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Molecular characterisation of *Neisseria gonorrhoeae* associated with disseminated gonococcal infections in Queensland, Australia: a retrospective surveillance study

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ABSTRACT

Objectives Gonorrhoea caused by *Neisseria gonorrhoeae* is the second most notified sexually transmitted infection (STI) in Australia and the case numbers for this STI have been increasing globally. Progressive gonococcal infection may lead to disseminated gonococcal infection (DGI), which causes significant morbidity among patients. This study aims to examine the genetic diversity of *N. gonorrhoeae* isolates collected in Queensland from January 2010 to August 2015 and to determine factors associated with DGI in Queensland.

Design Retrospective surveillance study for epidemiological purposes.

Setting All gonorrhoeae isolates referred by private and public pathology laboratories to the state of Queensland, Australia Neisseria reference laboratory.

Methods Between January 2010 and August 2015, 3953 *N. gonorrhoeae* isolates from both metropolitan and regional Queensland infections were typed with NG-MAST (*N. gonorrhoeae* multiantigen sequence typing) to assess the genetic diversity between strains. Whole-genome sequencing (WGS) was used to investigate strain-related factors associated with DGI.

Results ST6876 was the most common NG-MAST type, detected in 7.6% of the isolates. DGI was significantly more likely in females <30 years (OR 13.02, p<0.0001) and in older males >30 years (OR 6.04, p<0.0001), with most cases originating from North Queensland (OR 8.5, p<0.0001). Strains harbouring PIA class of *porB* type were associated with DGI (OR 33.23, p<0.0001).

Conclusion Genotyping techniques, such as NG-MAST and WGS, are proving instrumental in providing an insight into the population structure of *N. gonorrhoeae*, and genetic mechanisms of pathogenesis, such as for DGI.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Genetic diversity of over 3500 *Neisseria gonorrhoeae* isolates was assessed by *N. gonorrhoeae* multiantigen sequence typing genotyping.
⇒ Demographic factors associated with *N. gonorrhoeae* infection and disseminated gonococcal infection (DGI) cases were examined across all isolates.
⇒ Genotyping assisted in identification of populations associated with higher incidence of DGI.
⇒ Only a small number of strains were further characterised by whole-genome sequencing, meaning significance of associations with genetic markers such as pglA, PIA porB type and gonococcal genetic island could not be drawn and further studies with larger rates of whole-genome sequencing are needed to address this.

INTRODUCTION

Disseminated gonococcal infection (DGI) is a complication of gonorrhoea from bacteraemic spread of *Neisseria gonorrhoeae*. It typically presents as an arthritis-dermatitis syndrome but in rare cases can lead to death via septic shock.[1] DGI primarily occurs in individuals with an asymptomatic untreated primary infection, most often female, although some studies have shown an association with males.[2–4] Early diagnosis and treatment are required to avert significant morbidity.

Both host and *N. gonorrhoeae* strain-related factors can predispose to DGI. Host-associated risk factors include recent menstruation, pregnancy and complement deficiencies.[5–7] Several strain-related factors have been proposed including exhibiting an arginine–hypoxanthine–uracil (AHU) auxotype, expressing particular phase-variable variants of the pilus glycosyl transferase A (*pgtA*) gene, opacity genes and harbouring a Protein IA (PIA) class of *porB* gene (as opposed to PIB).[8–14] Gonococcal genetic island (GGI) is another speculated virulence factor encoding a type IV secretion system, which plays a role in horizontal gene transfer.[15] However, except for the PIA gene, evidence to support the strain-related factors is limited. For example, an early study of DGI-causing

Table 1 Demographics of *Neisseria gonorrhoeae* isolates, Queensland January 2010 to August 2015

Demographics		N	% of total
All cases		3953	100
porB Class	PIA*	1078	27.3
	PIB*	2875	72.7
Geographical location	NQ	1193	30.1
	SEQ	2403	60.7
	Others	357	9.0
Sex	Female	1055	26.6
	Male	2898	73.3
Age groups	<30 Female	808	20.4
	≥30 Female	234	5.9
	<30 Male	1754	44.3
	≥30 Male	1119	28.3
	Age not specified	38	0.9

*PorB class IA or IB
NQ, northern Queensland; SEQ, south-east Queensland.

gonococci in Australia found that none were of the AHU auxotype, while a later Australian study found no strong association with the phase-variable allele of the *pgtA* gene, subsequently referred to as the *pglA* gene.[16][17] Previous studies assessing stain-related factors associated with DGI are limited by sample size and/or lack of comparison to non-DGI isolates.

We sought to assess the genetic diversity of *N. gonorrhoeae* isolates collected in Queensland from January 2010 to August 2015 and to determine factors associated with DGI in Queensland. In addition, we used whole-genome sequencing (WGS) to gain insight to any existing strain-related factors which may have contributed to the occurrence of DGI.

METHODS

N. gonorrhoeae isolates from Queensland

From 2010 to 2015, the Australian state of Queensland reported 16 506 gonococcal infections over the 6 years, equivalent to an average notification rate of 60 cases per 100 000 population per year.[18] Approximately 75% of these notifications were diagnosed by Nucleic Acid Amplification Test (NAAT) only, with no isolate available for further testing. Diagnostic methods used varied across the state, with 80% of cases in the northern Queensland (NQ) regions diagnosed by NAAT only, as opposed to 70% of cases from the rest of the state; with larger variations of between 58% and to 84% for individual health service districts. A total of 3953 *N. gonorrhoeae* isolates were included in this study, isolated from specimens collected between January 2010 and August 2015 in pathology laboratories servicing Queensland and surrounding areas and subsequently referred to the state reference laboratory

at Queensland Health Forensic and Scientific Services. One isolate per patient episode, defined as not collected within 1 month of a previously included strain, was included. Data collected for each isolate included date of specimen collection, age, sex, postcode of residence and specimen type. To identify disease prevalence in specific age groups isolates from males and females were grouped into two groups, that is, <30 years and ≥30 years. Postcode of residence was used to assign broad geographical region categories of NQ and south-east Queensland (SEQ) and other (sparsely populated central Queensland, interstate or overseas). DGI was defined where the organism was isolated from blood culture, joint fluid and/or tissue.

N. gonorrhoeae multiantigen sequence typing typing and phylogenetic analysis

Crude DNA extracts of all the *N. gonorrhoeae* isolates received in the study period were routinely subjected to *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) as previously described.[19] These extracts were prepared by boiling 400 μL of TE buffer containing a loopful (1 μL) of pure *N. gonorrhoeae* colonies at 100°C for 10 min. NG-MAST comprises DNA sequencing of partial *tbpB* and *porB* genes and subsequent analysis via an online database to assign allele and sequence types (www.ng-mast.net). The *porB* sequence data from the NG-MAST was analysed to assign either PIA or PIB class.[20] The analysis was based on a phylogenetic assessment, and comparison with previously recognised PIA and PIB sequences from GenBank.

WGS and bioinformatic analysis

To investigate strain-related factors associated with DGI, we selected 16 isolates from 8 different NG-MAST types, which were prevalent in DGI, in this study. Two strains from each NG-MAST were selected, comprising both DGI and non-DGI strains. DNA was extracted from isolates using the QIAsymphony SP, using the DSP DNA minikit (Qiagen, Germany), as per manufacturer's guidelines. WGS was performed on the Illumina NextSeq 500 platform (Illumina, California, USA) using NextSeq 500 Mid Output V2 kit (Illumina) with Nextera XT library preparation. Reads were trimmed with Trimmomatic (V.0.36),[21] corrected and assembled with Spades (V.3.10.1) (https://github.com/ablab/spades),[22] and assemblies uploaded to pubMLST to determine presence/absence of the gonococcal genetic island,[23] and analysed with Ridom SeqSphere +4.10.0 (Ridom, Germany) using alleles from 1649 *N. gonorrhoeae* cgMLST v1.0 loci[24] and *Neisseria* spp. MLST.[25] WGS assemblies are available on pubMLST with ID numbers 52753-52768. WGS raw sequence files and associated metadata have been submitted to the European Nucleotide Archive with Project Accession number PRJEB52601.

Statistical analysis

Descriptive analysis was performed using Microsoft Excel. Annual rates of reported cases were computed by using the number of cases reported as numerators, and statistics

Table 2 NG-MAST typing of isolates collected in Queensland from January 2010 to August 2015

Year	No of *Neisseria gonorrhoeae* isolates	No of *porB* alleles	Most frequent *porB* alleles	No of *tbpB* alleles	Most frequent *tbpB* alleles	Number of NG-MAST types	Most frequent NG-MAST types	*porB* class	Invasive (DGI) isolates*
2010	782	165	porB 4101 (18.7%)	87	tbpB 29 (17.6%)	208	6876 (16.1%)	PIA*—255 (32.6%)	12 (1.5%)
			porB 2280 (5.3%)		tbpB 1330 (16.3%)		6863 (5.4%)	PIB†—527 (67.3%)	
			porB 908 (4.7%)		tbpB 1329 (8.4%)		1407 (4.7%)		
			porB 1808 (4.4%)		tbpB 110 (6.6%)		2992 (4.4%)		
			porB 4143 (3.7%)		tbpB 349 (6.1%)		6940 (3.7%)		
2011	754	157	porB 4101 (17.9%)	85	tbpB 29 (24.0%)	206	6876 (14.4%)	PIA—240 (31.8%)	8 (1.0%)
			porB 1808 (8.8%)		tbpB 1330 (15.5%)		2992 (8.8%)	PIB—514 (68.1%)	
			porB 4099 (5.3%)		tbpB 349 (7.0%)		6879 (4.7%)		
			porB 14 (4.7%)		tbpB 33 (6.7%)		21 (4.4%)		
			porB 4104 (3.7%)		tbpB 1329 (6.1%)		6937 (3.2%)		
2012	680	156	porB 4101 (12.2%)	91	tbpB 29 (19.7%)	203	21 (8.6%)	PIA—195 (28.6%)	10 (1.4%)
			porB 14 (8.6%)		tbpB 349 (12.3%)		2992 (7.6%)	PIB—485 (71.3%)	
			porB 4099 (7.7%)		tbpB 33 (10.8%)		6879 (7.5%)		
			porB 1808 (7.6%)		tbpB 1330 (9.1%)		6876 (7.0%)		
			porB 4104 (5.5%)		tbpB 1329 (7.0%)		6937 (4.1%)		
2013	650	167	porB 1808 (7.2%)	93	tbpB 29 (18.9%)	217	21 (6.0%)	PIA—160 (24.6%)	10 (1.5%)
			porB 4101 (6.7%)		tbpB 349 (8.3%)		4822 (6.0%)	PIB—490 (75.3%)	
			porB 1903 (6.1%)		tbpB 33 (7.5%)		6879 (5.3%)		
			porB 14 (6.0%)		tbpB 110 (6.9%)		4186 (5.2%)		
			porB 4099 (5.3%)		tbpB 241 (6.9%)		5533 (4.0%)		
2014	633	147	porB 1808 (14.6%)	90	tbpB 241 (8.5%)	201	4186 (7.1%)	PIA—140 (22.1%)	18 (2.8%)
			porB 2569 (7.2%)		tbpB 29 (7.8%)		9654 (5.6%)	PIB—493 (77.8%)	
			porB 147 (6.4%)		tbpB 4 (7.1%)		4244 (4.8%)		
			porB 4101 (4.5%)		tbpB 110 (6.1%)		10039 (3.7%)		
			porB 5912 (3.7%)		tbpB 1744 (6.0%)		5004 (2.3%), 6879 (2.3%)		
2015†	454	115	porB 1808 (12.1%)	74	tbpB 241 (16.5%)	154	4186 (10.1%)	PIA—87 (19.1%)	6 (1.3%)
			porB 2569 (10.1%)		tbpB 4 (6.4%)		4244 (5.2%)	PIB—367 (80.8%)	
			porB 147 (8.14%)		tbpB 29 (5.9%)		9654 (4.4%)		
			porB 2656 (5.0%)		tbpB 893 (5.7%)		9909 (3.9%)		
			porB 543 (4.1%)		tbpB 1744 (5.3%)		11821 (3.7%)		

PorB class IA or IB
*Percentage of total isolates.
†Data collected up until August 2015.
DGI, disseminated gonococcal infection; NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing.

Table 3 Demographic factors, *porB* type and NG-MAST types associated with disseminated gonococcal infection (DGI) in Queensland (January 2010 to August 2015)

Risk factor		Non-DGI	DGI	Total	DGI as %	Univariate OR	P value
porB class	PIA*	1019	59	1078	5.5	33.23	<0.0001
	PIB*	2870	5	2875	0.2	0.03	
Geographical location	NQ	1143	50	1193	4.2	8.5	<0.0001
	SEQ	2394	9	2403	0.4	0.1	
	Other	352	5	357	1.4	0.85	
Sex	Female	1015	40	1055	3.8	4.72	<0.0001
	Male	2874	24	2898	0.8	0.2	
Age group (years)	<30 female	779	29	808	3.58	13.02	<0.0001
	<30 male	1749	5	1754	0.2	0.07	
	≥30 female	223	11	234	4.7	2.85	0.01
	≥30 male	1100	19	1119	1.69	0.35	
	<30 female	779	29	808	3.58	0.75	0.44
	≥30 female	223	11	234	4.7	1.32	
	<30 male	1749	5	1754	0.2	0.16	<0.0001
	≥30 male	1100	19	1119	1.69	6.04	
	Age not specified	38	0	38	0.0		
Prevalent NG-MAST types	ST758	17	2	19	10.5	3.67	0.03
	ST6876	296	6	302	2.0	1.2	0.6
	ST6886	30	3	33	9.1	6.3	0.001
	ST6937	80	14	94	14.9	13.3	<0.0001
	ST6939	34	3	37	8.1	5.57	0.02
	ST7126	22	3	25	12.0	8.6	0.007
	ST8712	17	5	22	22.7	19.3	<0.0001
	ST10711	15	3	18	16.7	12.7	0.002
	Other STs	3378	25	3403	0.7	0.09	
	Total	3889	64	3953	1.6		

*PorB class IA or IB
NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing; NQ, northern Queensland; SEQ, south-east Queensland.

Queensland yearly population as denominators. Categorical variables were examined using the Fischer's exact test performed in GraphPad Prism V.7 (GraphPad Software, California, USA). ORs with 95% CIs were obtained from logistic regression models in Microsoft Excel to quantify associations between independent variables and outcome. P values of <0.05 were considered statistically significant.

Patient and public involvement
There was no patient or public involvement in the study.

RESULTS
Demographics and *N. gonorrhoeae* isolates
A total of 3953 *N. gonorrhoeae* isolates in this study from January 2010 to August 2015 consisted of genital (n=3099; 78.3%), invasive (n=64; 1.6%), anorectal (n=456; 11.5%), oropharyngeal (n=233; 5.8%), ocular (n=31; 0.7%) and other/not specified (n=70; 1.7%) specimen types,

as listed in online supplemental data table 1. Overall, majority of these isolates were reported in SEQ (n=2403; 60.7%), followed by NQ (n=1193; 30.1%) and other regions (sparsely populated central Queensland, interstate or overseas), which constituted of 9% (n=357) of the total cases. The isolates comprised 73% (n=2898) from males (62.9 cases per 100 000 population) and 27% (n=1055) from females (22.9 cases per 100 000 population). Further breakdown into age groups showed that 20.4% (n=808) gonococcal infections were represented by females <30 years of age and 5.9% of infections were reported in females ≥30 years of age. Similar trend was observed in males where 44.3% (n=1754) of *N. gonorrhoeae* infections were noted in males <30 years of age and 28.3% (n=1119) of infections were present in males ≥30 years of age. PIB class of porB was assigned to 72.7% (n=2875) of isolates whereas 27.3% (n=1078) of the isolates belonged
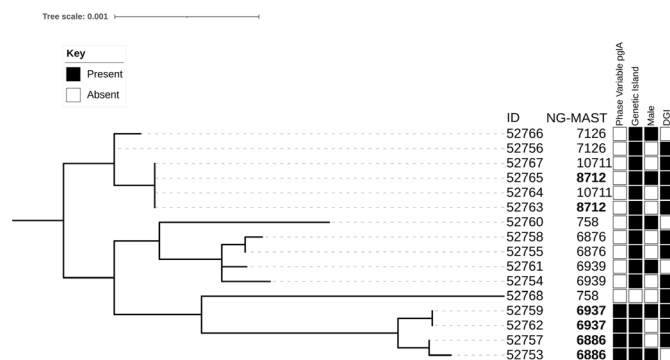
Figure 1 Core genome maximum likelihood phylogeny of 16 PIA strains of *Neisseria gonorrhoeae* from Queensland based on cgMLST. The tree is rooted at centre point and annotated with strain ID, NG-MAST associated with DGI, and sequence types derived from MLST and NG-MAST, with presence/absence of phase variable *pglA* and gonococcal genetic island. The phylogenetic distance is indicated by the length of the horizontal lines. Visualised with iTOL.[27] DGI, disseminated gonococcal infection; NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing.

to *porB* class PIA. Table 1 shows a breakdown for all strains by PIA/PIB, NQ/SEQ and age group.

NG-MAST typing and phylogenetic analysis

Among the 3953 isolates tested, 574 alleles of *porB* gene were identified, the most prevalent of which was the 4101 allele, present in 441 isolates (11.1%). The *tbpB* gene was represented by 250 alleles with the most frequent allele was 29, detected in 653 isolates (16.5%). Combinations of *porB* and *tbpB* alleles resulted in 823 NG-MAST types. Overall ST6876 was the most common NG-MAST type, detected in 302 isolates (7.6%). It was also the most prevalent in years 2010 and 2011, represented by 126 (16.1%) and 109 (14.4%) isolates, respectively. In 2012 and 2013, ST21 became more prevalent, represented by 8.6% and 6.0% of the isolates collected in those years. In 2014 and 2015 (up to August), ST4186 was found with high frequency with 7.1% and 10.1% of the isolates, respectively. 771 STs were represented by only one isolate, and these Sequence Types (STs) accounted for between 16.8% and 23.3% of total isolates each year. Table 2 shows a summary of the most frequent alleles and types over the 5 years.

Disseminated gonococcal infection

From January 2010 to August 2015, 64 DGI-related isolates were received by the reference laboratory, comprising 1.6% of total isolates; 49 cases (76.6%) were diagnosed from joint samples and 15 (23.4%) from blood samples. This study only had access to cultured gonococcal isolates, so any DGI cases diagnosed by NAAT only are not considered here. A summary of the demographics, *porB* class types and strain types (n=8) associated with DGI cases is provided in table 3. Even though the majority of total gonococcal isolates were from males, DGI was significantly more likely in females (OR 4.72, p<0.0001),

particularly in those aged <30 years (OR 13.02, p<0.0001) and in older males aged >30 years (OR 6.04, p<0.0001) when compared with their younger counterparts. The majority of DGI cases (n=50; 78%) originated from the north Queensland, and cases from this region had higher odds of being associated with DGI (OR 8.5, p<0.0001). A total of 31 STs of the total 823 were observed among the 64 DGI cases. PIA *porB* type was significantly associated with DGI (OR 33.23, p<0.0001), and accounted for 59 (92.2%) of the 64 DGI cases. Seven of the prevalent NG-MASTs in DGI (ST758, ST6886, ST6937, ST6939, ST7126, ST8712 and ST10711), all with PIA class of *porB*, were found to be individually associated with DGI (table 3). However, the most prevalent NG-MAST in this study ST6876 (n=302), which is also PIA class of *porB*, was not found to be associated with DGI (n=6) (OR 1.2, p=0.6).

Whole-genome sequencing

Figure 1 shows a core genome phylogeny of the 16 strains from 8 different NG-MAST, all of which were PIA types, selected for WGS. The four ST6886 and ST6937 strains contained phase variable *pglA* (NEIS0213) alleles with long homopolymeric tracts of Gs, while the others did not. These four strains grouped together by cgMLST, despite one of them sharing an MLST profile with other strains that did not form part of this group. Only 1 of the 16 strains sequenced did not possess the gonococcal genetic island.

DISCUSSION

This study investigated the burden of gonococcal infections in Queensland and identified those most at risk of developing DGI. The increasing gonorrhoea rates among males could be a result of rapidly increasing rates of gonorrhoea in the MSM population. Another explanation would be that gonorrhoea is more symptomatic in men and as a result they are more likely to seek healthcare. In this study, we applied *N. gonorrhoeae* NG-MAST genotyping to a large, diverse, consecutively collected *N. gonorrhoeae* isolate collection in a setting where DGI is not uncommon and used WGS to investigate other strain-related factors. We have used a dataset of strains that encompassed both metropolitan and regional populations and has subsequently highlighted different populations vulnerable to DGI. We observed a higher likelihood of DGI in females, in cases reported in NQ when compared with SEQ and with strains harbouring the PIA gene. This finding agrees with the previous studies suggesting female predominance[2] but contradicts a more recent study from the Northern Territory of Australia, which did not show any significant association between DGI and sex,[26] but did not examine strain-related factors such as PIA/PIB. Moreover, our data may indicate that DGI is associated with certain PIA gene positive NG-MAST types, suggesting that additional mechanisms possessed by particular PIA bearing genotypes may be at play. PIA has previously been reported to be associated with DGI due to a diminished

inflammatory response, which increases the chances that a mucosal infection may go untreated and, therefore, progress to DGI.[12]

Our data identified *pglA* phase variation present within certain NG-MAST types that were associated with DGI, however, further studies with larger diversity of strain collections are required, as this finding is inconsistent with the work of Power *et al*.[17] Our sequencing work did not show any evidence that DGI is associated with the gonococcal genetic island as seen by Dillard *et al*,[15] however, with the limited number of strains we sequenced, no statistical significance can be drawn and further studies are required to confirm or refute this. Data on the coexistence of genital infections for DGI cases were not consistently available, however, there were DGI cases that did not have a genital infection recorded in the dataset. Absence of a genital infection suggests cases may have cleared a mucosal infection before progressing to DGI, which would be more likely for asymptomatic infections in females rather than via anorectal injury infection in males.

The majority of gonorrhoea cases yielding an isolate are represented by males who continue to have higher notification rates than females in Queensland. DGI is not a rare occurrence, being noted in 1.6% of culture-positive cases and younger females showing higher rates than males. High DGI rates among younger women are concerning as infertility is one of the potential outcomes of untreated gonorrhoea infection, which has downstream social and economic impacts. Genotyping techniques, such as NG-MAST and WGS, are proving instrumental in providing an insight into the population structure of *N. gonorrhoeae*, and genetic mechanisms of pathogenesis, such as for DGI.

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Patient consent for publication Not applicable.

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Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information. WGS raw sequence files and associated metadata have been submitted to the European Nucleotide Archive with Project Accession number PRJEB52601.

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