PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Comparative assessment of CDS, CLSI disc diffusion and Etest techniques for antimicrobial susceptibility testing of Neisseria gonorrhoeae: A six year study
AUTHORS	Bala, Manju; Singh, Vikram; Kakran, Monika; Ramesh, V.

VERSION 1 - REVIEW

REVIEWER	Jo-Anne R Dillon, PhD Professor, Department of Biology, College of Arts and Science Research Scientist, Vaccine and Infectious Disease Organization Vaccine and Infectious Disease Organization University of Saskatchewan 120 Veterinary Road Saskatoon, SK S7N 5E3 Canada There are no competing interests with the authors of this paper
REVIEW RETURNED	21-Mar-2012

THE STUDY	A more thorough statistical review is required to ascertain whether the analytical methods used are appropriate.
REPORTING & ETHICS	An ethics approval number is not provided.
GENERAL COMMENTS	The article by Singh et al compares the Etest, the CLSI disk diffusion assay and the calibrated dichotomous sensitivity (CDS) test to determine the susceptibilities of 295 Neisseria gonorrhoeae isolates to penicillin, tetracycline, ceftriaxone, ciprofloxacin and spectinomycin. The Etest was used as the gold standard method of comparison. This study concludes that the CDS method compared better to Etest results than did the CLSI method and is best used in resource poor settings. A comparative study such as this one is needed as there is great interest in the use of the CDS method. Methods: • Were the 295 isolates tested the total number collected during the period or the total number recovered after storage and subsequently tested? o No storage methods are listed and the method of subculture prior to antimicrobial susceptibility testing is also not indicated. • The discussion states that all test methods were done from a “single” inoculum while the methods state that it was the “same” inoculum. This should be clarified. Since the length of time an isolate is in suspension before inoculation is important, details about the method of inoculum preparation and the number of isolates tested per batch should be provided. The suspending solution should also be indicated.

	o Were all three susceptibility tests run simultaneously or were tests run on different days? • What company supplied the GCMB? For CLSI and Etest, was the same medium with the identical supplement (i.e. Isovitalex or vitamin growth supplement) used in testing the same isolate on eth same day? • When one isolate failed to grow adequately, how was duplicate testing handled for the other tests? • Where were the various antibiotic-containing discs purchased? • Since the control strains must have been tested for each method on multiple occasions, what was the reproducibility of their MICs with each method? • Descriptions of what complete and essential agreement means (in Tables 2 and 3) are not described in the methods. • In the summary section, the authors describe susceptibility testing to cefixime, cefpedoxime, gentamicin and azithromycin which is not reported. As results are not presented or discussed in the paper and represent testing using only one method, these comments should be omitted. The same applies to comments about the use of nalidixic acid (appears in methods section). Results and conclusions: • The results are a little confusing and repetitive. It would be better to discuss one Table at a time. • Some numbers are difficult to extrapolate; for example it is not clear where the number 8.5% discrepancy for penicillin and Etest comes from. • In interpreting Tables 2 and 3, the major conclusion is that, there are no “very major” discrepancies between either the CDS or CLSI methods or the Etest. Except for results with tetracycline, there are no major discrepancies either. That is, as compared to the Etest, both methods correctly labeled isolates as being properly resistant or susceptible. The term minor discrepancy (“involving the less susceptible category”) is where differences arise between the CLSI method and the CDS method as compared to the Etest. Thus the % agreement really references largely a single category of interpretation. Since interpretive MIC criteria are not given, it would be important to further analyze what exactly the minor discrepancies are and to discuss how serious this might be when interpreting break points. Further, if tetracycline were removed from the CLSI analysis, then the correlation coefficients between the two methods and the Etest are identical. This should be discussed as well as the problems reported in the literature on the reproducibility of results with tetracycline, especially when different media are used. • The authors separate TRNG from other isolates. Were TRNG isolates verified by tetM analysis? Or were they identified on the basis of Etest MIC? • The authors should analyze PPNG isolates and non-PPNG (Table 1) isolates separately as inoculum size can have a large effect on susceptibility for PPNG. • The authors note that the MIC method is the recommended gold standard for reference laboratories in most parts of the world and then proceed to critique the method (Discussion, line 5). It still remains the gold standard although it is recognized that Etest is better suited to smaller scale testing. The purpose of the study is not to critique the agar dilution method per se, which in any case was not evaluated, but rather to compare the CDS and CLSI disc diffusion methods as compared to Etest.

REVIEWER	Athena E. Limnios

	Senior Scientist in the NSW Neisseria Reference Laboratory and Quality Officer in the WHO Collaborating Centre for STD, Sydney, for the Western Pacific and South East Asia Regions. Microbiology Department, SEALS The Prince of Wales Hospital Barker Street, Randwick, NSW, Australia.
REVIEW RETURNED	05-Apr-2012

THE STUDY	Page8 Line 6:Specify in introduction it is 'Etest MIC' Page8 Line 48: specify which agar base was used in chocolate agar plates in CDS technique. This was specified for the CLSI and Etest diffusion techniques. Should an explanation be provided to indicate that the differences in the composition of the media used explain the outcome of reading of results? Page9 Line 26: Include "Etest" in this heading to distinguish Etest MIC from agar dilution MIC method. Page9 Line 44: include 'N. gonorrhoeae' in WHO reference strains C,G..... Quality Control: Authors mention in the discussion that all test procedures were carefully controlled. The Etest diffusion method was used as the reference standard to supply the MIC values to compare CDS and CLSI test results . Need to show that the Etest MIC values were supported by satisfactory Quality Control (QC) measures. A table could be included to show the values and/or interpretation of some WHO N. gonorrhoeae controls on the reference standard Etest medium and on the two disc diffusion tests being compared. Page9 Line 44: include 'N. gonorrhoeae' in WHO reference strains C,G..... Suggest authors include the following reference which specifies acceptable MIC values and categories of susceptibilty for the 2008WHO control panel of n. gonorrhoeae; Unemo, M., O. Fasth, H. Fredlund, A. Limnios, and J. Tapsall. 2009. Phenotypic and genetic characterization of the 2008 WHO Neisseria gonorrhoeae reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. Journal of Antimicrobial Chemotherapy (2009) 63:1142–1151. Page18 Line13: suggest 'reference standard' replace 'gold standard', as latter generally refers to agar dilution MIC method. Page7 Line 44 & Page17 Line34: Sentences too long Acknowledgements: page20 Line 37: A/Professor John W. Tapsall AM, Ms Athena E. Limnios Line 39: A/Professor Monica M. Lahra, WHO Collaborating Centre for STD,
GENERAL COMMENTS	This is a well constructed study over the 6year period from a low level resourced microbiology laboratory, where the Etest diffusion

	technique was the reference standard method to which CDS and CLSI disc diffusion techniques with their interpretive criteria were compared. This sets a positive direction for any laboratory to obtain valid AST data to provide epidemiologically sound surveillance of AMR in Neisseria gonorrhoeae. data.

REVIEWER	Janice Lo Consultant Medical Microbiologist Public Health Laboratory Services Branch Centre for Health Protection Department of Health Hong Kong SAR China No competing interests declared.
REVIEW RETURNED	31-Mar-2012

THE STUDY	The authors selected five antimicrobials for the current study, namely penicillin, tetracycline, ciprofloxacin, ceftriaxone and spectinomycin. However, the rationale for testing these five antimicrobials was not given. It is essential to base the study on the currently recommended empirical treatment in the locality. Neisseria gonorrhoeae has developed resistance globally to several antimicrobial agents such as penicillin, tetracyclines and quinolones. Literature search revealed that in India, the antimicrobial non-susceptibility rate of N. gonorrhoeae to penicillin, tetracycline and ciprofloxacin were 92.2%, 82.8% and 100% respectively (Sexually Transmitted Diseases 2012; 39: 188-190). Furthermore, as pointed out in the introduction section of the manuscript, a similar study as the current study has previously been undertaken and published for the agents penicillin, ciprofloxacin and ceftriaxone, where 198, 301 and 128 isolates respectively were tested (Indian Journal of Medical Research 2005; 122; 48-51). When compared with 295 isolates in the current study, the numbers in the previous study were not limited, as claimed in the introduction of the current manuscript. Since the agents currently recommended for empirical treatment of gonorrhoea are injectable or oral cephalosporins, it will be much more informative to evaluate an oral extended-spectrum cephalosporin (e.g. cefixime) for which the resistance mechanism is mainly the presence of the mosaic penA gene and which is different from the injectable agent ceftriaxone.
RESULTS & CONCLUSIONS	In the current study, although the authors found that the CDS method correlated better than the CLSI method with the Etest, no very major discrepancies were detected with the CLSI method. In particular, for the two agents still effective for treatment of gonorrhoea, ceftriaxone and spectinomycin, the correlation among all three methods was excellent. As mentioned above, a similar study has previously been undertaken and published for the agents penicillin, ciprofloxacin and ceftriaxone (Indian Journal of Medical Research 2005; 122; 48-51), with the similar finding that the CDS method, being comparable to MIC determination by the Etest, was suitable for use in resource-poor areas.
GENERAL COMMENTS	Antimicrobial susceptibility testing of Neisseria gonorrhoeae is a very important subject due to the propensity for the organism to become resistant to agents used for its treatment. Evaluating for a suitable susceptibility testing method in resource poor areas is worthwhile.

	Overall, the current study did not appear to yield additional findings to the previous study (Indian Journal of Medical Research 2005; 122; 48-51), in that the CDS method, being comparable to MIC determination by the Etest, was suitable for use in resource-poor areas. Without evaluation of an oral extended-spectrum cephalosporin, the current standard recommended empirical treatment for gonorrhoea, the study as presented in the current manuscript does not appear to merit publication as a full paper.

VERSION 1 – AUTHOR RESPONSE

Point-by-point response to reviewers' comments:

Reviewer: Jo-Anne R Dillon, PhD, Professor, Department of Biology, College of Arts and Science
Research Scientist, Vaccine and Infectious Disease Organization
Vaccine and Infectious Disease Organization
University of Saskatchewan
Canada

Comment 1: A more thorough statistical review is required to ascertain whether the analytical methods used are appropriate.
Answer: As suggested, statistical methods were reviewed again and were found to be appropriate.

Comment 2: An ethics approval number is not provided.
Answer: Ethical approval was not required as this study was on Neisseria gonorrhoeae isolated in the laboratory and not on human subjects. This was mentioned while submitting the manuscript.

Comment 3: The article by Singh et al compares the Etest, the CLSI disk diffusion assay and the calibrated dichotomous sensitivity (CDS) test to determine the susceptibilities of 295 Neisseria gonorrhoeae isolates to penicillin, tetracycline, ceftriaxone, ciprofloxacin and spectinomycin. The Etest was used as the gold standard method of comparison. This study concludes that the CDS method compared better to Etest results than did the CLSI method and is best used in resource poor settings. A comparative study such as this one is needed as there is great interest in the use of the CDS method.
Answer: The authors are thankful to the reviewer for appreciating the fact that this study was needed because of great interest in the use of the CDS method in resource poor settings.

Methods:

Comment 4: Were the 295 isolates tested the total number collected during the period or the total number recovered after storage and subsequently tested?
Answer: A total number of 295 Neisseria gonorrhoeae were isolated during the period of study and were tested by three methods immediately at the time of isolation. It was a prospective study as was mentioned under ‘Design’ section of Abstract.

Comment 5: No storage methods are listed and the method of subculture prior to antimicrobial susceptibility testing is also not indicated.

Answer: In routine, this laboratory is preserving the isolates at -70°C in 20% glycerol broth and using chocolate agar with Columbia agar base (HiMedia Laboratories Pvt. Ltd., Mumbai, India) for subculture. In addition, preservation by lyophilization was started in this lab in 2009. As it was a prospective study, method of storage and method of subculture from stored isolates were not mentioned in the manuscript.

Comments 6: The discussion states that all test methods were done from a “single” inoculum while the methods state that it was the “same” inoculum. This should be clarified. Since the length of time an isolate is in suspension before inoculation is important, details about the method of inoculum preparation and the number of isolates tested per batch should be provided. The suspending solution should also be indicated.
Answer: As suggested, necessary corrections and additions have been made in the revised manuscript. Number of isolates tested per batch was not fixed as the isolates were not tested from the stored ones. All fresh isolates were tested.

Comment 7: Were all three susceptibility tests run simultaneously or were tests run on different days?
Answer: All three susceptibility tests were run simultaneously on same day. Necessary additions have been made in the revised manuscript.

Comment 8: What company supplied the GCMB? For CLSI and Etest, was the same medium with the identical supplement (i.e. Isovitalex or vitamin growth supplement) used in testing the same isolate on the same day?
Answer: GCMB supplied by Oxoid Ltd., Basingstoke, Hampshire, England was used. Necessary additions have been made in the revised manuscript.
Yes, for CLSI and Etest, the same medium with identical supplement (i.e. Isovitalex or vitamin growth supplement) was used in testing the same isolate on the same day.

Comment 9: When one isolate failed to grow adequately, how was duplicate testing handled for the other tests?
Answer: Duplicate testing was repeated for all the three methods whenever the isolate failed to grow adequately.

Comment 10: Where were the various antibiotic-containing discs purchased?
Answer: The antibiotic discs for CDS method (low concentration discs) were from Oxoid Ltd., Wade road, Basingstoke, Hants, UK and were provided by WHO collaborating centre for STD, Department of Microbiology, The Prince of Wales Hospital, Sydney, Australia as mentioned in acknowledgement section. The antibiotic discs for CLSI were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Necessary additions have been made in the revised manuscript.

Comment 11: Since the control strains must have been tested for each method on multiple occasions, what was the reproducibility of their MICs with each method?
Answer: The reproducibility of control strains tested for Etest, CDS and CLSI method was >95%.

Comment 12: Descriptions of what complete and essential agreement means (in Tables 2 and 3) are not described in the methods.
Answer: 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. Necessary additions have been

made in the revised manuscript.

Comment 13: In the summary section, the authors describe susceptibility testing to cefixime, cefpedoxime, gentamicin and azithromycin which is not reported. As results are not presented or discussed in the paper and represent testing using only one method, these comments should be omitted. The same applies to comments about the use of nalidixic acid (appears in methods section).
Answer: As per earlier reviewer's comment i.e "Importantly, the study does not assess susceptibility testing for the oral cephalosporins (cefixime or cefpodoxime) which are in widespread use across the world to treat gonorrhoea and gentamicin or azithromycin either, which are the other drugs which may be potentially used alone or in combination to treat cephalosporin resistant gonococci in the future", this limitation "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" was included in the 'Strengths and limitations' section of 'Summary' of this study. Its results were not presented or discussed in the paper as comparison of three techniques for cefixime, cefpedoxime, gentamicin and azithromycin could not be carried out. We may kindly be allowed to retain this as limitation because Reviewer 2 has also commented that "it will be much more informative to evaluate an oral extended-spectrum cephalosporin (e.g. cefixime)".
Quinolone testing in CDS technique is performed using a combination of both ciprofloxacin and nalidixic acid (ref 16). Susceptibility to nalidixic acid was not reported as it was used in the CDS technique to determine less susceptible isolates to ciprofloxacin.

Results and conclusions:

Comment 14: The results are a little confusing and repetitive. It would be better to discuss one Table at a time.
Answer: In first paragraph of 'Results' section, Table 1 has been described first followed by Table 2 and 3. Even under each antibiotic, Table 1 has been discussed first. Some changes have been made in the revised manuscript.

Comment 15: Some numbers are difficult to extrapolate; for example it is not clear where the number 8.5% discrepancy for penicillin and Etest comes from.
Answer: In Table 2, complete % agreement for penicillin was 91.5%. The number 8.5% discrepancy mentioned for penicillin is 100-91.5%.
Comment 16: In interpreting Tables 2 and 3, the major conclusion is that, there are no "very major" discrepancies between either the CDS or CLSI methods or the Etest. Except for results with tetracycline, there are no major discrepancies either. That is, as compared to the Etest, both methods correctly labeled isolates as being properly resistant or susceptible. The term minor discrepancy ("involving the less susceptible category") is where differences arise between the CLSI method and the CDS method as compared to the Etest. Thus the % agreement really references largely a single category of interpretation. Since interpretive MIC criteria are not given, it would be important to further analyze what exactly the minor discrepancies are and to discuss how serious this might be when interpreting break points. Further, if tetracycline were removed from the CLSI analysis, then the correlation coefficients between the two methods and the Etest are identical. This should be discussed as well as the problems reported in the literature on the reproducibility of results with tetracycline, especially when different media are used.
Answer: Agreed, there were minor discrepancies between the CLSI method and the CDS method as compared to the Etest and there were no "very major" discrepancies except for results with tetracycline.
As suggested, additions and changes have been made in the revised manuscript.

Comment 17: The authors separate TRNG from other isolates. Were TRNG isolates verified by tetM analysis? Or were they identified on the basis of Etest MIC?
Answer: TRNG isolates were identified on the basis of Etest MICs, CDS and CLSI interpretative criteria as shown in Table 1.

Comment 18: The authors should analyze PPNG isolates and non-PPNG (Table 1) isolates separately as inoculum size can have a large effect on susceptibility for PPNG.
Answer: It was mentioned in the results under section 'Penicillin', that 95 (32.2%) isolates 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. PPNG and non-PPNG were not included separately in the Table because of difficulty in making more columns in Table 1.

Comment 19: The authors note that the MIC method is the recommended gold standard for reference laboratories in most parts of the world and then proceed to critique the method (Discussion, line 5). It still remains the gold standard although it is recognized that Etest is better suited to smaller scale testing. The purpose of the study is not to critique the agar dilution method per se, which in any case was not evaluated, but rather to compare the CDS and CLSI disc diffusion methods as compared to Etest.
Answer: Agreed, agar dilution method still remains the gold standard. Necessary changes have been carried out in the revised manuscript.

Reviewer: Janice Lo
Consultant Medical Microbiologist
Public Health Laboratory Services Branch
Centre for Health Protection
Department of Health
Hong Kong SAR
China

Comment 1: The authors selected five antimicrobials for the current study, namely penicillin, tetracycline, ciprofloxacin, ceftriaxone and spectinomycin. However, the rationale for testing these five antimicrobials was not given. It is essential to base the study on the currently recommended empirical treatment in the locality. Neisseria gonorrhoeae has developed resistance globally to several antimicrobial agents such as penicillin, tetracyclines and quinolones. Literature search revealed that in India, the antimicrobial non-susceptibility rate of N. gonorrhoeae to penicillin, tetracycline and ciprofloxacin were 92.2%, 82.8% and 100% respectively (Sexually Transmitted Diseases 2012; 39: 188-190). Furthermore, as pointed out in the introduction section of the manuscript, a similar study as the current study has previously been undertaken and published for the agents penicillin, ciprofloxacin and ceftriaxone, where 198, 301 and 128 isolates respectively were tested (Indian Journal of Medical Research 2005; 122; 48-51). When compared with 295 isolates in the current study, the numbers in the previous study were not limited, as claimed in the introduction of the current manuscript. Since the agents currently recommended for empirical treatment of gonorrhoea are injectable or oral cephalosporins, it will be much more informative to evaluate an oral extended-spectrum cephalosporin (e.g. cefixime) for which the resistance mechanism is mainly the presence of the mosaic penA gene and which is different from the injectable agent ceftriaxone.

Answer: Agreed, it is essential to base the study on the currently recommended empirical treatment. That is why ceftriaxone and spectinomycin, two antibiotics still effective for treatment of gonorrhea are also included in the study. In the present study 295 isolates were tested for ceftriaxone in comparison to 128 isolates in the previous study and this number is significantly much more. Spectinomycin, an alternative drug of choice for treatment was not tested in the earlier study of 2005.
It is also agreed, the mechanisms of resistance to ceftriaxone may be different to oral cephalosporins. Information on comparison of Etest, CDS and CLSI could not be included in this article as we are carrying out susceptibility testing for cefixime, oral cephalosporin by CLSI method only and for cefpodoxime, another oral cephalosporin by CDS technique. Etest testing was not performed for both the antimicrobials because of limitation of resources. Moreover interpretive criteria for cefixime by CDS method are not available till now. Interpretive criteria for cefpodoxime by CDS technique were devised in 2009 and were not available during most of the study period. Therefore, comparison of disc diffusion techniques with MIC values could not be carried out. It was included in this study as a limitation "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" in the 'Strengths and limitations' section of 'Summary' of this study.
Necessary changes have been made in the revised manuscript.

Comment 2: In the current study, although the authors found that the CDS method correlated better than the CLSI method with the Etest, no very major discrepancies were detected with the CLSI method. In particular, for the two agents still effective for treatment of gonorrhoea, ceftriaxone and spectinomycin, the correlation among all three methods was excellent. As mentioned above, a similar study has previously been undertaken and published for the agents penicillin, ciprofloxacin and ceftriaxone (Indian Journal of Medical Research 2005; 122; 48-51), with the similar finding that the CDS method, being comparable to MIC determination by the Etest, was suitable for use in resource-poor areas.
Answer: The correlation among all three methods was excellent for the two agents still effective for treatment of gonorrhoea, ceftriaxone and spectinomycin, It was discussed in the present study that reason for this can be that resistance was not reported for these antimicrobial agents in the present study. This is under "Discussion' section on page 19 of the original manuscript and now page 20 of the revised manuscript. In the 2005 study, CLSI method was not compared with the Etest. Tetracycline and Spectinomycin (an alternative drug of choice for treatment) were not tested in the earlier study.
Necessary changes have been made in the revised manuscript.

Comment 3: Antimicrobial susceptibility testing of Neisseria gonorrhoeae is a very important subject due to the propensity for the organism to become resistant to agents used for its treatment. Evaluating for a suitable susceptibility testing method in resource poor areas is worthwhile.
Answer: The authors are thankful to the reviewer for appreciating the fact that evaluating for a suitable susceptibility testing method in resource poor areas is worthwhile and this was the main objective of the present study.

Comment 4: Overall, the current study did not appear to yield additional findings to the previous study (Indian Journal of Medical Research 2005; 122; 48-51), in that the CDS method, being comparable to MIC determination by the Etest, was suitable for use in resource-poor areas. Without evaluation of an oral extended-spectrum cephalosporin, the current standard recommended empirical treatment for gonorrhoea, the study as presented in the current manuscript does not appear to merit publication as a full paper.
Answer: Interpretive criteria by CDS method for cefixime, oral extended-spectrum cephalosporin are

not available till now. Interpretive criteria for cefpodoxime (another oral extended-spectrum cephalosporin) by CDS technique were devised in 2009 and were not available during most of the study period. Therefore, comparison of disc diffusion techniques with MIC values could not be carried out. In the present study 295 isolates were tested for ceftriaxone (injectable extended-spectrum cephalosporin), the current recommended empirical treatment for gonorrhoea in comparison to 128 isolates in the previous study and this number is significantly higher. Tetracycline and Spectinomycin (an alternative drug of choice for treatment) were not tested in the earlier study.
This is the first study in literature to determine the comparability of two disc diffusion techniques i.e. CDS and CLSI method with Etest MICs. Even Reviewer 1 has commented that “A comparative study such as this one is needed as there is great interest in the use of the CDS method.” Reviewer 3 has also commented that “This is a well constructed study over the 6year period from a low level resourced microbiology laboratory, where the Etest diffusion technique was the reference standard method to which CDS and CLSI disc diffusion techniques with their interpretive criteria were compared. This sets a positive direction for any laboratory to obtain valid AST data to provide epidemiologically sound surveillance of AMR in Neisseria gonorrhoeae. data.”In view of the comments of Reviewer 1 & 3, authors would appreciate if this manuscript can be considered for publication as a full paper.

Reviewer: Athena E. Limnios

Senior Scientist in the NSW Neisseria Reference Laboratory and Quality Officer in the WHO Collaborating Centre for STD, Sydney, for the Western Pacific and South East Asia Regions.
Microbiology Department, SEALS
The Prince of Wales Hospital
Barker Street, Randwick, NSW, Australia.

Comment 1: Page8 Line 6: Specify in introduction it is 'Etest MIC'
Answer: As suggested, necessary additions have been made in the revised manuscript.

Comment 2: Page8 Line 48: specify which agar base was used in chocolate agar plates in CDS technique. This was specified for the CLSI and Etest diffusion techniques.
Answer: Necessary additions have been made in the revised manuscript.

Comment 3: Should an explanation be provided to indicate that the differences in the composition of the media used explain the outcome of reading of results?
Answer: Agreed, the differences in the composition of the media used influence the outcome of results and this was discussed in the ‘Discussion’ section in line13-15 on page 18 of the original manuscript.

Comment 4: Page9 Line 26: Include "Etest" in this heading to distinguish Etest MIC from agar dilution MIC method.
Answer: Necessary addition has been carried out in the revised manuscript.

Comment 5: Page9 Line 44: include 'N. gonorrhoeae' in WHO reference strains C,G....
Answer: Necessary addition has been carried out in the revised manuscript.

Comment 6: Authors mention in the discussion that all test procedures were carefully controlled. The Etest diffusion method was used as the reference standard to supply the MIC values to compare CDS and CLSI test results. Need to show that the Etest MIC values were supported by satisfactory Quality Control (QC) measures.
Answer: Necessary changes have been carried out in the revised manuscript.

Comment 7: A table could be included to show the values and/or interpretation of some WHO N. gonorrhoeae controls on the reference standard Etest medium and on the two disc diffusion tests being compared.
Answer: A reference has been included in the revised manuscript for WHO N. gonorrhoeae control strains.

Comment 8: Page9 Line 44: include 'N. gonorrhoeae' in WHO reference strains C,G....
Answer: Necessary addition has been carried out in the revised manuscript.

Comment 9: Suggest authors include the following reference which specifies acceptable MIC values and categories of susceptibilty for the 2008WHO control panel of n. gonorrhoeae;
Unemo, M., O. Fasth, H. Fredlund, A. Limnios, and J. Tapsall. 2009. Phenotypic and genetic characterization of the 2008 WHO Neisseria gonorrhoeae reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. Journal of Antimicrobial Chemotherapy (2009) 63:1142–1151.
Answer: As suggested, this reference has been included in the revised manuscript.

Comment 10: Page18 Line13: suggest 'reference standard' replace 'gold standard', as latter generally refers to agar dilution MIC method.
Answer: Necessary changes have been carried out in the revised manuscript.

Comment 11: Page7 Line 44 & Page17 Line34: Sentences too long.
Answer: Necessary changes have been carried out in the revised manuscript.

Comment 12: Acknowledgements: page20
Line 37: A/Professor John W. Tapsall AM, Ms Athena E. Limnios
Line 39: A/Professor Monica M. Lahra,
WHO Collaborating Centre for STD,
Answer: Necessary changes have been carried out in the revised manuscript.

Comment 13: This is a well constructed study over the 6year period from a low level resourced microbiology laboratory, where the Etest diffusion technique was the reference standard method to which CDS and CLSI disc diffusion techniques with their interpretive criteria were compared. This sets a positive direction for any laboratory to obtain valid AST data to provide epidemiologically sound surveillance of AMR in Neisseria gonorrhoeae data.
Answer: The authors are thankful to the reviewer for appreciating the fact that this is a well constructed study from a low level resourced microbiology laboratory.

VERSION 2 – REVIEW

REVIEWER	Jo-Anne R Dillon, PhD Professor, Dept of Biology College of Arts and Science Research Scientist Vaccine and Infectious Disease Organization University of Saskatchewan 120 Veterinary Road

	Saskatoon, SK, S7N 5E3
REVIEW RETURNED	22-May-2012

THE STUDY	The attached file will explain inclusions that should be added to the abstract. A change in title for clarification is also requested. Many minor errors/omissions in grammar appear throughout the text.
RESULTS & CONCLUSIONS	The results could be more nuanced by clearly spearating tetracylcine results from the rest. Please see attached comments.
GENERAL COMMENTS	The paper by Vikram et al has considered all comments by reviewers and adequately answered these comments. However, a few modifications would strengthen and focus the article. 1. The title should be changed to comparative assessment of CDS and CLSI *disk diffusion* and Etest techniques…The CLSI agar dilution method is a gold standard method for the determination of the antimicrobial susceptibility of N. gonorrhoeae isolates. Therefore, the method evaluated should be clearly specified. 2. In following up comments from Dr Lo, the major antimicrobials which are tested for gonococcal susceptibility to-day are third generation cephalosporins and spectinomycin. In some jurisdictions, ciprofloxacin may be used providing that susceptibility testing is carried out to establish susceptibility. Thus, combining tetracycline results with the overall results skews the agreement between methods. Tetracycline susceptibility testing is no longer carried out in many areas. On page 20 of the discussion the authors have stated that "if tetracycline were removed from the CLSI analysis, then the correlation coefficients between the two methods are identical". This is important because, in fact, no difference between the two methods was found for antimicrobial agents presently recommended for treatment (i.e. ceftriaxone and spectinomycin). The above statement regarding the effect of the inclusions of tetracycline results on the data overall should appear in the abstract. The authors might also consider providing data in the tables when tetracycline is excluded from the analysis. 3. The statement on page 20 " emergence of resistance to (ceftriaxone and spectinomycin) may lead to poor agreement between the CLSI and Etest" is purely speculative and should be deleted. 4. The previous statement (included in point 3) alludes to a consistent error in the paper – the term CLSI must be defined as CLSI disc diffusion assay. Otherwise, this statement could be misinterpreted as CLSI agar dilution assay (a more reasonable comparison with Etest). This omission occurs in several places throughout the text. 5. The authors should include in the text a statement that that the reproducibility of control strains was greater than 95% for all methods. (Comment 11 for JRD review).

	6. Nalidixic acid is not mentioned in results and should be deleted from the methods section. 7. The results are sometimes hard to follow as the authors jump between the three tables, often without an appropriate reference to the relevant table. E.g. Page 12 line 14-15, etc. 8. The Tables should have a subscript which indicates the meaning of minor, major and very major for easier reading and reference.

REVIEWER	Athena Limnios Senior Hospital Scientist/ Quality Officer for EQA Programmes, WHO Collaborating Centre for STD, Microbiology Department, The Prince of Wales Hospital, Randwick, Sydney, NSW, 2035, AUSTRALIA.
REVIEW RETURNED	07-May-2012

THE STUDY	a couple of minor grammatical errors not corrected since the first review: P4 Line30 and P8 Line 29 A total of.....was (not were) included.... "total" the subject of sentence is singular P13 Line18 spelling of Pearson's

REVIEWER	Janice Lo Consultant Medical Microbiologist Public Health Laboratory Services Branch Centre for Health Protection Department of Health Hong Kong SAR China
REVIEW RETURNED	03-May-2012

THE STUDY	Q.12: Not applicable, as no supplemental documents given.
RESULTS & CONCLUSIONS	The authors did not specifically address the previous comment "However, the rationale for testing these five antimicrobials was not given. It is essential to base the study on the currently recommended empirical treatment in the locality." The authors did not give the rationale for choosing penicillin, tetracycline and quinolones for this study, and did not provide information on the standard recommended therapy for gonorrhoea in India, to support the choice of antimicrobial agents for the current study. The authors

	did not address the previous comment that "Literature search revealed that in India, the antimicrobial non-susceptibility rate of N. gonorrhoeae to penicillin, tetracycline and ciprofloxacin were 92.2%, 82.8% and 100% respectively (Sexually Transmitted Diseases 2012; 39: 188-190)." With such high resistance rates and the lack of clinical utility for these agents, efforts and resources would better be focussed on evaluation of antimicrobials of more clinical relevance. Of the two remaining agents evaluated in the current study, ceftriaxone has been addressed in an earlier publication (Indian Journal of Medical Research 2005; 122; 48-51, as mentioned in the previous comments). Although the current study comprised 295 isolates while the earlier study tested 128 isolates, there was no new finding from the current study. Only spectinomycin testing yielded novel findings. Nevertheless, the two disk diffusion susceptibility methods yielded excellent concordance, and the CDS method was not shown to be superior. Overall, the manuscript did not provide novel findings to the field in terms of practical applications.

VERSION 2 – AUTHOR RESPONSE

Point-by-point response to reviewers' comments:

Reviewer: Janice Lo
Consultant Medical Microbiologist
Public Health Laboratory Services Branch
Centre for Health Protection
Department of Health
Hong Kong SAR
China

Comment 1: The authors did not specifically address the previous comment "However, the rationale for testing these five antimicrobials was not given. It is essential to base the study on the currently recommended empirical treatment in the locality." The authors did not give the rationale for choosing penicillin, tetracycline and quinolones for this study, and did not provide information on the standard recommended therapy for gonorrhoea in India, to support the choice of antimicrobial agents for the current study. The authors did not address the previous comment that "Literature search revealed that in India, the antimicrobial non-susceptibility rate of N. gonorrhoeae to penicillin, tetracycline and ciprofloxacin were 92.2%, 82.8% and 100% respectively (Sexually Transmitted Diseases 2012; 39: 188-190)." With such high resistance rates and the lack of clinical utility for these agents, efforts and resources would better be focussed on evaluation of antimicrobials of more clinical relevance.

Answer: These five antimicrobials were tested because during the study period, antimicrobial susceptibility testing was being carried out by this centre for these five antibiotics as recommended by WHO Collaborating Centre for STDs, Sydney, Australia. Penicillin, tetracycline and quinolones were tested because of epidemiological reasons. Ciprofloxacin although not recommended for treatment in India, it may be used in some countries providing that susceptibility testing is carried out to establish susceptibility. This has been mentioned by the third reviewer too. We agree with the reviewer that it is essential to base the study on the currently recommended empirical treatment. That is why ceftriaxone and spectinomycin, two antibiotics still effective for treatment of gonorrhea are also included in the study. In India, according to National management guidelines, syndromic management (cefixime and azithromycin) is being carried out for urethral discharge and cervical discharge cases.

Information on comparison of Etest, CDS and CLSI for cefixime and azithromycin could not be included in this article as we are carrying out susceptibility testing for cefixime, oral cephalosporin by CLSI method only and for cefpodoxime, another oral cephalosporin by CDS technique. Etest testing was not performed for both the antimicrobials because of limitation of resources. Moreover interpretive criteria for cefixime by CDS method are not available till now. Interpretive criteria for cefpodoxime by CDS technique were devised in 2009 and were not available during most of the study period. Interpretation criteria for azithromycin by CDS have been established in September 2011 and are not available by CLSI technique. Therefore, comparison of disc diffusion techniques with MIC values could not be carried out for cefixime and azithromycin. It was included in this study as a limitation. Considering the high percentage of resistance reported for penicillin, tetracycline and ciprofloxacin as mentioned by the reviewer in the article “Sexually Transmitted Diseases 2012; 39: 188-190” this centre has already started testing of antimicrobials of more clinical relevance such as gentamicin, cefixime and azithromycin in the year 2011.

Comment 2: Of the two remaining agents evaluated in the current study, ceftriaxone has been addressed in an earlier publication (Indian Journal of Medical Research 2005; 122; 48-51, as mentioned in the previous comments). Although the current study comprised 295 isolates while the earlier study tested 128 isolates, there was no new finding from the current study. Only spectinomycin testing yielded novel findings. Nevertheless, the two disk diffusion susceptibility methods yielded excellent concordance, and the CDS method was not shown to be superior.

Answer: Excellent correlation was observed for ceftriaxone and spectinomycin by all the susceptibility testing methods, reason being that resistance was not reported for these antimicrobial agents in the present study. Emergence of resistance to both these antibiotics may lead to poor agreement between the disc diffusion technique and Etest. This was discussed in the ‘Discussion’ section.

Reviewer: Athena Limnios
Senior Hospital Scientist/Quality Officer for EQA Programmes,
WHO Collaborating Centre for STD, Microbiology Department,
The Prince of Wales Hospital, Randwick,
Sydney, NSW, 2035, AUSTRALIA.

Comment 1: A couple of minor grammatical errors not corrected since the first review:
P4 Line30 and P8 Line 29
A total of.....was (not were) included....
"total" the subject of sentence is singular
P13 Line18 spelling of Pearson's
Answer: Necessary corrections have been made in the revised manuscript.

Reviewer: Jo-Anne R Dillon, PhD
Professor, Dept of Biology, College of Arts and Science
esearch Scientist, Vaccine and Infectious Disease Organization
University of Saskatchewan, 20 Veterinary Road
Saskatoon, SK

Comment : The results could be more nuanced by clearly separating tetracycline results from the rest.
Answer: Necessary corrections have been made in the revised manuscript.

The paper by Vikram et al has considered all comments by reviewers and adequately answered these comments. However, a few modifications would strengthen and focus the article.

Comment 1: The title should be changed to comparative assessment of CDS and CLSI disk diffusion and Etest techniques…The CLSI agar dilution method is a gold standard method for the determination of the antimicrobial susceptibility of N. gonorrhoeae isolates. Therefore, the method evaluated should be clearly specified.
Answer: As suggested, the title has been changed.

Comment 2: In following up comments from Dr Lo, the major antimicrobials which are tested for gonococcal susceptibility to-day are third generation cephalosporins and spectinomycin. In some jurisdictions, ciprofloxacin may be used providing that susceptibility testing is carried out to establish susceptibility. Thus, combining tetracycline results with the overall results skews the agreement between methods. Tetracycline susceptibility testing is no longer carried out in many areas. On page 20 of the discussion the authors have stated that "if tetracycline were removed from the CLSI analysis, then the correlation coefficients between the two methods are identical". This is important because, in fact, no difference between the two methods was found for antimicrobial agents presently recommended for treatment (i.e. ceftriaxone and spectinomycin). The above statement regarding the effect of the inclusions of tetracycline results on the data overall should appear in the abstract. The authors might also consider providing data in the tables when tetracycline is excluded from the analysis.
Answer: Necessary corrections have been made in the revised manuscript.

Comment 3: The statement on page 20 "emergence of resistance to (ceftriaxone and spectinomycin) may lead to poor agreement between the CLSI and Etest" is purely speculative and should be deleted.
Answer: As suggested, the above sentence has been deleted.

Comment 4: The previous statement (included in point 3) alludes to a consistent error in the paper – the term CLSI must be defined as CLSI disc diffusion assay. Otherwise, this statement could be misinterpreted as CLSI agar dilution assay (a more reasonable comparison with Etest). This omission occurs in several places throughout the text.
Answer: Necessary corrections have been made at several places throughout the text in the revised manuscript.

Comment 5: The authors should include in the text a statement that that the reproducibility of control strains was greater than 95% for all methods. (Comment 11 for JRD review).
Answer: As suggested, necessary additions have been made in the revised manuscript.

Comment 6: Nalidixic acid is not mentioned in results and should be deleted from the methods section.
Answer: Quinolone testing in CDS technique is performed using a combination of both ciprofloxacin and nalidixic acid (ref 16). Therefore, it won't be appropriate to delete it from 'Methods' section. Susceptibility to nalidixic acid was not reported as it was used in the CDS technique to determine less susceptible isolates to ciprofloxacin.

Comment 7: The results are sometimes hard to follow as the authors jump between the three tables, often without an appropriate reference to the relevant table. E.g. Page 12 line 14-15, etc.
Answer: As suggested, necessary additions have been made in the revised manuscript.

Comment 8: The Tables should have a subscript which indicates the meaning of minor, major and very major for easier reading and reference.
Answer: Necessary additions have been made in the revised manuscript.