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Characterization of *Neisseria gonorrhoeae* strains isolated from patients attending the Sexually Transmitted Diseases (STD) and gynaecology clinics in Yangon

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A total of 42 *N. gonorrhoeae* strains were isolated from 200 patients (76 men and 124 women) attending STD and gynaecology clinics in Yangon during 2006 to 2008. All the strains belonged to WI serogroup. On the basis of plasmid-mediated antimicrobial resistance, they were characterized into 14 (33.3%) penicillinase producing *N. gonorrhoeae* (PPNG) strains, 6 (14.3%) tetracycline resistant *N. gonorrhoeae* (TRNG) strains, 10 (23.9%) PP/TRNG strains and 12 (28.6%) quinolone resistant *N. gonorrhoeae* (QRNG) strains. The plasmid profiles revealed as all PPNG isolates carried a 4.4 MDa penicillinase plasmid and 3 PP/TRNG isolates carried both 4.4 MDa penicillinase plasmid and 2.6 MDa cryptic plasmid. The majority of the *N. gonorrhoeae* isolates were susceptible to azithromycin, cefixime, ceftriaxone and spectinomycin. High-level ciprofloxacin resistant strains (ciprofloxacin minimum inhibitory concentration ≥4 µg/ml) and strains possessing multidrug resistance to first-line antibiotics, prescribed in the WHO recommended treatment regimen for gonorrhoea, were found among the tested isolates. Precise characterization of Myanmar gonococcal isolates provides benefits for the management and control of gonococcal infection and also elucidates the molecular epidemiology of gonorrhoea to some extent in Myanmar.

INTRODUCTION

Gonorrhoea, a classical sexually-transmitted disease (STD) caused by the bacterium *Neisseria gonorrhoeae*, still remains one of the common STDs in developing countries. Approximately 62 million new cases of gonorrhoea occurred worldwide in 1999 [1]. There are some prevalence studies of gonorrhoea in Myanmar. A study carried out on 426 symptomatic women attending various clinics in Mandalay, showed that the prevalence of gonorrhoea was 2.7% in general clinics and 14.4% in STD clinics [2]. Mar Mar Nyein *et al*. reported 2.2% of gonorrhoea cases among 90 symptomatic women at the Central Women's Hospital, Yangon [3]. Thein Myint Thu *et al.* reported the prevalence of gonorrhoea was 3.8% in married women in a closed sub-urban military community [4]. The global health problem of gonorrhoea is also concerned with the development of antimicrobial resistance in *N. gonorrhoeae*. The worldwide prevalence of gonorrhoea and the emergence of antibiotic resistant *N. gonorrhoeae* reinforce the need for surveillance of its susceptibility to antibiotics commonly used for treatment. The antibiotics included in the currently recommended WHO regimen are ciprofloxacin, ceftriaxone, cefixime, azithromycin and spectinomycin. Trimethoprim/sulphamethoxazole and kanamycin are the drugs for the alternative regimen. A large portion of gonococcal isolates worldwide are now resistant to penicillin and tetracycline [5].

Antimicrobial resistance is widespread among the strains of *N. gonorrhoeae* and occur both as chromosomally mediated resistance to a variety of agents and as plasmid-mediated resistance to penicillin (penicillinase or betalactamase producing *N. gonorroeae*/

PPNG) and to tetracycline (tetracycline resistant *N. gonorrhoeae*/TRNG). PPNG were first isolated in 1976 in South-east Asia and TRNG emerged there in 1980. Fluoroquinolone resistant gonococci (QRNG) appeared in several Asian countries during the early 1990s [6].

A variety of typing methods for gonococci have been used to monitor the antibiotic resistant strains, to identify the characteristics of the organisms causing the outbreaks and to recognize the geographically predominant strains. Auxotyping, serotyping, plasmid analysis and genotyping such as DNA amplification fingerprinting, ribotyping, *opa* typing, pulse field gel electrophoresis and sequencing of specific gene have been used as epidemiological tools [7].

Precise characterization of *N. gonorrhoeae* can provide valuable information on the gonococcal strain population in the community and the emergence and spread of antibiotic resistant strains. Since no vaccine exists for *N. gonorrhoeae* infection due to lack of a suitable animal model and considerable data regarding antigenic variability of the bacterium, a better knowledge of the molecular epidemiology of gonorrhoea infection will contribute to effective prevention and control measures. Thus, an in-depth study on characteristics such as serotypes, plasmid profiles and antibiotic susceptibility pattern of Myanmar gonococcal isolates will greatly benefit the management and control of this problem and also elucidate the molecular epidemiology of gonorrhoea to some extent in Myanmar.

MATERIALS AND METHODS

Study design

A cross-sectional descriptive study

Study population and study area

This study was carried out on patients attending Central STD Clinic and gynaecology out-patient departments (OPDs) of Central Women's Hospital and Thingangyun Sanpya Hospital in Yangon, Myanmar. Informed consent was obtained from eligible patients. Total study population was 200 patients comprising 76 males and 124 females, 110 were STD clinic attendees comprising 76 males and 34 females and 90 were women attending gynaecology OPDs.

Inclusion criteria

- Patients presenting with urethral/vaginal discharge, urinary symptoms, inguinal lymphadenopathy and other STI symptoms
- Both sexes
- Patients aged 18 years and above
- Patients who gave informed consent

Exclusion criteria

- Female patients who had menstruation at the time of specimen collection

Study period

From September 2006 to August 2008

Specimen collection

Demographic and clinical data of eligible patients were recorded in a proforma. Three urethral swab specimens from male patients and three endocervical swabs from female patients were collected. Urethral swab specimens were taken at least one hour after the patient had urinated. One swab was applied on two glass slides for direct smears and a second swab was inoculated into Amies transport medium for culture. The specimens were transported in an ice box to the Bacteriology Research Division, Department of Medical Research (Lower Myanmar) within three hours.

Direct microscopic examination

Gram-stained smears were examined to detect gram-negative extracellular and intracelullar diplococci within polymorphonuclear leucocytes.

Primary isolation of bacteria by culture

The specimens collected in Amies transport medium were inoculated onto Modified Thayer Martin media and Chocolate agar and incubated at 35-36˚C in a humid atmosphere containing 3-7% carbon dioxide for

24-48 hours. Colonies likely to be gonococci were identified by gram stain, oxidase test, catalase test, superoxol test and carbohydrate degradation test [8].

Serogroping of isolated N. gonorrhoeae strains

The Phadebact monoclonal GC test kit (Boule Diagnostics, Sweden) including gonococcal reagent which contains murine monoclonal antibodies to Protein IA and IB bound to non-viable staphylococci indicating WI and WII/WIII serogroups, was used to determine the serotypes.

Antibiotic susceptibility testing

Disc diffusion test and the E test (epsilometer gradient agar diffusion test) were used to determine antibiotic susceptibility and minimum inhibitory concentration (MIC) of *N. gonorrhoeae* isolates.

Disc diffusion method

Antibiotic discs (Oxoid, Hampshire, England) containing pencillin G (10 IU), sulphamethoxazole/trimethoprim (25 µg), chloramphenicol (30 µg), tetracycline (30 µg), gentamycin (10 µg), kanamycin (30 µg), amikacin (30 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), cefixime (5 µg) and spectinomycin (100 µg) were used. The results were interpreted as susceptible, intermediate and resistant according to Clinical and Laboratory Standards Institute [9].

E test

The susceptibility as well as the minimum inhibitory concentration (MIC) of penicillin, ciprofloxacin, ceftriaxone and azithromycin was determined by using E test on 42 culture-confirmed *N. gonorrhoeae* isolates. Based on MIC values described by the manufacturer (AB Biodisk, Sweden), the isolates were interpreted as susceptible, intermediate and resistant. Both disc diffusion and E test were performed simultaneously with the same inoculums. Reference strain of *N. gonorrhoeae* ATCC 49226 was used for the quality control of disc diffusion and E test.

Detection of penicillinase - producing N. gonorrhoeae (PPNG) and tetracycline resistant N. gonorrhoeae (TRNG)

The isolated colonies were tested by chromogenic cephalosporin test using nitrocefin disc, BBL, USA, to detect penicillinase-producing *N. gonorrhoeae* (PPNG). Plasmid-mediated resistance to tetracycline (TRNG) was detected by an agar diffusion susceptibility test using a disc containing 30 µg of tetracycline. TRNG produce very small zone of inhibition or none at all. The corresponding MIC of TRNG is ≥16 µg/ml.

Analysis of plasmid profiles in antibiotic resistant N. gonorrhoeae isolates

Plasmid DNA from *N. gonorrhoeae* isolates was extracted by alkaline lysis method and subjected to plasmid analysis by agarose gel electrophoresis with ethidium bromide staining. The separated plasmid DNA bands were viewed by UV transilluminator. Plasmid profile was distinguished among drug resistant isolates [10].

Data analysis

Data entry and analysis were carried out by using SPSS version 11. Univariate analysis was done to describe distribution of *N. gonorrhoeae* infection among the study population according to sex, age, occupation and education level. The culture and smear-positive cases were presented in percentages. Drug sensitivity rates of culture-positive cases were also described in percentages. Plasmid profiles of drug resistant cases were analyzed according to the pattern of drug resistance.

Ethical consideration

Research and Ethical Committee, University of Medicine (1) approved the study.

RESULTS

Detection of N. gonorrhoeae infection among the study population

Of 200 specimens tested, *N. gonorrhoeae* was identified from 14 urethral swab speci-

mens from male patients and 28 endocervical swab specimens from female patients comprising a total of 42 isolates. Thus, overall detection rate of *N. gonorrhoeae* infection was found to be 21%.

Serotypes of isolated N. gonorrhoeae strains

All tested 42 *N. gonorrhoeae* isolates showed WI serogroup.

Antibiotic susceptibility pattern by disc diffusion method

N. gonorrhoeae isolates were resistant to penicillin (≅81%), tetracycline (83.3%), chloramphenicol (83.3%) and septrin (78.6%). They showed moderate resistance to injection form antibiotics such as gentamycin (52.3%), kanamycin (47.6%) and amikacin (42.8%). The isolates were sensitive to azithromycin (88.1%), cefixime (80.9%), ceftriaxone (>73%), spectinomycin (76.2%) and ciprofloxacin (>52%).

MIC values determined by E test

Anti-gonococcal activities of azithromycin, ciprofloxacin, ceftriaxone and penicillin by E test were expressed in range of minimum inhibitory concentrations as shown in Table 1. High-level ciprofloxacin resistance (MIC ≥4 µg/ ml) was seen in 12 (28.6%) out of 42 tested isolates.

Table 1. Range of MIC values of penicillin, ciprofloxacin, ceftriaxone and azithromycin determined by E test

Antibiotic (MIC range on E test strip)	Sensitivity pattern	No. of strains	MIC values of tested antibiotic (µg/ ml)
Azithromycin (0.016-256)	Susceptible	37	0.25-0.5
	Resistant	5	2-6
Ciprofloxacin (0.002-32)	Susceptible	22	0.032-0.064
	Intermediate	8	0.125-0.5
	Resistant	12	4-8
Ceftriaxone (0.002-32)	Susceptible	31	0.094-0.25
	Resistant	11	0.75-1.5
Penicillin (0.002-32)	Susceptible	3	0.064
	Intermediate	5	0.125-1
	Resistant	34	2-32

Multiple drug resistant N. gonorrhoeae strain

N. gonorrhoeae strains that were resistant to at least 2 or more antibiotics prescribed in the current treatment regimen namely: ciprofloxacin, ceftriaxone, cefixime, spectinomycin and azithromycin, were analyzed. A total of 7 multidrug resistant *N. gonorrhoeae* strains were detected among the drug resistant isolates. Two *N. gonorrhoeae* isolates were resistant to ciprofloaxin, ceftriaxone and spectinomycin, one isolate was resistant to ciprofloxacin, azithromycin and spectinomycin and four isolates were resistant to ciprofloxacin and ceftriaxone.

Distribution of multidrug resistant strains according to the gender is shown in Table 2. Four multidrug resistant strains were found in males and 3 multidrug resistant strains were found in females. All 7 patients were STD clinic attendees and 2 women were commercial sex workers.

Table 2. Drug resistance pattern of *N. gonorrhoeae* showing multiple resistance to antibiotics prescribed in current treatment regimen

Gender	Resistant antibiotics	No. of *N. gonorrhoeae* isolates	Sub - total	Total
Male	Cipro+ Ceftria+Spectino	1		
	Cipro+Azithro+Spectino	1	4	
	Cipro+Ceftria	2		7
Female	Cipro+Ceftria+Spectino	1		
	Cipro+Ceftria	2	3	

Cipro = Ciprofloxacin, Ceftria = Ceftriaxone,
Azithro = Azthrimycin, Spectino = Spectinomycin

Characterization of plasmid-mediated antibiotic resistant N. gonorrhoeae isolates

Characteristics of drug resistant isolates according to the following categories are shown in Table 3.

1. PPNG (beta-lactamase positive, tetracycline MIC ≤16 µg/ml)
2. TRNG (beta-lactamase negative tetracycline MIC ≥16 µg/ml)
3. PP/TRNG (beta-lactamase positive tetracycline MIC ≥16 µg/ml)
4. QRNG (ciprofloxacin MIC of ≥1 µg/ml)

Plasmid-mediated resistance to penicillin and/ or tetracycline was found in 30 (71.4%) of total 42 *N. gonorrhoeae* isolates. Twenty-

Table 3. Categories of antibiotic resistant *N. gonorrhoeae* isolates (n=42)

Categories	Criteria	No. (%) of *N. gonorrhoeae* isolates
PPNG	Beta-lactamase (+), tetracycline MIC ≤16 µg/ml	14 (33.3%)
PP/TRNG	Beta-lactamase (+), tetracycline MIC ≥16 µg/ml	10 (23.9%)
TRNG	Beta-lactamase (-), tetracycline MIC <16 µg/ml	6 (14.3%)
QRNG	Ciprofloxacin MIC of ≥1 µg/ml	12 (28.6%)

four *N. gonorrhoeae* isolates were found to be PPNG and 16 isolates were found to be TRNG. QRNG (quinolone resistant *N. gonorrhoeae* which showed ciprofloxacin MIC of ≥1 µg/ml) was detected by determination of MIC using E test. High-level ciprofloxacin resistance (MIC ≥4 µg/ml**)** was seen in 12 isolates.

Plasmid profiles among antibiotic resistant N. gonorrhoeae isolates

Plasmid analysis was carried out on 30 isolates exhibiting plasmid-mediated resistance to penicillin and/or tetracycline. All 14 PPNG *N. gonorrhoeae* isolates carried 4.4 MDa penicillinase plasmids and 3 PP/TRNG isolates carried both 4.4 MDa penicillinase plasmid as well as 2.6 MDa cryptic plasmid (Plate 1).

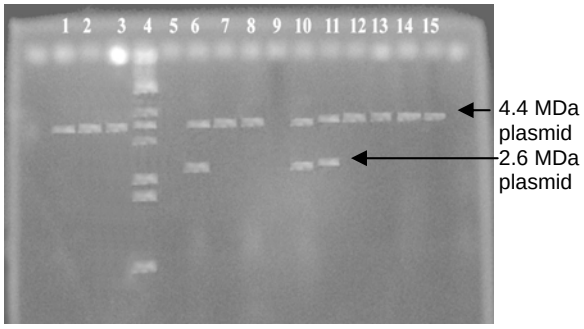

Plate 1. Plasmid analysis of *N. gonorrhoeae* isolates

Lane 1-3 - Positive samples showing 4.4 MDa plasmid
Lane 4 - Lambda HindIII DNA marker
Lane 5 - Negative control
Lane 6, 10 & 11 - Positive samples showing 4.4 MDa and 2.6 MDa plasmids
Lane 7-8 &12-15 - Positive samples showing 4.4 MDa plasmid

DISCUSSION

In the present study, 21% of the symptomatic patients were found to have gonococcal infection. The demographic data showed gonorrhoea patients were within the age of 24-42 years which is the reproductive age group. The people in this age group can transmit infection to sexual partners and newborns and they are also highly mobile group which can increase the spread of infection within the community.

The high infection rate was found among commercial sex workers, labourer and sedentary workers with low level of education. The similar finding was also seen in a study which stated core transmitters of gonorrhoea were of young age, low socio-economic status and high risk group for STDs eg. commercial sex workers [11].

All 42 *N. gonorrhoeae* isolates were typed as WI serogroup. WI serogroup contains protein IA, outer membrane protein. Although the present study had limitations to proceed to further serovar typing on isolated strains, the results obtained so far indicate the predominant serotype existing in our environment.

It was found that most isolates showed high resistance to oral antibiotics and moderate resistance to injection form antibiotics that were used in old STD treatment regimens. Most isolates were sensitive to antibiotics like azithromycin, ceftriaxone, cefixime and spectinomycin that are prescribed in the currently used STD treatment guidelines and >52% of the isolates were sensitive to ciprofloxacin which is a commonly used oral antibiotic in this treatment regimen.

In a report, 6.7% of the gonococcal isolates from STD patients in Hlinethaya Township in Yangon were found to be less sensitive to penicillin, but susceptible to ciprofloxacin and ceftriaxone [12]. The present study showed decreased susceptibility of gonococcal isolates to ciprofloxacin.

In 2007, CDC no longer recommended fluoroquinolones for the treatment of gono-

coccal infection and associated pelvic inflammatory diseases [13]. Recently, there were reports on occurrence of high-level resistance to ciprofloxacin (MIC ≥4 µg/ml) in Argentina, Israel and Taiwan [14]. In Myanmar, although there have been no reported high-level ciprofloxacin resistant cases previously, the present study showed that the emergence of high-level ciprofloxacin resistance (MIC ≥4 µg/ml) which accounts for 28.6% (12/42) of tested isolates.

Although quinolones such as ciprofloxacin are recommended as the first-line of therapy for gonorrhoea in developing countries, the emergence of significant resistance to ciprofloxacin and presence of high-level resistant strains will limit the usefulness of this drug.

Emergence of multidrug resistant strains of *N. gonorrrhoeae* is a serious threat in control of gonococcal infections. Wang *et al*. reported that clinical cases which showed multidrug resistance (penicillin, tetracycline and ciprofloxacin) and decreased susceptibility to cefixime occurred in Hawaii [15].

The present study was carried out cross-sectionally and all patients were out-patients, so the clinical outcome of multi-drug resistant cases cannot be determined. However, all multidrug resistant patients were STD clinic attendees including 2 commercial sex workers. If they were not treated properly and effectively, the multi-drug resistant strains can be transmitted to other persons and will circulate in the community. Studies of antibiotic resistant plasmids found in *N. gonorrhoae* can predict associated antibiotic resistant genes which can provide epidemiological data for outbreak analysis and transmission of antibiotic resistant genes in the regions.

A study in Bangladesh reported 23.4% PPNG among penicillin resistant isolates and 17.5% TRNG among tetracycline resistant isolates [16]. In a study in Thailand, all penicillin resistant *N. gonorrhoeae* isolates in Bangkok Hospital during 2000-2002 produced β lactamase [17]. There are limited studies on plasmid-mediated gonococcal resistance in Myanmar. Thida *et al*. reported that 45.5% PPNG, 59.1% TRNG, 36.4% PP/TRNG strains were observed among 22 *N. gonorrhoeae* strains isolated from Mandalay [18].

In the present study, isolated *N. gononor-rhoeae* strains from STD were categorized into 14 (33.3%) PPNG strains, 6 (14.3%) TRNG strains, 10 (23.9%) PP/TRNG strains and 12 (28.6%) QRNG strains. The findings from the present study and previous studies in Yangon and Mandalay indicate the occurrence of plasmid-mediated resistance among gonococcal isolates in Myanmar. The 4.4 MDa plasmid is the epidemic β lactamase plasmid which predominated in Asia and North America. The cryptic 2.6 MDa plasmid was found in 96% of clinical isolates of *N. gonorrhoeae*. These cryptic plasmids are not associated with the virulence of gonococcal strains, pilus production, or the pilin protein and their functions are still unknown [19].

In the present study, plasmid analysis was carried out on 30 isolates exhibiting plasmid-mediated resistance to penicillin and/or tetracycline. All 14 PPNG *N. gonorrhoeae* isolates carried a 4.4 MDa penicillinase plasmid and 3 PP/TRNG isolates carried both 4.4 MDa penicillinase plasmid and 2.6 MDa cryptic plasmid. When plasmid patterns were compared to the previous study in Mandalay, the plasmid profiles of *N. gonorrhoeae* isolates from Yangon and Mandalay were found to be different as isolates from Mandalay carried 25.2 MDa tetracycline resistant plasmid and 2.6 MDa cryptic plasmid. The present information on the plasmid patterns of gonococcal isolates in Myanmar provides preliminary baseline data.

In the present study, serotyping, determination of antimicrobial susceptibility pattern and plasmid analysis methods were used to characterize the isolated *N. gonor-rhoeae* strains. All the isolated strains belonged to serogroup WI and most of the antibiotic resistant strains exhibited plasmid-mediated antimicrobial resistance. The

present study also highlighted the occurrence of high-level ciprofloxacin resistant strains and multidrug resistant strains.

Further area of research on gonococcal infection including the elucidation of associated different plasmids and antibiotic resistant genes can provide epidemiological data for outbreak analysis and transmission of antibiotic resistant genes in the regions. Since no vaccine exists for *N. gonorrhoeae* infection, a better knowledge of molecular epidemiology of gonorrhoea will contribute to effective prevention and control measures.

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