Serological classification in relation to auxotypes, plasmid contents, and susceptibilities to antimicrobials of PPNG and non-PPNG strains isolated in Greece

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SUMMARY Thirty three penicillinase producing *Neisseria gonorrhoeae* (PPNG) and 152 non-PPNG strains were serologically classified in relation to their patterns of resistance to antimicrobials, nutritional requirements (auxotypes), and plasmid contents. Of the 185 strains, 65 (35%) belonged to the WI and 120 (65%) to the WII/III serogroup, the predominant serovars of which were Arostr and Bropt, respectively. Associations between serotype and susceptibility to antimicrobial agents, auxotype, and plasmid content were observed. Of the 152 non-PPNG strains, 112 (74%) belonged to serogroup WI. The WI non-PPNG strains were more sensitive to penicillin, thiamphenicol, tetracycline, and cefotaxime than the WII/III non-PPNG strains. Auxotyping and serogrouping the strains showed no differentiation other than that arginine, hypoxanthine, and uracil dependent (AHU⁻) strains belonged to serogroup WI. The proline, citrulline, and uracil dependent (PCU⁻) strains belonged, as expected, to serogroup WII/III. Analysing the 33 PPNG strains showed that of 21 carrying the 4.5 megadalton (Asian type) penicillin resistance plasmid, 17 (81%) belonged to serogroup WI, with serovar Arstr predominating, and only four (19%) to serogroup WII/III. Of 11 isolates carrying the 3.2 megadalton (African type) resistance plasmid, seven (64%) belonged to serogroup WI (serovar Av predominating) and four (36%) to WII/III (with serovar Bropt predominating). One strain carried the 3.05 megadalton penicillin resistance plasmid (Toronto type), belonged to the WI serogroup, and had serovar Arostr.

To obtain a better understanding of the epidemiology of gonococcal infections, reliable methods based on the different characteristics of strains of *Neisseria gonorrhoeae* are necessary. The discovery by Catlin that *N gonorrhoeae* strains could be divided into groups based on their nutritional requirements provided the first practical method for typing gonococci, which has become invaluable in epidemiological investigation.¹ Knowledge of the composition of the gonococcal outer membrane also led to the production of monoclonal antibodies specific for *N gonorrhoeae* and to its classification into WI and WII/III serogroups.²⁻⁵ Thus in any given geographical area or clinical situation a few dominant serovars, or reaction patterns, have been shown by these monoclonal antibodies,⁵ which are therefore

valuable epidemiological tools. Assessing the resistance of *N gonorrhoeae* to antimicrobials and studying its genetic basis is also important not only for successful treatment but also as an epidemiological marker.

These typing techniques have been invaluable in tracking microepidemics within countries as well as in monitoring the international dissemination of gonococcal strains, particularly penicillinase producing *N gonorrhoeae* (PPNG) strains⁴⁻⁶⁻⁸ and also those chromosomally resistant to antibiotics.⁹

The present study was undertaken to characterise the Greek PPNG and non-PPNG strains by auxotype, plasmid content, and serovar; to develop a data base for future comparisons; and to ascertain, if possible, the origin of certain strains.

Patients, materials, and methods

BACTERIAL STRAINS

Thirty three PPNG and 152 non-PPNG strains were isolated from men with acute urethritis who attended the Andreas Syngros venereal hospital as outpatients.

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As control strains for auxotyping and for analysing plasmid DNA, we used 1347 arginine requiring strains (auxotype Arg⁻) with the 3.2 Mdalton penicillin resistance plasmid and the 2.6 Mdalton cryptic plasmid and 1745 prototrophic (auxotype Zero) strains with the 24.5 Mdalton conjugative plasmid in association with the 4.5 Mdalton penicillin resistance plasmid and the cryptic plasmid. To measure minimum inhibitory concentrations (MICs) of antibiotics we used the five reference strains proposed by the World Health Organisation.¹⁰

We stored all strains at -70°C in heart infusion broth (Oxoid) supplemented with 20% (v/v) glycerol.

GROWTH AND IDENTIFICATION

We used selective and non-selective media and the substrates designated by Diagnostics Pasteur under appropriate conditions of temperature, humidity, and carbon dioxide.

MEASURING MIC

We used the agar dilution method with the non-selective medium provided by Diagnostics Pasteur.¹¹ The antibiotics used were penicillin (Sigma), spectinomycin (Upjohn), thiamphenicol (Clin-Midy), tetracycline (Hoechst), and cefotaxime (Roussel).

DETECTING BETA-LACTAMASE

We tested for β -lactamase activity using the chromogenic cephalosporin substrate, Nitrocefin (Oxoid).

AUXOTYPING

For auxotyping we used the chemically defined media and the technical procedures described by Catlin¹² and Hendry and Stewart.¹³

ELECTROPHORETIC ANALYSIS OF PLASMID DNA

We undertook agarose gel (0.7%, Beecham) electrophoresis of plasmid DNA according to Portnoy¹⁴ and partial DNA purification as described by Maniatis.¹⁵

SEROTYPING BY COAGGLUTINATION

We performed serological characterisation of the gonococcal strains by coagglutination using the Ph panel of monoclonal antibodies to protein I and the methods described by Bygdeman *et al.*² We thought that the Ph panel would be sufficient for our serological classification because the specificity of the two existing panels, GS and Ph, and the designation of specific serovars have already been compared.^{16,17} The serotyping procedure was as follows: whole cells of *N. gonorrhoeae* were suspended in phosphate buffered saline (PBS) and heated at 100°C for 30 minutes. One drop of coated staphylococci was mixed with an equal volume of boiled gonococci on a microtitre bioplate

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(Lindigö, Sweden), which was rocked gently for two minutes. The degree of agglutination was then noted. Control tests performed with uncoated protein A staphylococci were always included.

Results

Of our 185 gonococcal isolates examined, 65 (35.1%) were in the WI and 120 (64.9%) in the WII/III serogroup. Thirty three were PPNG strains, of which 25 (75.8%) belonged to the WI and eight (24.2%) to the WII/III serogroup. Of the 152 non-PPNG isolates, 40 (26.3%) were in the WI and 112 (73.7%) in the WII/III serogroups. The serovars are shown in table 1.

AUXOTYPES, SEROGROUPS, AND SEROVARs

The 185 PPNG and non-PPNG strains isolated were of 11 different auxotypes. Table 2 shows the relation between auxotypes and serogroups.

In the 152 non-PPNG strains, the Zero auxotype predominated (in 92) followed by the proline requiring (Pro⁻) auxotype in 18 strains. The serovars predominating in strains of the Zero auxotype were Arost in the WI and Bropt in the WII/III serogroups, whereas the Pro⁻ non-PPNG strains belonged almost exclusively (17/18) in the WII/III serogroup with serovar Bropt predominating; the only strain in the WI serogroup was of serovar Arst (data not shown in table 2). Of the 12 strains requiring arginine, hypoxanthine, and uracil (AHU⁻), eight (66.7%) belonged to serogroup WI (six of which were of serovar Arost) and four belonged to serogroup WII/III and Brpyut was the predominant serovar. In contrast, most (7/9) of the strains requiring proline, citrullin, and uracil (PCU⁻) were in serogroup WII/III and Brpyut was the predominant serovar; the remaining two were in serogroup WI and were of serovars Arost and Arst.

Table 1 Serogroups and serovars of 185 penicillinase producing *Neisseria gonorrhoeae* (PPNG) and non-PPNG strains (figures are numbers (percentages) of strains)

Serovars	Non-PPNG (n = 152)	PPNG (n = 33)	Total (n = 185)
Serogroup WI:	40 (26.3)	25 (75.8)	65 (35.1)
Arost	24 (60.0)	1 (4.0)	25 (38.5)
Arot	0	1 (4.0)	1 (1.5)
Arst	7 (17.5)	17 (68.0)	24 (36.9)
Av	9 (22.5)	6 (24.0)	15 (23.1)
Serogroup WII/III:	112 (73.7)	8 (24.2)	120 (64.9)
Bpyut	20 (17.9)	1 (12.5)	21 (17.5)
Bropt	31 (27.7)	2 (25.0)	33 (27.5)
Broptst	0	2 (25.0)	2 (1.7)
Brpyut	12 (10.7)	0	12 (10.0)
Av/Bx	11 (9.8)	0	11 (9.2)
Other	38 (33.9)	3 (37.5)	41 (34.2)

*Percentages of serovars estimated from No of strains in corresponding serogroup, percentages of serogroups estimated from total No of PPNG or non-PPNG strains.

Table 2 Auxotypes of 33 penicillinase producing *Neisseria gonorrhoeae* (PPNG) and 152 non-PPNG strains in relation to WI and WII/III serogroups

Auxotypes	Non-PPNG strains			PPNG strains		
	No	WI	WII/III	No	WI	WII/III
Zero	92	19 (20.7)	73 (79.3)	15	11 (73.3)	4 (26.7)
Pro ⁻	18	1 (5.6)	17 (94.4)	16	13 (81.2)	3 (18.8)
Arg ⁻	12	5 (41.7)	7 (58.3)	1	1 (4)	0
AHU ⁻	12	8 (66.7)	4 (33.3)	0	0	0
PCU ⁻	9	2 (22.2)	7 (77.8)	0	0	0
Others*	9	5 (55.6)	4 (44.4)	1	0	1 (12.5)
Total	152	40	112	33	25	8

Zero, prototrophic; Pro⁻, proline requiring; Arg⁻, arginine requiring; AHU⁻, arginine, hypoxanthine, and uracil requiring; PCU⁻, proline, citrulline, and uracil requiring auxotypes.

*Other auxotypes: Pro⁻Arg⁻ (proline and arginine requiring); Ura⁻, (uracil requiring); His⁻Leu⁻, (histidine and leucine requiring); Pro⁻Arg⁻Hyx⁻, (proline, arginine, and hypoxanthine requiring); His⁻, (histidine requiring); Arg⁻Leu⁻Hyx⁻Ura⁻, (arginine, leucine, hypoxanthine, and uracil requiring).

On the other hand, the 33 PPNG strains were almost equally of Pro⁻ and Zero auxotypes, except two that were of auxotype Arg⁻ and Pro⁻Arg⁻ (proline and arginine requiring). Most (24/31, 77.4%) of the Pro⁻ and Zero, as well as the one Arg⁻, PPNG strains belonged to serogroup WI and Arst was the predominant serovar. The one Pro⁻Arg⁻ PPNG strain belonged to serogroup WII/III and was of serovar Broptyst.

SEROGROUPS AND SUSCEPTIBILITY TO ANTIMICROBIAL OF NON-PPNG STRAINS

Table 3 shows the susceptibility to five antimicrobial agents of the 152 non-PPNG strains tested, in relation to their serogroups. The strains most susceptible to penicillin, cefotaxime, tetracycline, and thiamphenicol belonged to serogroup WI, whereas the strains belonging to serogroup WII/III were less susceptible. The strains in WI and WII/III serogroups were equally susceptible to spectinomycin.

SEROGROUPS, SEROVARS, AND PLASMID CONTENTS

Table 4 presents the relation between plasmid contents, serogroups, and serovars.

Electrophoretic analysis of the 152 non-PPNG strains showed that 95 harboured only the 2.6 megadalton cryptic plasmid, 20 harboured the 24.5

megadalton conjugative plasmid in conjunction with the 2.6 megadalton cryptic plasmid, two carried the 24.5 megadalton conjugative plasmid only, and 35 were plasmid free. The plasmid content findings, in relation to the coagglutination tests carried out for serogrouping, showed that most (67/95, 70.5%) of the non-PPNG strains carrying only the 2.6 megadalton cryptic plasmid belonged to serogroup WII/III, whereas 28/95 (29.5%) were in serogroup WI. As for the 20 isolates carrying both the 24.5 megadalton and the 2.6 megadalton plasmids, 16 (80%) were in the WII/III and four (20%) in the WI serogroups. The two strains carrying only the 24.5 megadalton conjugative plasmid were in serogroup WII/III. A similar distribution was seen in the 35 plasmid free strains, with 27 (77.1%) belonging to the WII/III and eight (22.9%) to the WI serogroup.

In terms of serovars, most (24) of the 40 WI non-PPNG strains in all four plasmid content groups were of serovar Arost, whereas various serovars dominated the 112 non-PPNG strains in the WII/III serogroup (Bropt (31), Bpyvut (20), and Brpyust (12)) depending on their plasmid content.

Electrophoretic analysis of the 33 PPNG strains divided them into two main groups, based on the type of penicillin resistance plasmid that they harboured: 21 carrying the 4.5 megadalton (Asian type), 11

Table 3 Correlation between serogroups and susceptibility to antimicrobials (minimum inhibitory concentrations (MIC) for 50% and 90% of strains) in 152 non-PPNG strains

Antimicrobial	WI serogroup			WII/III serogroup		
	MIC range (mg/l)	MIC ₅₀ (mg/l)	MIC ₉₀ (mg/l)	MIC range (mg/l)	MIC ₅₀ (mg/l)	MIC ₉₀ (mg/l)
Penicillin	≤0.015 – 8	0.064	1	≤0.015 – 8	0.26	1
Cefotaxime	≤0.0005– 0.128	0.008	0.128	≤0.0005– 0.128	0.032	0.128
Spectinomycin	≤4 –64	16	32	≤4 –64	16	32
Tetracycline	≤0.125 – 2	0.25	1	≤0.125 –16	0.5	2
Thiamphenicol	≤0.125 – 2	0.5	1	≤0.125 – 8	0.5	2

Table 4 Correlation between plasmid contents, serogroups, and serovars of 185 PPNG and non-PPNG strains (figures are numbers (percentages) of strains)

Serovars	Non-PPNG strains with plasmids:				PPNG strains with resistance plasmids:		
	Cryptic (2.6 Mdalton) (n = 95)	Conjugative (24.5 Mdalton) (n = 2)	Both (n = 20)	Neither (n = 35)	African (3.2 Mdalton) (n = 11)	Asian (4.5 Mdalton) (n = 21)	Toronto (3.05 Mdalton) (n = 1)
Serogroup WI:	28 (29.5)	0	4 (20.0)	8 (22.9)	7 (63.6)	17 (81.0)	1 (100)
Arost	15	0	2	7	0	0	1
Arot	0	0	0	0	1	0	0
Arst	6	0	0	1	1	16	0
Av	7	0	2	0	5	1	0
Serogroup WII/III:	67 (70.5)	2 (100.0)	16 (80.0)	27 (77.1)	4 (36.4)	4 (19.0)	0
Bopst	0	0	0	0	0	1	0
Bpyvut	12	0	8	0	0	1	0
Bropt	18	0	5	8	1	1	0
Bropyst	0	0	0	0	2	0	0
Brpyt	0	0	0	0	0	1	0
Brpyust	0	2	0	10	0	0	0
Bys	0	0	0	0	1	0	0
Av/Bx	11	0	0	0	0	0	0
Other	26	0	3	9	0	0	0

carrying the 3.2 megadalton (African type), and only one harbouring the 3.05 megadalton (Toronto type) penicillin resistance plasmids. Correlation between plasmid content and serogroups and serovars was also observed (table 4). Of the 21 PPNG strains carrying the 4.5 megadalton resistance plasmid, 17 (81%) belonged to the WI and only four (19%) to the WII/III serogroup. Most (16/17, of the 4.5 megadalton WI strains were of serovar Arst, whereas each of the four 4.5 megadalton WII/III strains had a different serovar. The 11 PPNG strains harbouring the 3.2 megadalton resistance plasmid were distributed as follows: seven (63.6%) were in the WI serogroup and Av was the predominant serovar; the remaining four (36.4%) belonged to serogroup WII/III and were of Bropyst (2), Bropt (1), and Bys (1) serovars. The one strain harbouring the 3.05 megadalton resistance plasmid belonged to serogroup WI and was of serovar Arost.

Discussion

Classifying gonococcal strains, based on their nutritional requirements, serogroups, serovars, and plasmid content, is a powerful tool for analysing the epidemiology of infections with non-PPNG and PPNG strains.^{3 18 19} These traits not only play an important part in studying the epidemiology of outbreaks of infections with PPNG strains and strains resistant to other antibiotics such as tetracycline,^{8 20-22} but also constitute excellent epidemiological markers for monitoring changes in the phenotypes of gonococci.^{20 22 23}

Our results agree with those of previous observations regarding the serological classification of gonococci in Canada, Thailand, Singapore, Korea, areas of

Europe, the United States of America, Australia, and Africa, where most non-PPNG strains belong to the WII/III serogroup.^{4 19 20 22 24-29} Indeed, 73.7% of the non-PPNG Greek isolates were in serogroup WII/III, whereas only 26.3% belonged to serogroup WI. In terms of serovars, the most common in serogroup WI was Arost (38.5%), which was rarely associated with infections by PPNG strains—results similar to those observed by other workers.^{19 25} On the other hand, the most common serovar among our WII/III gonococcal strains was Bropt (27.5%). The incidence (25%) of serovar Bropt in our PPNG strains was similar to that observed in South East Asia (Bangkok 23%, Korea 21%).²⁵

Our findings regarding their nutritional requirements show that the Zero and Pro⁻ strains predominated, followed by Arg⁻ strains, which confirms observations made by other workers in areas such as Canada, the USA, and Europe.^{13 30 31} Most of the prototrophic, Pro⁻, Arg⁻, and PCU⁻ isolates belonged to serogroup WII/III, as expected. Most of the AHU⁻ strains, however, were in serogroup WI, which confirmed Danielson's and Sandström's finding of a strong correlation between AHU⁻ strains and serogroup WI.^{3 31 32} The observation of other workers that there is a greater diversity of serovars in serogroup WII/III than in serogroup WI agrees with our results.

Differences in susceptibility to antimicrobial agents between serogroups have been also noted by other workers.^{23 25 33} Our WI serogroup strains were more sensitive to penicillin, thiamphenicol, tetracycline, and cefotaxime, whereas our strains in the WI and WII/III serogroups were equally sensitive to spectinomycin. The strains of the Arost serovar, which predominated in serogroup WI, showed an overall sensitivity,

whereas serovar Bropt strains were appreciably more resistant to antimicrobial agents (data not shown); similar findings have also been reported previously.¹⁹

Correlation between serogroup and plasmid content showed that the WII/III serogroup predominated in non-PPNG strains, regardless of their plasmid content. In the WII/III strains lacking the 2.6 megadalton cryptic plasmid (those that were plasmid free or harbouring the 24.5 megadalton conjugative plasmid only) the predominant serovar was Brpyust, which was rare in the other plasmid content groups of the WII/III strains.

Most (25/33, 75.8%) PPNG (in contrast to non-PPNG) strains were shown to belong to serogroup WI. When serovars were analysed, five out of the seven strains belonging to the WI serogroup and harbouring the African type resistance plasmid (3.2 megadalton) were of serovar Av and auxotype Zero, which has also been found in Canada, Sweden, and The Netherlands.^{6,19,20} Though 3.2 megadalton Av serovar PPNG strains have been isolated in Sweden, they had been imported there from Africa and the same serovar was also probably imported to Greece. On the other hand, the combinations of serovar and auxotype in the four 3.2 megadalton WII/III strains suggested that PPNG strains had also been imported from Asia, which confirms the observations made by other workers.²⁵

The combination of serovar and auxotype in the 4.5 megadalton WI strains, such as Arst/Pro⁻ and Arst/Zero, shows that those strains were of Asian origin,²⁵ though the Arst/Pro⁻ combination has also been found in Africa.

It is worth noting that the combinations Bopst/Zero and Bpyvut/Zero were isolated for the first time in Greece, whereas the Bropt/Pro⁻ and Brpyt/Pro⁻ combinations have also been found in Asia and Europe (data not shown).

In conclusion, our results confirm correlation between serotype, auxotype, susceptibility to antimicrobials, and plasmid content noted by other workers.^{2-4,6,18-20,25} They also suggest that most of our PPNG strains were imported from Asia, although some were imported from Africa and Europe. As Greece is a naval and tourist country, these findings are not surprising. This study has also shown that certain WII/III serovars correlated highly with resistance to antibiotics, that the presence or absence of transfer and cryptic plasmids correlated with serovar, and that the distribution of PPNG strains can be characterised in part by analysing serovar, auxotype, and plasmid content.

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