Isolation and identification of multiple drug resistant Neisseria gonorrhoeae *from urethritis patients.*

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Abstruct:
Gonorrhea caused by *Neisseria gonorrhoeae* is one of the most common STIs and is a global health problem because of emerging antibiotic resistant strains that compromise the effectiveness of treatment. This study was carried out to determine the susceptibility and resistance of the most effective antibiotic to *Neisseria gonorrhoeae* isolated from urethritis patients. Thirty five patients with urethritis were included in this study. Bacterial isolates were identified by standard procedures resistance patterns were detected by disk diffusion test (DDT) and minimum inhibitory concentration (MIC). The minimum inhibitory concentration of all antibiotic used in this study were determined by an agar dilution method as complementary test to the previous sensitivity test to verify the rate of resistance. The results showed that *N. gonorrhoeae* were completely resistance to cephalexin, gentamicin and trimethoprim with high rate of resistance to rifampicin, doxycycline (84.21%), azithromycin (73.68%) and amikacine (68.42%) and moderate to low rate of resistance to ciprofloxacin (52.63%) and cefotaxime (42.1%). Result showed that *N. gonorrhoeae* isolates had high rate of sensitivity to levofloxacin and imipenem (94.73%) and 4 out of 19 isolate showed resistances to ceftriaxone with sensitivity rate 78.95%. The present study made to prove that there was only limited number of drugs effective against *N. gonorrhoeae*, and most probably in near future, if irrational use of antibiotic is not stopped the rate of resistance will increase.

Keywords: *Neisseria gonorrhoeae*, sexually transmitted diseases (STDs), disk diffusion test (DDT), minimum inhibitory concentration (MIC).

عزل وتشخيص البكتريا البنيه *Neisseria gonorrhoeae* المقاومة للمضادات الحيوية

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الخلاصة

يعتبر مرض السيلان الناجم عن الاصابة ببكتريا النيسيريه البنيه (*N.gonorrhoeae*) هو واحد من الامراض المنقولة بالاتصال الجنسي الاكثر شيوعا و يمثل مشكلة صحيه عالميه بسبب ظهور سلالات مقاومة للمضادات الحيوية التي تؤثر سلبا على فعاليه المضادات الحياتيه. أجريت هذه الدراسة لتحديد قابلية ومقاومة المضاد الحيوي الاكثر فعالية لنيسيريه السيلان المعزوله من مرضى التهاب الاحليل . أدرج خمسة و ثلاثين من المرضى الذين يعانون من التهاب الاحليل في هذه الدراسة. وقد تم تحديد العزلات البكتيرية ثم تم الكشف عن مقاومة هذه البكتريا للمضادات الحياتية عن طريق أختبار انتشار القرص (DDT) والحد الادنى من تركيز المثبط الادنى (MIC). تم تحديد الحد الأدنى للتراكيز المثبطة لجميع المضادات الحيوية المستخدمة في هذه الدراسة من خلال وسيلة نشر أجار كما تم استعمال اختبار الحد الادنى من تركيز المثبط للتحقق من معدل المقاومة. وقد تميزت النيسيرية البنيه كمقاومة أذا كان MIC اكبر من تركيز المثبط الادنى نقطة توقف في حين سيكون عرضة اذا كان أقل من نقطة توقف. أظهرت هذه الدراسة ازدياد معدل عزلات هذه البكتريا المقاومة للمضادات التقليدية كما تم تسجيل حالات مقاومة للادوية الحديثة.

الكلمات المفتاحية : بكتريا النيسيرية البنيه، الامراض المنقولة جنسيا، الكشف بواسطة اختبار الهلام، الحد الادنى من تركيز المثبط.

Introductions

Among etiological agents of treatable sexually transmitted diseases (STDs), *Neisseria gonorrhoeae* is considered to be most important because of emerging antibiotic resistant strains that compromise the effectiveness of treatment [1]. However, treatment options for gonorrhea are diminishing as *N. gonorrhoeae* have developed resistance to several antimicrobial drugs such as sulfonamides, penicillin, tetracyclines and quinolones. Antimicrobial resistance (AMR) surveillance of *N. gonorrhoeae* helps establish and maintain the efficacy of standard treatment regimens [2]. Gonorrhea caused by *N. gonorrhoeae* is one of the most common sexually transmitted infections and is a global health problem [3]. Undetected and untreated infections can lead to complications like pelvic inflammatory disease, ectopic pregnancy, tubal factor infertility, adverse pregnancy outcomes in females, testicular, prostate infections and infertility in males. Also, asymptomatic patients, unaware of their infection, may serve as a reservoir of infection to their partners [4]. A major contributing factor to the continued spread of gonococcal infections is the remarkable ability of *N. gonorrhoeae* to acquire resistance to antibiotics [5]. Over the last two decades; *N. gonorrhoeae* strains were developed high level of resistance against several antimicrobial agents in different countries [6]. The emergence of strains resistant to extended-spectrum cephalosporins, the antibiotics used as the first line treatment for uncomplicated gonococcal infections, is a serious concern worldwide as it may pose a problem in the management of gonorrhea [7]. The emergence and spread of resistance in *N. gonorrhoeae* has occurred mainly by the acquisition of new DNA via conjugation and transformation and determinants may be located on thechromosome or on extra chromosomal elements [8]. In contrast to plasmid-mediated resistance, chromosomal resistance often occurs incrementally; Chromosomal alterations can affect permeability and simultaneously reduce susceptibility to penicillin, tetracycline and macrolides [9]. For the penicillin and quinolones there are multiple resistance mechanisms, involving porins (uptake), efflux pumps and the cellular targets of these antibiotics [10]. High-level resistance to spectinomycin and aminoglycosides probably occurs by point mutations affecting their ribosomal target sites [3].

Materials and Methods

From April 2013 to March 2014, thirty five patients with urethritis were included in this study. All patients attended private clinical laboratories in Abu-Ghraib province and outpatient visitor to dermatology and venereology in Abu-Ghraib hospital, Baghdad. Urethral discharge from male was

collected by using cotton swab andspecific small swabs were inserted into the urethral canal if there is no discharge.Female samples were taken from vagina, endocervix and urethra; specimen was inoculated immediately into Amies transport medium and transferred to thelaboratory.

Samples were culture immediately on chocolate agar under 8 %CO_2 condition at 37°C for 24 hs. The isolated colony identified by gram stain and biochemical tests included oxidase test and sugar (glucose) fermentation test. Resistance patterns of *N. gonorrheae*to various antibiotics were determined by disk diffusion test (DDT) and minimum inhibitory concentration (MIC). The following antimicrobial discs were tested cephalexin (30µg), cefotaxime (30µg), ceftriaxone (30µg), amikacine (30µg), gentamycin (10µg), imipenem (10µg), tetracycline (30µg), co-trimoxazole (25µg), ciprofloxacin(5µg), levofloxacin (10µg), azithromycin (30µg), rifampicin (30µg), when the incubation was complete, the diameter of the inhibition zone around the disks was measured and compared with the break points of clinical laboratory institute (CLSI) [11]. The minimum inhibitory concentration was performed by a standard agar dilution method and has been applied for determination the lowest antibiotics concentration that inhibits growth of *N. gonorrhoeae*. Stock solutions of each antibiotic at concentrations of 10 mg/ml, 1mg/ml, and 0.1 mg/ml; then two fold dilutions from 0.5-512µg/ml for all antibiotics were prepared. Muller Hinton agar medium was prepared, sterilized by autoclaving, after cooling, 25 ml were added to each antibiotic container; the content mixed well and poured into petri dishes. The inoculum density was adjusted by using 0.5 McFarland standard tubes (1.5×10^8 colony-forming units/ml) and then 20 µl of each inoculum were spotted on the agar surface of Muller Hinton agar medium and incubated at 37°C for 24 h under CO_2 conditions [12].

Results

This study included 35 patients with profuse, scanty, moderate watery mucopurulent or purulent urethral or vaginal discharge they were 25 (71.42%) males and 10 (28.57%) females, the age ranged from 18-30 years. Nineteen strains of *N. gonorrhoeae* were identified (out of 35 patients samples) by using chocolate agar medium, oxidase test and glucose fermentation test. By the disc-diffusion methods, all isolates (n, 19) were tested for their sensitivity to cephalexin, cefotaxime, ceftriaxone, amikacine, gentamicin, imipenem, doxycycline, trimethoprim, ciprofloxacin, levofloxacin, azithromycin and rifampicin. The result showed that *N. gonorrhoeae* isolates were completely resistant to cephalexin, gentamicin and trimethoprim, high rate of

resistance to rifampicin and doxycycline (84.21%), azithromycin(73.68%) and amikacine (68.42 %) with moderate to low rate of resistance to ciprofloxacin (52.63%) and cefotaxime (42.1%). The results also showed that the isolates had high rate of sensitivity to levofloxacin and imipenem (94.73%). Furthermore, it was found that four isolates out of nineteen showed resistances to ceftriaxone (78.95%) (Table 1).

In current study, all *N. gonorrhoeae* isolates (n,19) were resistance to more than three patterns of antibiotic which suggest that strains were multiple drug resistance (MDR), a novel four ceftriaxone resistant isolates were identified, and one strain resistant to levofloxacin and imipenem (Table 1).

Minimum inhibitory concentration of *N. gonorrhoeae* isolates

The MIC of all antibiotic used in this study were determined by an agar dilution method as complementary test to the previous sensitivity test to verify the rate of resistance. *N. gonorrhoeae* was characterized as resistant if breakpoint was greater than MIC defined by clinical laboratory institute (CLSI) while it will be susceptible if break point was less than the MIC (Table 2).

Results of MIC revealed that highly resistant to cephalexin (MIC 512 µg/ml) and five isolates with MIC 256 µg/ml while remain isolates with MIC 128µg/ml; for trimethoprim and gentamicin. MIC determination showed that all the isolates were highly resistant to the antibiotic (MIC, 512µg/ml). When cefotaxime is considered, out of the eight resistant isolates two isolates were highly resistant (MIC, 512 µg/ml), one isolate with MIC 256 µg/ml and remain five isolates with MIC 128µg/ml. One isolate out of the four resistant to ceftriaxone showed an MIC32 µg/ml while the remaining three had MIC 16 µg/ml. For amikacine, nine isolates had MIC 512 µg/ml,one isolate with MIC 256 µg/ml, and three isolates with MIC 128 µg/ml. Only one isolate of *N. gonorrheae* exhibiting highly resistance to imipenem (MIC, 512 µg/ml). MIC of doxycycline showed that 13 out of 16 isolates resistance to this drug had MIC 512 µg/ml and remain (3 isolates) had MIC 256 µg/ml respectively. Determination of MIC of ciprofloxacin revealed that all 10 isolates resistances to ciprofloxacin had MIC 512 µg/ml. One levofloxacin resistant isolate with MIC 128 µg/ml. The MIC results of fourteen resistant isolates to azithromycin were eleven isolates had MIC 512 µg/ml; one isolate with MIC 256 µg/ml and two with MIC 128 µg/ml respectively. Out of sixteen rifampin resistant isolates fifteen isolates with MIC 512 µg/ml and only one isolate with MIC 128 µg/ml.

Discussion

Among the etiological agents of treatable STDs, *N. gonorrhoeae* stands out because of

the extent to which antibiotic resistance compromises the effectiveness of individual case management and resistance also affects control programs [13]. *N. gonorrhoeae* causes infections principally the urethra in men and the endocervix in women, although it may also infect extra genital mucosal sites. Genital infection in men usually presents with a urethral discharge, but silent infections are common in women case [14]. In the present study, antimicrobial resistance and multiple drug resistance were found in all *N. gonorrhoeae* isolates; and 100% were resistant to more than three antimicrobial agents. This finding was also observed by Tapsall *et al*. [4]. They reported that many *N. gonorrhoeae* isolates were multidrug- and extensively drug-resistant. Concurrent resistance of *N. gonorrhoeae* to a large number of antimicrobials was noted in Iraq after 1994 by Azhar A.F. impending an alarming signal [15].

The current study showed that all *N. gonorrhoeae* isolates presented similar resistance profile and they were fully resistant to cephalexin, gentamycin and trimethoprim. This result was compatible with study of Jo-anne *et al.* [16]. In a marked consistency with the results of Unemo *et al.* [17]. The resistance patterns of *N. gonorrhoeae* to several antibiotics, rifampicin (89.4%), doxycycline (84.2%), azithromycin (73.6%) and amikacine (68.4%). Fluoroquinolones showed a broad spectrum antimicrobial activity including activity against *N. gonorrhoeae* [18]. These drugs have been demonstrated to be effective in treating gonococcal urethritis which may explain the extensive use of these drugs for treating gonococcal infections and the emerging resistance. Moreover, the use fluoroquinolones to treat other community infections might also complicated the situation [18]. Our study revealed that 10 isolates (52.6%) were highly resistant to ciprofloxacinand had MIC 512 µg/ml and this observation was in line with the findings in most previous studies [19,20]. Regarding levofloxacin, a newly introduced therapeutic agent inIraq, 94.7 % of the isolates were sensitive to levofloxacin; the high sensitivity to levofloxacin might be attributed to the recent introduction of this agent for the treatment of gonococcal. *N. gonorrhoeae* developed resistance to multiple classes of β-lactams antimicrobial drug such as ceftriaxone, cefotaxime and imipenem and the emergence of new generation β-lactams resistant *N. gonorrhoeae* threatens effective disease control [21]. There were three sporadic reports of two ceftriaxone-resistant strains of *N. gonorrhoeae* in the past 5 years: H041 and F89. H041 was identified in only a single case involving a female sex worker in Japan in 2009 [22,23]. F89 was initially reported in France in 2010 in a man who had sex with men [22]. Subsequently, resistant strain was detected in Spain in two

homosexual men [23]. In Australia, ceftriaxone resistant *N. gonorrhoeae* was identified and called A8806 strain [24].In this study, we identified four novel ceftriaxone resistant isolates, one of these isolate had an MIC 32 µg/ml and the remaining three isolates with MIC 16 µg/ml. The emergence of ceftriax resistant of *N. gonorrhoeae* raises concerns for controlling gonorrhea and it was stated that it is due to the availability of ceftriaxone and their abuse by general public [25]. The result of the current study revealed high sensitivity to imipenem (94.7 %) and this might be explained by the high cost of the agent which limits its use.

Conclusion

Our study proved that there was only limited number of drugs effective against *N. gonorrhoeae* and most probably in near future, if irrational use of antibiotic is not stopped the rate of resistance will increase. Susceptibility testing should be carried out on all clinical isolates, and the empirical antibiotic treatment changed accordingly.

References

1. Cohen,M.S.(1998)Sexually transmitteddiseases enhance HIVtransmission: Nolongera hypothesis, LANCET,; 351(Suppl III), 5–7.

2. Chen, P., Roberts,M., and Elwell, L.P.(2008)High Prevalenceofmutationin thequinolonepersistence- determine region and MTRR lociin*Neisseriagonorrhea* isolatedin a tertiary hospitalinsouthernTaiwan, J. of American Society for Microbiology, 138:69–75

3. Tapsall,J.W. (2001) Antimicrobial resistance in Neisseria gonorrhoeae. WHO/CDS/CSR/DRS/2001.3 World Health Organization, Geneva, Switzerland:http://www.who.int/entity/drugresistance/Antimicrobial_resistance_in_Neisseria_gonorrhoeae.pdf

4. Tapsall, J.W.,Ndowa, F., Lewis,D.A. and Unemo,M. (2009)Meeting the public health challenge ofmultidrug-and extensivelydrug resistance Neisseria *gonorrhea.* Expert review antimicrobialInfectiontherapy J. 7:821–34.

5. Wang, B.,Xu, J.S., Wang,C.X.,MiZ.H.,Pu,Y.P and Hui, M.(2006)Antimicrobial susceptibility of *Neisseria gonorrhea* isolatedin Jiangsuprovince , China, withafocus on Fluoroquinolone resistance . J. Medical Microbiology 55:1251–5.

6. Stathi, M.,Flemetakis, A.,Mirlagou, V., Avgerinou, H.,Kyriakis,K.P., and Maniatis, A. N. (2006)antimicrobial susceptibility of Neisseria gonorrhea in Greece: data forthe years 1994 - 2004. J.ANTIMICROBlOGY CHEMOTHERPY 57:775–9.

7. Bala,M. and Sood, S. (2010)Cephalosporin resistance in*Neisseria gonorrhea*. J.Global INFECTSDISEASE 2(3), 284–290.

8. Brannigan,J.A.,Tirodimos,I.A.,Zhang,Q.Y.,Dowson,C.G and Spratt, B.G.(1990)Insertionof an extra amino acidis the main cause of the low affinity of penicillin –binding protein 2 in penicillin resistance strain of Neisseria gonorrhea . MOLECULAR MICROBIOLOGY J. 4:913–9.

9. Hagman,K.E., Pan, W., Spratt,B.G.,Bathazar, J.T., Judd,R.C., and Shafer W.M.(1995)Resistance of Neisseria gonorrhea Hydrophobic Agents is Modulated bythe MTRRCDE effluxsystem . Microbiology J. 41:611–22.

10. Ropp, P.A., Hum, O.M., Nicholas, R.A.(2002)Mutations in*PON*A, thegeneencoding penecilin pending protein 1, and a novel locus , *PEN*C, arerequired for high level Chromosomally mediated penicillin resistance in*Neisseria gonorrhea* . Antimicrobial agentsChemotherapy J. 46:769–77.

11. Clinical laboratory Standard Institute Performancestandards forantimicrobial susceptibilitytesting,[19 TH] Informational Supplement. CLSI Document, 2009; M100–S19. WAYNE (PA).

12. Wiegand, I.,Hilpert,K.,Hancock R.E.,(2008)Agarand broth diffusion methodstodetermine theminimalinhibitory concentration (MIC) ofantimicrobial substance. Jan;3(2), 163-175. availablefrom:www//:ptth.nature.com/nature protocols**.**

13. Vanessa, G., David,J.,Anuradha,R.and TAN,J.(2010)molecularanalysis ofantimicrobial resistance mechanisms in*Neisseria gonorrhea* isolatedfrom ontario, Canada. J.ofAmiricansocietyformicrobiology, 178-185.

14. Workowski, K.A.,berman,S.M., Douglas, J.M.(2008)Emergence antimicrobialresistance in*Neisseria gonorrhea*:Urgent needtostrengthenpreventionstrategies. Annual INTERNATIONAL MEDICAL J. 148:606–13.

15. Azhar AbdulFattah. Epidemiology and screening of plasmid content of penicillin- sensitive and resistant *Neisseria gonorrhoeae*.Microbiol. MSc. Thesis College of Medicine, Al-Nahrain University, Baghdad, Iraq. (1994).

16. Jo-Anne,Dillon ,S.;Rajinder,P. and Parti,S.(2015)Antibiotic resistance in Neisseria gonorrhoeae :Will Infections be Untreatable in the Future? Culture J. volume 25 number (1)

17. Unemo M., Golparian D., Nicholas R., Ohnishi M., and Gallay A.,(2012)high-level Cefixime-andCeftriaxone-resistant*Neisseria gonorrhea*in France: novel*PEN*A mosaicalleleinasuccessful internationalclonecauses treatment failure. Antimicrobial. Agents' chemotherapy J.56, 1273-80.

18. Hooper,D.C.and Wolfson, J.S. (1991)Fluoroquinoloneantimicrobial agents. J. of National ENGELAND MEDICIN 324, 384–394.

19. Linder J.A., Huang E.S., Steinman,M.A., Gonzales R., and Stafford,R.S.(2005)Fluoroquinoloneprescribing intheUnited States: 1995 TO 2002. AM. J. MED. 118, 259–68.

20. Perez-Losada,M., Crandall,K.A., Bash,M.C., Dan,M., Zenilman,J., and Viscidi R.P,(2007)distinguishing importation fromdiversificationofquinolone-resistant*Neisseria gonorrhea* bymolecular evolutionaryanalysis . BMC EVOL. BIOL. 7:84.

21. Fenton,K.A., Ison, C., Johnson,A.P., Rudd,E., Soltani, M., and Martin,I. (2003)Ciprofloxacinresistance in *Neisseria gonorrhea* inEngland and Walesin 2002. LANCET,361:1867–9.

22. Onnishi, M.(2011). Emerging Ceftriaxone- resistance*Neisseria gonorrhea,* Japan. Emerging infectious disease J. 17 (1), 40-42.

23. Ohnishi,M., Golparian, D., Shimuta,K., (2011)Is *Neisseria gonorrhea* initiatingafuture eraof untreatable gonorrhea? Detailed characterization of the first strain with high level - resistance to ceftriaxone. Antimicrobial. Agents chemotherapy J. 55, 3538-45.

24. Manzano,S.and Lacroix, L.E. (2014): A newmultidrug strain of*Neisseria gonorrhea* in Australia. The new England J.of medicine.1850-53.

25. Tomberg, J., Unemo,M., Ohnishi,M., Davies,C., and Nicholas,R.A. (2013):Identification of expanded –spectrum cephalosporinin the*PEN*A gene fromamino acids conferringhigh - levelresistance *Neisseria gonorrhea* strain H041. Antimicrobial. Agents chemotherapy J .57, 3029-36**.**

Table 1: Antibiotic sensitivity of different isolates of *Neisseria gonorrhea*

Isolate N=19	Cephalexin	Cefotaxime	ceftriaxone	Amikacine	gentamicin	imipenem	Doxycyline	Trimethoprim	ciprofloxacin	levofloxacin	azithromycin	rifampicin
1	R	S	S	R	R	S	R	R	R	S	R	R
2	R	S	S	R	R	S	R	R	R	S	R	R
3	R	S	S	S	R	S	R	R	S	R	R	R
4	R	S	S	R	R	S	S	R	R	S	S	R
6	R	R	R	S	R	S	R	R	R	S	R	S
18	R	R	S	S	R	S	R	R	R	S	R	R
19	R	S	R	R	R	S	R	R	R	S	R	S
13	R	S	S	R	R	S	R	R	S	S	R	R
14	R	S	S	R	R	S	S	R	S	S	R	R
15	R	S	R	R	R	S	R	R	S	S	R	R
20	R	S	S	R	R	S	R	R	S	S	R	R
21	R	S	S	R	R	S	S	R	S	S	R	R
22	R	R	S	R	R	S	R	R	R	S	R	R
26	R	R	S	S	R	S	R	R	R	S	R	S
29	R	R	S	R	R	S	R	R	S	S	S	R
30	R	R	R	R	R	S	R	R	R	S	S	R
31	R	R	S	S	R	S	R	R	R	S	S	R
32	R	S	S	R	R	R	R	R	S	S	S	R
35	R	R	S	S	R	S	R	R	S	S	R	R

R: Resistant

S: Sensitive

Table 2: serial two fold antibiotics dilution to determine the MIC using agar dilution method.

Antimicrobial concentration Mg/ml	Volume of antibiotic Stock solution (µl)	Final concentration When adding 25 ml agar
0.1	125	0.5
0.1	250	1
1	50	2
1	100	4
1	200	8
10	40	16
10	80	32
10	160	64
10	320	128
10	640	256
10	1280	512