regional Publication, South East Asia series no 33*: World Health Organisation, Geneva, 1999.
<http://w3.whosea.org/book33/> [accessed 22 December, 2011]
- 16 Bell SM, Pham JN, Fisher GT. Antibiotic susceptibility testing by the CDS method. *A manual for medical and veterinary laboratories*. Fifth edition. 2009; P42-58.
<http://web.med.unsw.edu.au/cdstest> [accessed 22 December, 2011]
- 17 Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. Seventeenth Informational Supplement. *CLSI document M100-S17*. Wayne, USA: Clinical and Laboratory Standards Institute 2007.

- 1
2
3
4 18 Unemo M, Fasth O, Fredlund H, *et al*. Phenotypic and genetic characterization of the
5
6 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality
7
8 assurance and quality control of gonococcal antimicrobial resistance surveillance for
9
10 public health purposes. *J Antimicrob Chemother* 2009;**63**:1142–51.
11
12
13 19. U.S. Food and Drug Administration. Class II Special Controls Guidance Document:
14
15 Antimicrobial Susceptibility Test (AST) Systems. U.S. Department of Health and
16
17 Human Services Food and Drug Administration, Center for Devices and Radiological
18
19 Health. Issued March 5, 2007.
20
21
22 [http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocum](http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080187.htm)
23
24 [ents/ucm080187.htm](http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080187.htm) [accessed 28 December, 2011]
25
26
27
28 20 Biedenbach DJ, Jones RN. Comparative Assessment of Etest for Testing
29
30 Susceptibilities of *Neisseria gonorrhoeae* to Penicillin, Tetracycline, Ceftriaxone,
31
32 Cefotaxime, and Ciprofloxacin: Investigation Using 510(k) Review Criteria,
33
34 recommended by the Food and Drug Administration. *J Clin Microbiol* 1996;**32**:14-7.
35
36
37
38 21 Guoming L, Qun C, Shengchun W. Resistance of *Neisseria gonorrhoeae* epidemic
39
40 strains to antibiotics: report of resistant isolates and surveillance in Zhanjiang, China:
41
42 1998 to1999. *Sex Transm Dis* 2000;**27**:115-8.
43
44
45 22 Jones RN, Gerlach EH, Koontz FP, *et al*. Development of *Neisseria gonorrhoeae* in
46
47 vitro susceptibility test methods for cefixime including quality control guidelines.
48
49 *Diagn Microbiol Infect Dis* 1990;**14**:383-8.
50
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52
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56
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