BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-061040
Article Type:	Original research
Date Submitted by the Author:	14-Jan-2022
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Keywords:	Epidemiology < TROPICAL MEDICINE, INFECTIOUS DISEASES, MOLECULAR BIOLOGY

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Disseminated gonococcal infection associated with *Neisseria gonorrhoeae* genotypes harbouring the PIA class of *porB* in Queensland, Australia

Running Title: DGI and *porB* genotype

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Abstract word count:227

Text word count: 2113

ABSTRACT

Objectives. Gonorrhoea caused by *Neisseria gonorrhoeae* is the second most notified sexually transmitted infection in Australia and 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 aimed 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.

Methods. Between January 2010 and August 2015, 3953 *Neisseria gonorrhoeae* isolates from both metropolitan and regional Queensland infections were typed with NG-MAST (*N. gonorrhoeae* multi antigen 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). WGS demonstrated that NG-MAST types having a *plgA* phase variation were more commonly detected in DGI.

***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.

Keywords: Neisseria gonorrhoeae; Gonorrhoea; DGI; Australia; Genotype; NG-MAST; WGS

Strengths and Limitations of this study

- This study investigated the burden of gonococcal infections in Queensland and identified those most at risk of developing DGI.
- Young women (<30 years) and older men (>30 years) were determined to be at higher risk of developing DGI.
- The PIA class of *porB* type was determined to be more common in the DGI cohort.
- The NG-MAST genotyping was performed on a large, diverse, consecutively collected *N. gonorrhoeae* isolate collection that encompassed both metropolitan and regional populations, which would assist in best capturing high risk populations for DGI.
- Only a small number of isolates were subject to whole genome sequencing so no statistical significance can be drawn from analysis of plgA phase variation and role of the gononococcal genetic island.

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 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 (T4SS) 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 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 utilised 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 NAAT only, with no isolate available for further testing. Diagnostic methods used varied across the state, with 80% of cases in the northern Queensland 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 one 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, <30 years and ≥30 years. Postcode of residence was used to assign broad geographic region categories of NQ (northern Queensland) and SEQ (south-east Queensland) 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.

NG-MAST typing and Phylogenetic analysis

Crude DNA extracts of all the *N. gonorrhoeae* isolates received in the study period were routinely subjected to *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) as previously described [19]. These extracts were prepared by boiling 400µL of TE buffer containing a loopful (1µL) of pure *N. gonorrhoeae* colonies at 100°C for 10 minutes. 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]. *porB* and *tbpB* sequences were concatenated for each different NG-MAST, aligned in Geneious R11 with MUSCLE [21] and a maximum likelihood phylogenetic tree was generated with RAxML software version 8.0 [22].

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, CA, USA) using NextSeq 500 Mid Output V2 kit (Illumina) with Nextera XT library preparation. Reads were trimmed with Trimmomatic [23], corrected and assembled with Spades [24], and assemblies uploaded to pubMLST to determine presence/absence of the gonococcal genetic island, and analysed with Ridom SeqSphere+ (Ridom GmbH, Germany) using alleles from 1,649 *N. gonorrhoeae* cgMLST v1.0 loci [25], and *Neisseria* spp. MLST [26]. WGS assemblies are available on pubMLST with ID numbers 52753-52768.

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 Queensland yearly population as denominators. Categorical variables were examined using the Fischer's Exact test performed in GraphPad Prism 7(GraphPad Software Inc., California). Odds ratios (OR) with 95% confidence intervals 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

No patients involved.

RESULTS

Demographics and *N. gonorrhoeae* isolates

The total 3,953 *N. gonorrhoeae* isolates in this study from January 2010 to August 2015 consisted of genital (n=3,099; 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 Supplementary 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 year 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 to *porB* class PIA. Table 1 shows a breakdown for all strains by PIA/PIB, NQ/SEQ, and age group.

Table 1: Demographics of *N. gonorrhoeae* isolates, Queensland January 2010 to August 2015. Created by the authors.

Demographics	n	Percentage (%) of total
All cases	3953	100

porB Class	PIA	1078	27.3
	PIB	2875	72.7
Geographic location	NQ	1193	30.1
	SEQ	2403	60.7
	Others	357	9.0
Sex	F	1055	26.6
	M	2898	73.3
Age groups	<30 Female	808	20.4
	≥30 Female	1754	44.3
	<30 Male	234	5.9
	≥30 Male	1119	28.3
	Age not specified	38	0.9

NG-MAST typing and Phylogenetic analysis

Among the 3,953 isolates tested, 574 alleles of *porB* gene were identified, the most prevalent of which was ST4101, 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 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 five years.

Table 2: NG-MAST typing of isolates collected in Queensland from January 2010 to August 2015. Created by the authors.

Year	Number of *N. gonorrhoea* isolates	Number of *porB* alleles	Most frequent *porB* alleles	Number 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%) porB 2280 (5.3%) porB 908 (4.7%) porB 1808 (4.4%) porB 4143 (3.7%)	**87**	tbpB 29 (17.6%) tbpB 1330 (16.3%) tbpB 1329 (8.4%) tbpB 110 (6.6%) tbpB 349 (6.1%)	**208**	6876 (16.1%) 6863 (5.4%) 1407 (4.7%) 2992 (4.4%) 6940 (3.7%)	**PIA - 255 (32.6%)** **PIB - 527 (67.3%)**	**12 (1.5%)**
2011	**754**	**157**	porB 4101 (17.9%) porB 1808 (8.8%) porB 4099 (5.3%) porB 14 (4.7%) porB 4104 (3.7%)	**85**	tbpB 29 (24.0%) tbpB 1330 (15.5%) tbpB 349 (7.0%) tbpB 33 (6.7%) tbpB 1329 (6.1%)	**206**	6876 (14.4%) 2992 (8.8%) 6879 (4.7%) 21 (4.4%) 6937 (3.2%)	**PIA - 240 (31.8%)** **PIB - 514 (68.1%)**	**8 (1.0%)**
2012	**680**	**156**	porB 4101 (12.2%) porB 14 (8.6%) porB 4099 (7.7%) porB 1808 (7.6%) porB 4104 (5.5%)	**91**	tbpB 29 (19.7%) tbpB 349 (12.3%) tbpB 33 (10.8%) tbpB 1330 (9.1%) tbpB 1329 (7.0%)	**203**	21 (8.6%) 2992 (7.6%) 6879 (7.5%) 6876 (7.0%) 6937 (4.1%)	**PIA - 195 (28.6%)** **PIB - 485 (71.3%)**	**10 (1.4%)**
2013	**650**	**167**	porB 1808 (7.2%) porB 4101 (6.7%) porB 1903 (6.1%) porB 14 (6.0%) porB 4099 (5.3%)	**93**	tbpB 29 (18.9%) tbpB 349 (8.3%) tbpB 33 (7.5%) tbpB 110 (6.9%) tbpB 241 (6.9%)	**217**	21 (6.0%) 4822 (6.0%) 6879 (5.3%) 4186 (5.2%) 5533 (4.0%)	**PIA - 160 (24.6%)** **PIB - 490 (75.3%)**	**10 (1.5%)**
2014	**633**	**147**	porB 1808 (14.6%) porB 2569 (7.2%) porB 147 (6.4%) porB 4101 (4.5%) porB 5912 (3.7%)	**90**	tbpB 241 (8.5%) tbpB 29 (7.8%) tbpB 4 (7.1%) tbpB 110 (6.1%) tbpB 1744 (6.0%)	**201**	4186 (7.1%) 9654 (5.6%) 4244 (4.8%) 10039 (3.7%) 5004 (2.3%),	**PIA - 140 (22.1%)** **PIB - 493 (77.8%)**	**18 (2.8%)**
2015**	**454**	**115**	porB 1808 (12.1%) porB 2569 (10.1%) porB 147 (8.14%) porB 2656 (5.0%) porB 543(4.1%)	**74**	tbpB 241 (16.5%) tbpB 4 (6.4%) tbpB 29 (5.9%) tbpB 893 (5.7%) tbpB 1744 (5.3%)	**154**	4186 (10.1%) 4244 (5.2%) 9654 (4.4%) 9909 (3.9%) 11821 (3.7%)	**PIA - 87 (19.1%)** **PIB - 367 (80.8%)**	**6 (1.3%)**

*percentage of total isolates

**Data Collected up until August 2015

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 to 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 amongst 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, $p=0.6$).

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

Risk factor		Non-DGI	DGI	Total	DGI as percentage (%)	Univariate OR	*p* value
porB class	PIA	1019	59	1078	5.5	33.23	<0.0001
	PIB	2870	5	2875	0.2	0.03	
Geographic 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	ST758	17	2	19	10.5	3.67	0.03
	ST6876	296	6	302	2.0	1.2	0.6

types	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%**		

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 one 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 health care. 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 to SEQ, and with strains harbouring the PIA gene. This finding agrees with 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 [15], 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 [27].

Our data identified *plgA* 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 co-existence of genital infections for DGI cases was 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.

Contributorship statement

Amy Jennison and David Whiley conceptualised the study. Christine Guglielmino conducted the laboratory investigation. Christine Guglielmino, Sumeet Sandhu, Colleen Lau and Ella Trembizki performed formal data analysis. All authors contributed to the writing and review of the manuscript.

Acknowledgments

This publication made use of the Neisseria Multi Locus Sequence Typing website (https://pubmlst.org/neisseria/) developed by Keith Jolley and sited at the University of Oxford [28]. The development of this site has been funded by the Wellcome Trust and European Union. We thank all laboratories for referring isolates included in this study, and Public Health Microbiology Staff for technical work.

Conflicts of interest. None

Data Availability

No additional data available

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Transparency declarations: D.M.W. reports research funding from SpeeDx Pty Ltd and is supported by a Queensland Advancing Clinical Research Fellowship from the Queensland Government. E.T is holding an NHMRC Early Career Fellowship. CLL is supported by an NHMRC Investigator Grant (APP1193826).

Ethics Approvals: The study was approved by Forensic and Scientific Services Human Ethics Committee (FSS-HEC, EC00305). HEC Ref Number HEC18_01.

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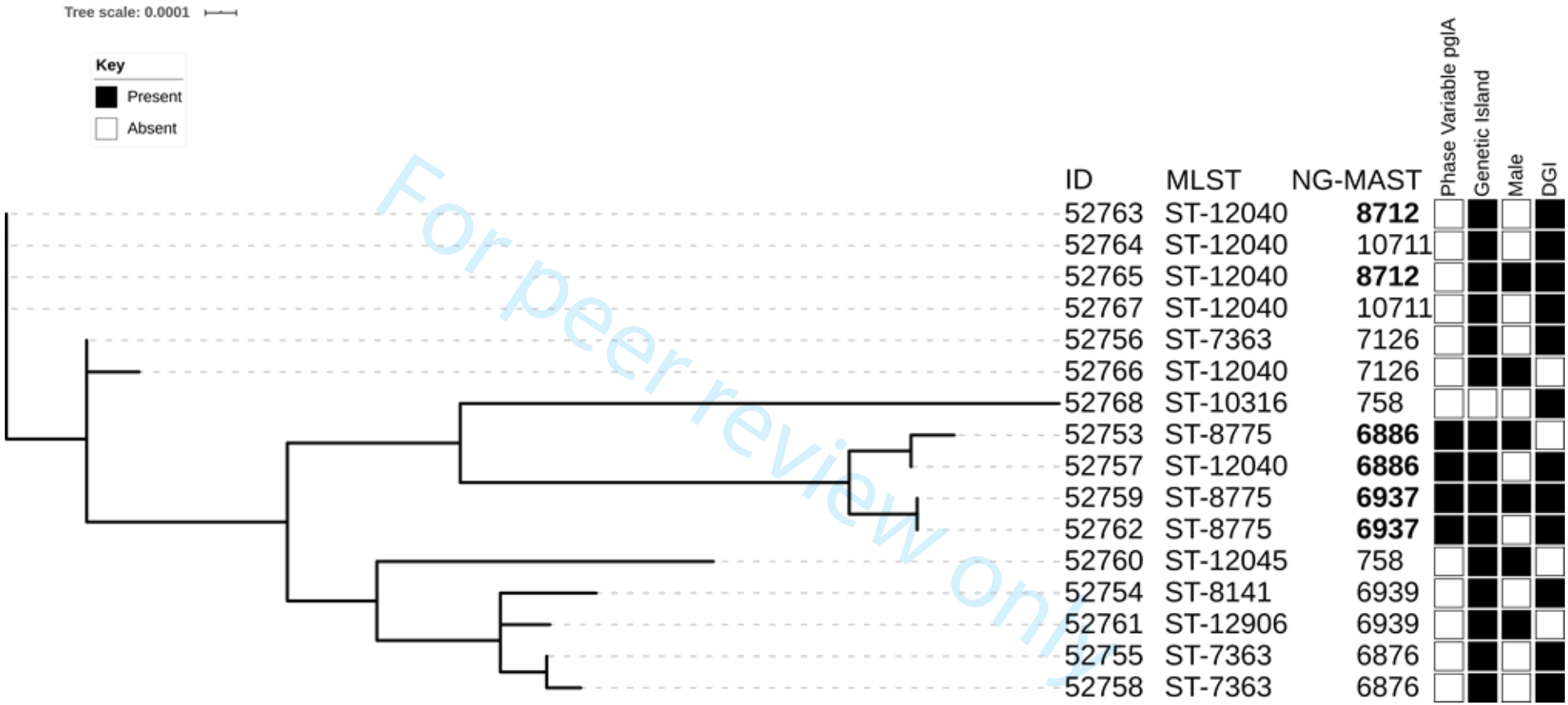
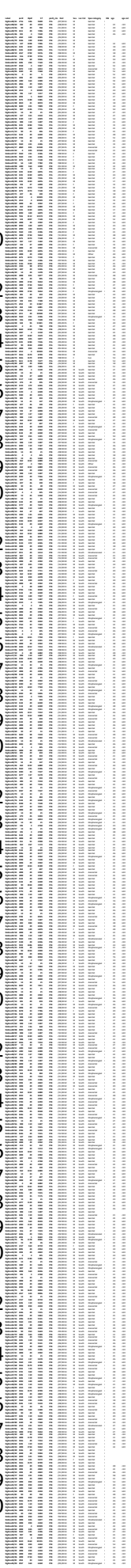
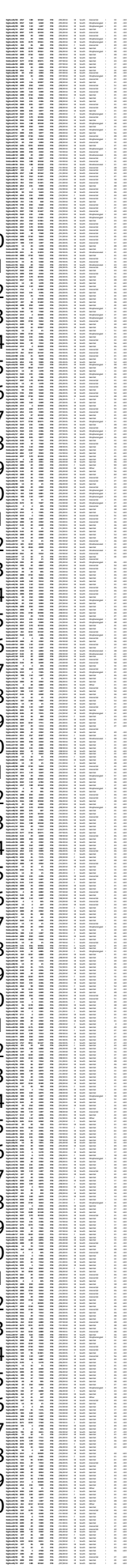
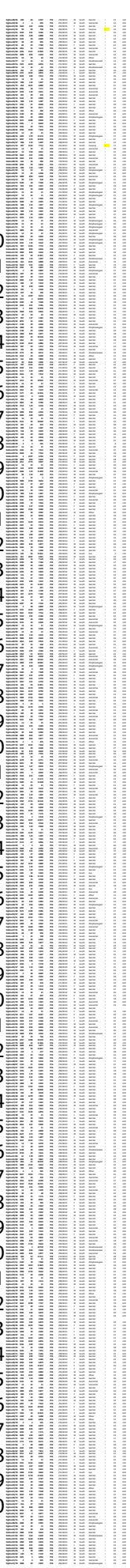
Figure Legend

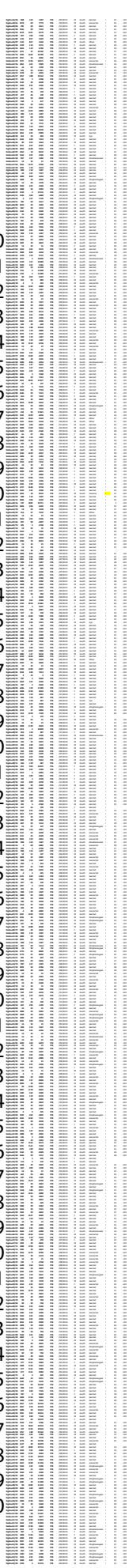
Figure 1: Core genome maximum likelihood phylogeny of 16 PIA strains of *N. gonorrhoeae* from Queensland based on cgMLST. The tree is annotated with strain ID, NG-MAST associated with DGI, and sequence types derived from MLST and NG-MAST, with presence/absence of phase variable *pglA* and gonococcal genetic island. Visualised with iTOL [29]. Created by the authors.

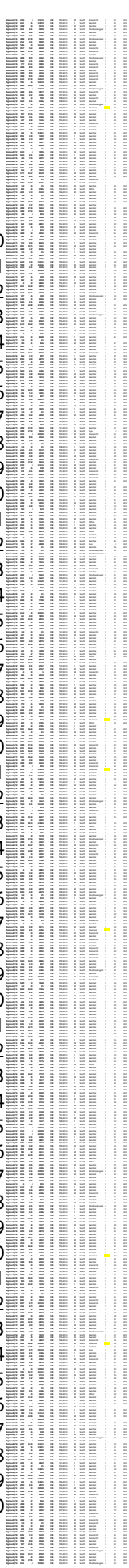


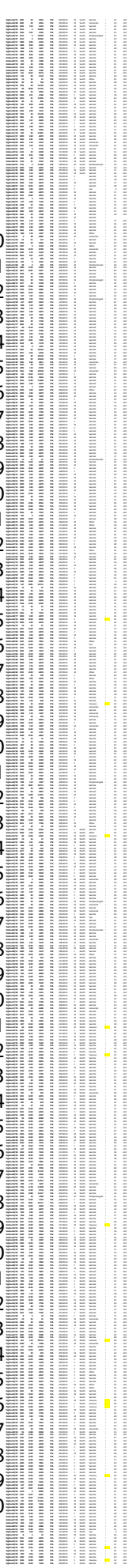


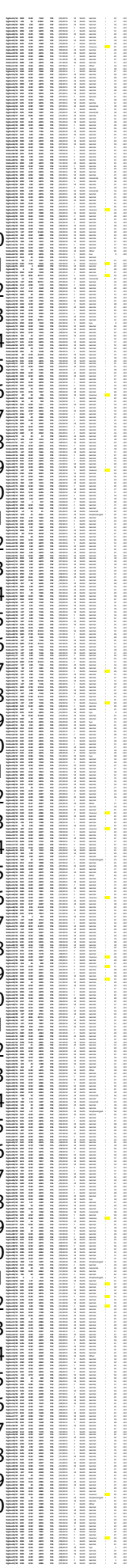


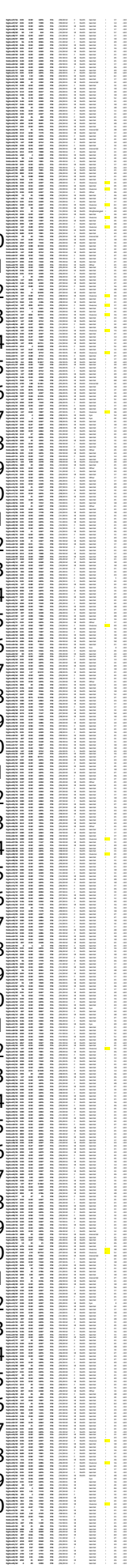












STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4-5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*4*
Bias	9	Describe any efforts to address potential sources of bias	6, 12
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5,6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	5
		(*b*) Describe any methods used to examine subgroups and interactions	5
		(*c*) Explain how missing data were addressed	NA
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	NA
		(*e*) Describe any sensitivity analyses	NA
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	4
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	4
		(b) Indicate number of participants with missing data for each variable of interest	4
Outcome data	15*	Report numbers of outcome events or summary measures	8-11
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make	6,8,10,11

		clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	10
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	10
Discussion			
Key results	18	Summarise key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12
Generalisability	21	Discuss the generalisability (external validity) of the study results	12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Molecular characterisation of Neisseria gonorrhoeae associated with disseminated gonococcal infections in Queensland, Australia

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-061040.R1
Article Type:	Original research
Date Submitted by the Author:	26-May-2022
Complete List of Authors:	Guglielmino, Christine; Health Support Queensland Forensic and Scientific Services Sandhu, Sumeet; Health Support Queensland Forensic and Scientific Services Lau, Colleen L.; Australian National University Research School of Population Health Buckely, Cameron; UQ Centre for Clinical Research, University of Queensland Trembizki, Ella; UQ Centre for Clinical Research, University of Queensland Whiley, David; UQ Centre for Clinical Research, University of Queensland Jennison, Amy; Health Support Queensland Forensic and Scientific Services
Primary Subject Heading:	Sexual health
Secondary Subject Heading:	Public health, Infectious diseases, Epidemiology
Keywords:	Epidemiology < TROPICAL MEDICINE, INFECTIOUS DISEASES, MOLECULAR BIOLOGY

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Molecular characterisation of *Neisseria gonorrhoeae* associated with disseminated gonococcal infections in Queensland, Australia

Running Title: DGI and *porB* genotype

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Abstract word count:227

Text word count: 2113

ABSTRACT

Objectives: Gonorrhoea caused by *Neisseria gonorrhoeae* is the second most notified sexually transmitted infection in Australia and 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 aimed 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 *Neisseria gonorrhoeae* isolates from both metropolitan and regional Queensland infections were typed with NG-MAST (*N. gonorrhoeae* multi antigen 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.

Keywords: *Neisseria gonorrhoeae*; Gonorrhoea; DGI; Australia; Genotype; NG-MAST; WGS

Strengths and Limitations of this study

- Genetic diversity of over 3500 *N. gonorrhoeae* isolates were assessed by NG-MAST genotyping.
- Demographic factors associated with NG infection and 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 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 (T4SS) 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 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 *pglA* 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 utilised 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 NAAT only, with no isolate available for further testing. Diagnostic methods used varied across the state, with 80% of cases in the northern Queensland 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 one 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, <30 years and ≥30 years. Postcode of residence was used to assign broad geographic region categories of NQ (northern Queensland) and SEQ (south-east Queensland) 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.

NG-MAST typing and Phylogenetic analysis

Crude DNA extracts of all the *N. gonorrhoeae* isolates received in the study period were routinely subjected to *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) as previously described [19]. These extracts were prepared by boiling 400µL of TE buffer containing a loopful (1µL) of pure *N. gonorrhoeae* colonies at 100°C for

10 minutes. 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, CA, USA) using NextSeq 500 Mid Output V2 kit (Illumina) with Nextera XT library preparation. Reads were trimmed with Trimmomatic (Version 0.36) [21], corrected and assembled with Spades (Version 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+ 4.1.0 (Ridom GmbH, Germany) using alleles from 1,649 *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 Queensland yearly population as denominators. Categorical variables were examined using the Fischer's Exact test performed in GraphPad Prism 7(GraphPad Software Inc., California). Odds ratios (OR) with 95% confidence intervals 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

No patients involved.

RESULTS

Demographics and *N. gonorrhoeae* isolates

The total 3,953 *N. gonorrhoeae* isolates in this study from January 2010 to August 2015 consisted of genital (n=3,099; 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 Supplementary 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 year 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 to *porB* class PIA. Table 1 shows a breakdown for all strains by PIA/PIB, NQ/SEQ, and age group.

Table 1: Demographics of *N. gonorrhoeae* isolates, Queensland January 2010 to August 2015. Created by the authors.

Demographics		n	Percentage (%) of total
All cases		3953	100
porB Class	PIA	1078	27.3
	PIB	2875	72.7

Geographic location	NQ	1193	30.1
	SEQ	2403	60.7
	Others	357	9.0
Sex	F	1055	26.6
	M	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

NG-MAST typing and Phylogenetic analysis

Among the 3,953 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 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 five years.

Table 2: NG-MAST typing of isolates collected in Queensland from January 2010 to August 2015. Created by the authors.

Year	Number of *N. gonorrhoeae* isolates	Number of *porB* alleles	Most frequent *porB* alleles	Number 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%) porB 2280 (5.3%) porB 908 (4.7%) porB 1808 (4.4%) porB 4143 (3.7%)	**87**	tbpB 29 (17.6%) tbpB 1330 (16.3%) tbpB 1329 (8.4%) tbpB 110 (6.6%) tbpB 349 (6.1%)	**208**	6876 (16.1%) 6863 (5.4%) 1407 (4.7%) 2992 (4.4%) 6940 (3.7%)	**PIA - 255 (32.6%)** **PIB - 527 (67.3%)**	**12 (1.5%)**
2011	**754**	**157**	porB 4101 (17.9%) porB 1808 (8.8%) porB 4099 (5.3%) porB 14 (4.7%) porB 4104 (3.7%)	**85**	tbpB 29 (24.0%) tbpB 1330 (15.5%) tbpB 349 (7.0%) tbpB 33 (6.7%) tbpB 1329 (6.1%)	**206**	6876 (14.4%) 2992 (8.8%) 6879 (4.7%) 21 (4.4%) 6937 (3.2%)	**PIA - 240 (31.8%)** **PIB - 514 (68.1%)**	**8 (1.0%)**
2012	**680**	**156**	porB 4101 (12.2%) porB 14 (8.6%) porB 4099 (7.7%) porB 1808 (7.6%) porB 4104 (5.5%)	**91**	tbpB 29 (19.7%) tbpB 349 (12.3%) tbpB 33 (10.8%) tbpB 1330 (9.1%) tbpB 1329 (7.0%)	**203**	21 (8.6%) 2992 (7.6%) 6879 (7.5%) 6876 (7.0%) 6937 (4.1%)	**PIA - 195 (28.6%)** **PIB - 485 (71.3%)**	**10 (1.4%)**
2013	**650**	**167**	porB 1808 (7.2%) porB 4101 (6.7%) porB 1903 (6.1%) porB 14 (6.0%) porB 4099 (5.3%)	**93**	tbpB 29 (18.9%) tbpB 349 (8.3%) tbpB 33 (7.5%) tbpB 110 (6.9%) tbpB 241 (6.9%)	**217**	21 (6.0%) 4822 (6.0%) 6879 (5.3%) 4186 (5.2%) 5533 (4.0%)	**PIA - 160 (24.6%)** **PIB - 490 (75.3%)**	**10 (1.5%)**
2014	**633**	**147**	porB 1808 (14.6%) porB 2569 (7.2%) porB 147 (6.4%) porB 4101 (4.5%) porB 5912 (3.7%)	**90**	tbpB 241 (8.5%) tbpB 29 (7.8%) tbpB 4 (7.1%) tbpB 110 (6.1%) tbpB 1744 (6.0%)	**201**	4186 (7.1%) 9654 (5.6%) 4244 (4.8%) 10039 (3.7%) 5004 (2.3%),	**PIA - 140 (22.1%)** **PIB - 493 (77.8%)**	**18 (2.8%)**
2015**	**454**	**115**	porB 1808 (12.1%) porB 2569 (10.1%) porB 147 (8.14%) porB 2656 (5.0%) porB 543(4.1%)	**74**	tbpB 241 (16.5%) tbpB 4 (6.4%) tbpB 29 (5.9%) tbpB 893 (5.7%) tbpB 1744 (5.3%)	**154**	4186 (10.1%) 4244 (5.2%) 9654 (4.4%) 9909 (3.9%) 11821 (3.7%)	**PIA - 87 (19.1%)** **PIB - 367 (80.8%)**	**6 (1.3%)**

*percentage of total isolates

**Data Collected up until August 2015

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 to 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 amongst 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, $p=0.6$).

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

Risk factor		Non-DGI	DGI	Total	DGI as percentage (%)	Univariate OR	*p* value
porB class	PIA	1019	59	1078	5.5	33.23	<0.0001
	PIB	2870	5	2875	0.2	0.03	
Geographic 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	ST758	17	2	19	10.5	3.67	0.03
	ST6876	296	6	302	2.0	1.2	0.6

types	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%**		

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 one 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 health care. 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 to SEQ, and with strains harbouring the PIA gene. This finding agrees with 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 co-existence of genital infections for DGI cases was 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.

Contributorship statement

Amy Jennison and David Whiley conceptualised the study. Christine Guglielmino conducted the laboratory investigation. Christine Guglielmino, Sumeet Sandhu, Colleen Lau, Cameron Buckley and Ella Trembizki performed formal data analysis. All authors contributed to the writing and review of the manuscript.

Acknowledgments

This publication made use of the Neisseria Multi Locus Sequence Typing website (https://pubmlst.org/neisseria/) developed by Keith Jolley and sited at the University of Oxford [23]. The development of this site has been funded by the Wellcome Trust and European Union. We thank all laboratories for referring isolates included in this study, and Public Health Microbiology Staff for technical work.

Conflicts of interest. None

Data Availability

WGS raw sequence files and associated metadata have been submitted to the European Nucleotide Archive with Project Accession number PRJEB52601.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Transparency declarations: D.M.W. reports research funding from SpeeDx Pty Ltd and is supported by a Queensland Advancing Clinical Research Fellowship from the Queensland Government. E.T is holding an NHMRC Early Career Fellowship. CLL is supported by an NHMRC Investigator Grant (APP1193826).

Ethics Approvals: The study was approved by Forensic and Scientific Services Human Ethics Committee (FSS-HEC, EC00305). HEC Ref Number HEC18_01.

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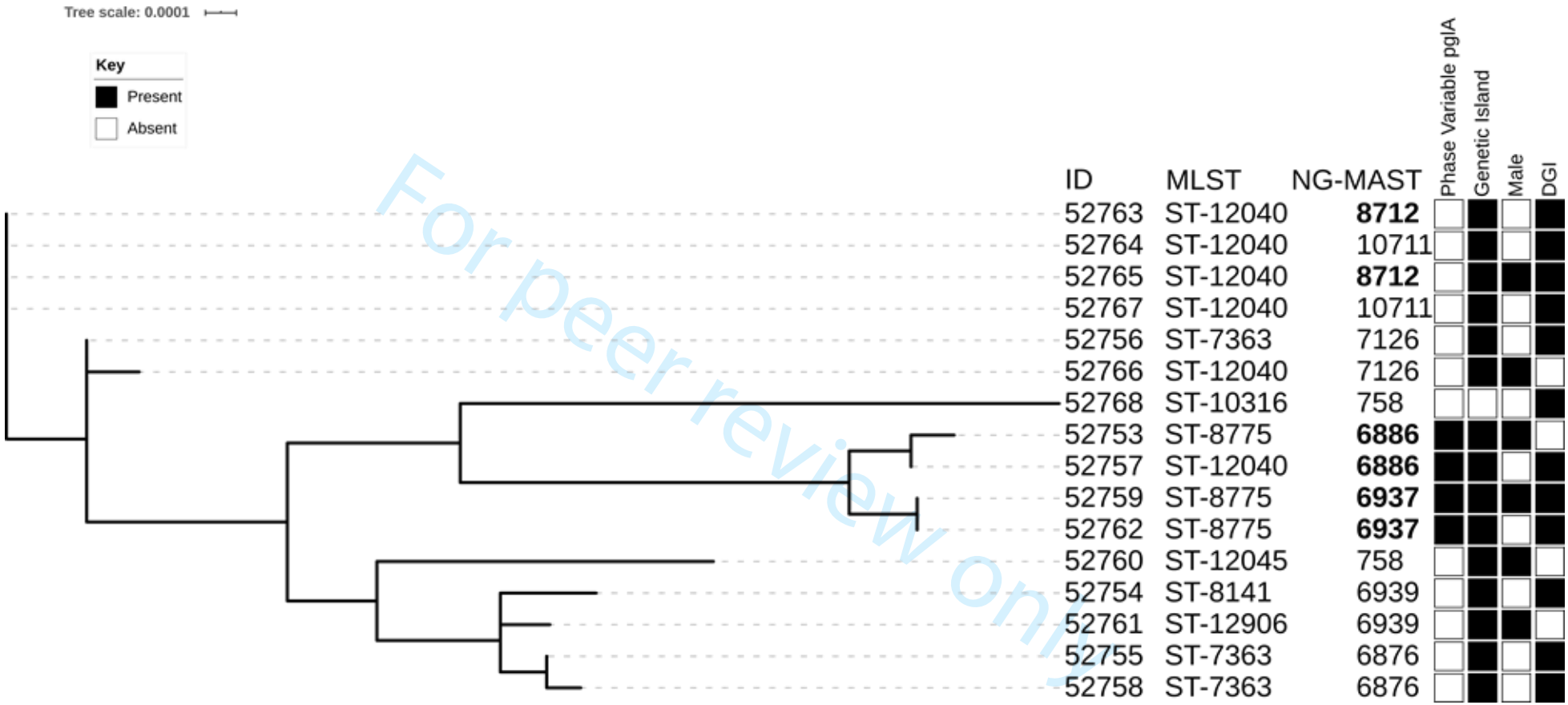
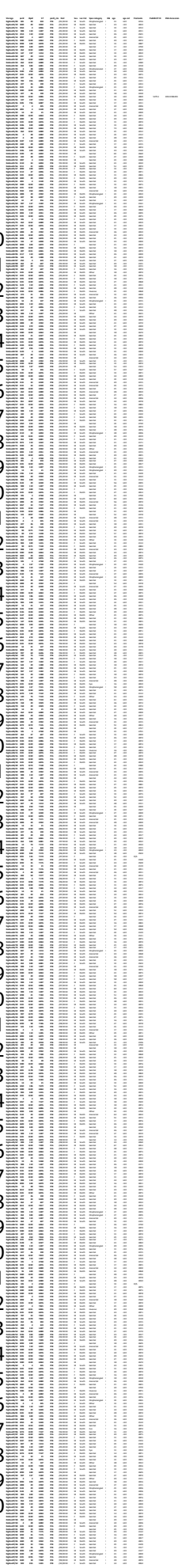
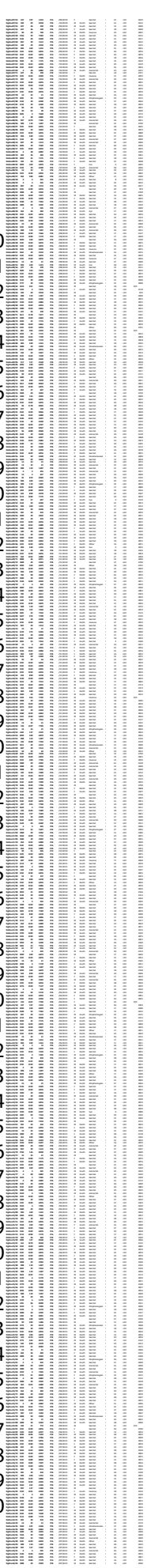
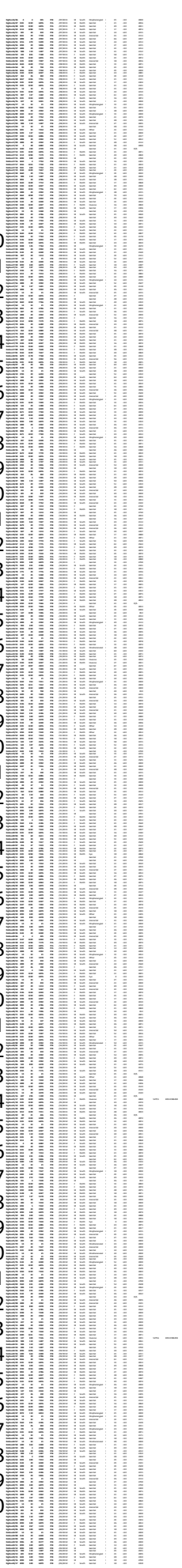
Figure Legend

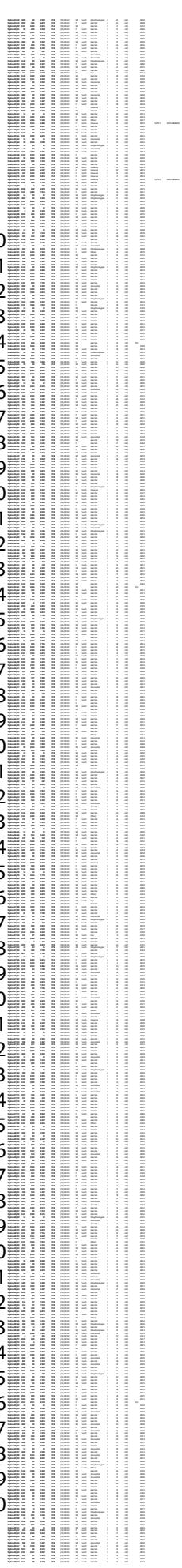
Figure 1: Core genome maximum likelihood phylogeny of 16 PIA strains of *N. gonorrhoeae* from Queensland based on cgMLST. The tree is rooted at centre-point and annotated with strain ID, NG-MAST associated with DGI, and sequence types derived from MLST and NG-MAST, with presence/absence of phase variable *pglA* and gonococcal genetic island. The phylogenetic distance is indicated by the length of the horizontal lines. Visualised with iTOL [27]. Created by the authors.

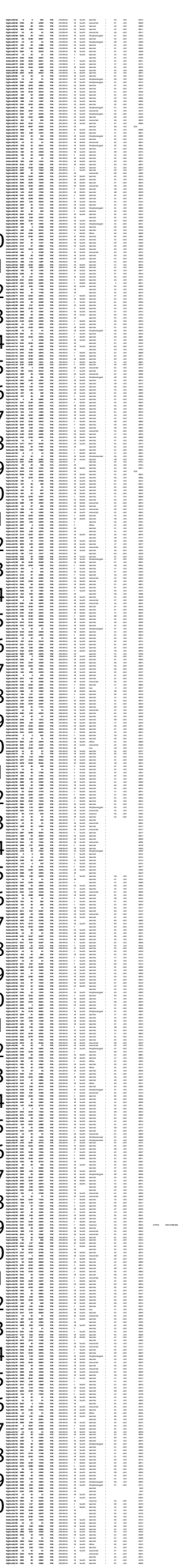


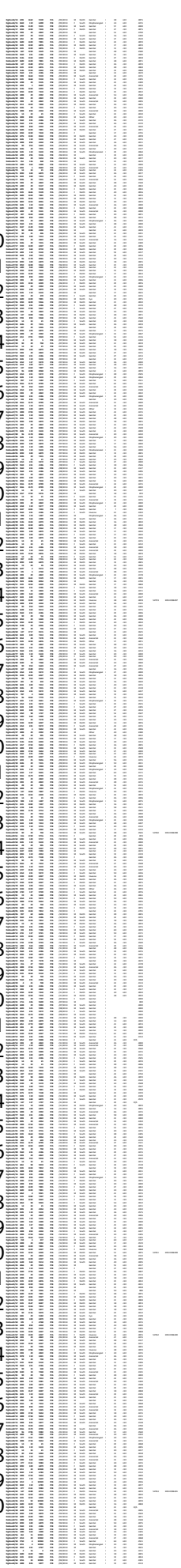


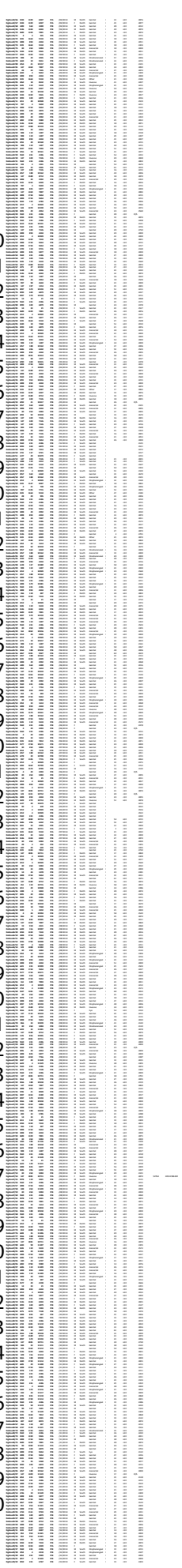


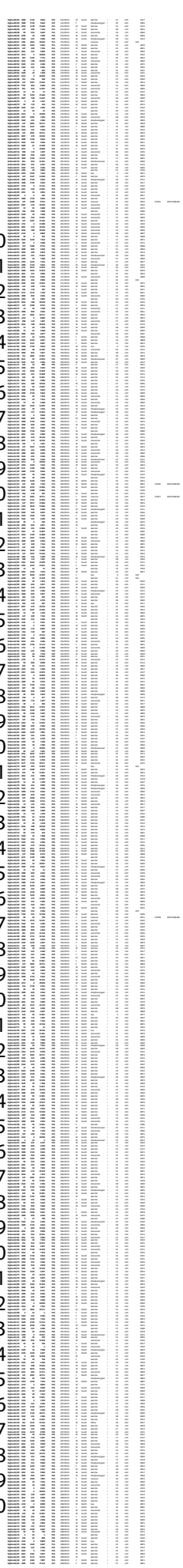












STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4-5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*4*
Bias	9	Describe any efforts to address potential sources of bias	6, 12
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5,6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	5
		(*b*) Describe any methods used to examine subgroups and interactions	5
		(*c*) Explain how missing data were addressed	NA
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	NA
		(*e*) Describe any sensitivity analyses	NA
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	4
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	4
		(b) Indicate number of participants with missing data for each variable of interest	4
Outcome data	15*	Report numbers of outcome events or summary measures	8-11
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make	6,8,10,11

		clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	10
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	10
Discussion			
Key results	18	Summarise key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12
Generalisability	21	Discuss the generalisability (external validity) of the study results	12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

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

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2022-061040.R2
Article Type:	Original research
Date Submitted by the Author:	30-Jun-2022
Complete List of Authors:	Guglielmino, Christine; Health Support Queensland Forensic and Scientific Services Sandhu, Sumeet; Health Support Queensland Forensic and Scientific Services Lau, Colleen L.; Australian National University Research School of Population Health Buckely, Cameron; UQ Centre for Clinical Research, University of Queensland Trembizki, Ella; UQ Centre for Clinical Research, University of Queensland Whiley, David; UQ Centre for Clinical Research, University of Queensland Jennison, Amy; Health Support Queensland Forensic and Scientific Services
Primary Subject Heading:	Sexual health
Secondary Subject Heading:	Public health, Infectious diseases, Epidemiology
Keywords:	Epidemiology < TROPICAL MEDICINE, INFECTIOUS DISEASES, MOLECULAR BIOLOGY

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

Running Title: DGI and *porB* genotype

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Abstract word count:227

Text word count: 2113

ABSTRACT

Objectives: Gonorrhoea caused by *Neisseria gonorrhoeae* is the second most notified sexually transmitted infection in Australia and 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 aimed 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 *Neisseria gonorrhoeae* isolates from both metropolitan and regional Queensland infections were typed with NG-MAST (*N. gonorrhoeae* multi antigen 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.

Keywords: Neisseria gonorrhoeae; Gonorrhoea; DGI; Australia; Genotype; NG-MAST; WGS

Strengths and limitations of this study

- Genetic diversity of over 3500 *N. gonorrhoeae* isolates were assessed by NG-MAST genotyping.
- Demographic factors associated with NG infection and 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 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 (T4SS) 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 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 utilised 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 NAAT only, with no isolate available for further testing. Diagnostic methods used varied across the state, with 80% of cases in the northern Queensland 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 one 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, <30 years and ≥30 years. Postcode of residence was used to assign broad geographic region categories of NQ (northern Queensland) and SEQ (south-east Queensland) 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.

NG-MAST typing and phylogenetic analysis

Crude DNA extracts of all the *N. gonorrhoeae* isolates received in the study period were routinely subjected to *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) as previously described [19]. These extracts were prepared by boiling 400µL of TE buffer containing a loopful (1µL) of pure *N. gonorrhoeae* colonies at 100°C for 10 minutes. 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, CA, USA) using NextSeq 500 Mid Output V2 kit (Illumina) with Nextera XT library preparation. Reads were trimmed with Trimmomatic (Version 0.36) [21], corrected and assembled with Spades (Version 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+ 4.1.0 (Ridom GmbH, Germany) using alleles from 1,649 *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 Queensland yearly population as denominators. Categorical variables were examined using the Fischer's Exact test performed in GraphPad Prism

7 (GraphPad Software Inc., California). Odds ratios (OR) with 95% confidence intervals 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

The total 3,953 *N. gonorrhoeae* isolates in this study from January 2010 to August 2015 consisted of genital (n=3,099; 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 Supplementary 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 year 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 to *porB* class PIA. Table 1 shows a breakdown for all strains by PIA/PIB, NQ/SEQ, and age group.

Table 1: Demographics of *N. gonorrhoeae* isolates, Queensland January 2010 to August 2015

Demographics	n	Percentage (%) of total
All cases	3953	100

porB Class	PIA	1078	27.3
	PIB	2875	72.7
Geographic location	NQ	1193	30.1
	SEQ	2403	60.7
	Others	357	9.0
Sex	F	1055	26.6
	M	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

NG-MAST typing and phylogenetic analysis

Among the 3,953 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 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 five years.

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

Year	Number of *N. gonorrhoeae* isolates	Number of *porB* alleles	Most frequent *porB* alleles	Number 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%) porB 2280 (5.3%) porB 908 (4.7%) porB 1808 (4.4%) porB 4143 (3.7%)	**87**	tbpB 29 (17.6%) tbpB 1330 (16.3%) tbpB 1329 (8.4%) tbpB 110 (6.6%) tbpB 349 (6.1%)	**208**	6876 (16.1%) 6863 (5.4%) 1407 (4.7%) 2992 (4.4%) 6940 (3.7%)	**PIA - 255 (32.6%)** **PIB - 527 (67.3%)**	**12 (1.5%)**
2011	**754**	**157**	porB 4101 (17.9%) porB 1808 (8.8%) porB 4099 (5.3%) porB 14 (4.7%) porB 4104 (3.7%)	**85**	tbpB 29 (24.0%) tbpB 1330 (15.5%) tbpB 349 (7.0%) tbpB 33 (6.7%) tbpB 1329 (6.1%)	**206**	6876 (14.4%) 2992 (8.8%) 6879 (4.7%) 21 (4.4%) 6937 (3.2%)	**PIA - 240 (31.8%)** **PIB - 514 (68.1%)**	**8 (1.0%)**
2012	**680**	**156**	porB 4101 (12.2%) porB 14 (8.6%) porB 4099 (7.7%) porB 1808 (7.6%) porB 4104 (5.5%)	**91**	tbpB 29 (19.7%) tbpB 349 (12.3%) tbpB 33 (10.8%) tbpB 1330 (9.1%) tbpB 1329 (7.0%)	**203**	21 (8.6%) 2992 (7.6%) 6879 (7.5%) 6876 (7.0%) 6937 (4.1%)	**PIA - 195 (28.6%)** **PIB - 485 (71.3%)**	**10 (1.4%)**
2013	**650**	**167**	porB 1808 (7.2%) porB 4101 (6.7%) porB 1903 (6.1%) porB 14 (6.0%) porB 4099 (5.3%)	**93**	tbpB 29 (18.9%) tbpB 349 (8.3%) tbpB 33 (7.5%) tbpB 110 (6.9%) tbpB 241 (6.9%)	**217**	21 (6.0%) 4822 (6.0%) 6879 (5.3%) 4186 (5.2%) 5533 (4.0%)	**PIA - 160 (24.6%)** **PIB - 490 (75.3%)**	**10 (1.5%)**
2014	**633**	**147**	porB 1808 (14.6%) porB 2569 (7.2%) porB 147 (6.4%) porB 4101 (4.5%) porB 5912 (3.7%)	**90**	tbpB 241 (8.5%) tbpB 29 (7.8%) tbpB 4 (7.1%) tbpB 110 (6.1%) tbpB 1744 (6.0%)	**201**	4186 (7.1%) 9654 (5.6%) 4244 (4.8%) 10039 (3.7%) 5004 (2.3%),	**PIA - 140 (22.1%)** **PIB - 493 (77.8%)**	**18 (2.8%)**
2015**	**454**	**115**	porB 1808 (12.1%) porB 2569 (10.1%) porB 147 (8.14%) porB 2656 (5.0%) porB 543(4.1%)	**74**	tbpB 241 (16.5%) tbpB 4 (6.4%) tbpB 29 (5.9%) tbpB 893 (5.7%) tbpB 1744 (5.3%)	**154**	4186 (10.1%) 4244 (5.2%) 9654 (4.4%) 9909 (3.9%) 11821 (3.7%)	**PIA - 87 (19.1%)** **PIB - 367 (80.8%)**	**6 (1.3%)**

*Percentage of total isolates

**Data collected up until August 2015

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 to 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 amongst 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, $p=0.6$).

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 percentage (%)	Univariate OR	*p* value
porB class	PIA	1019	59	1078	5.5	33.23	<0.0001
	PIB	2870	5	2875	0.2	0.03	
Geographic 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	ST758	17	2	19	10.5	3.67	0.03
	ST6876	296	6	302	2.0	1.2	0.6

types	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%**		

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 one 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 health care. 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 to SEQ, and with strains harbouring the PIA gene. This finding agrees with 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 co-existence of genital infections for DGI cases was 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.

Contributors

Amy Jennison and David Whiley conceptualised the study. Christine Guglielmino conducted the laboratory investigation. Christine Guglielmino, Sumeet Sandhu, Colleen Lau, Cameron Buckley and Ella Trembizki performed formal data analysis. All authors contributed to the writing and review of the manuscript.

Acknowledgments

This publication made use of the Neisseria Multi Locus Sequence Typing website (https://pubmlst.org/neisseria/) developed by Keith Jolley and sited at the University of Oxford [23]. The development of this site has been funded by the Wellcome Trust and European Union. We thank all laboratories for referring isolates included in this study, and Public Health Microbiology Staff for technical work.

Competing interests

None.

Data availability statement

WGS raw sequence files and associated metadata have been submitted to the European Nucleotide Archive with Project Accession number PRJEB52601.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Transparency declarations

D.M.W. reports research funding from SpeeDx Pty Ltd and is supported by a Queensland Advancing Clinical Research Fellowship from the Queensland Government. E.T is holding an NHMRC Early Career Fellowship. CLL is supported by an NHMRC Investigator Grant (APP1193826).

Ethics approval

The study was approved by Forensic and Scientific Services Human Ethics Committee (FSS-HEC, EC00305). HEC Ref Number HEC18_01.

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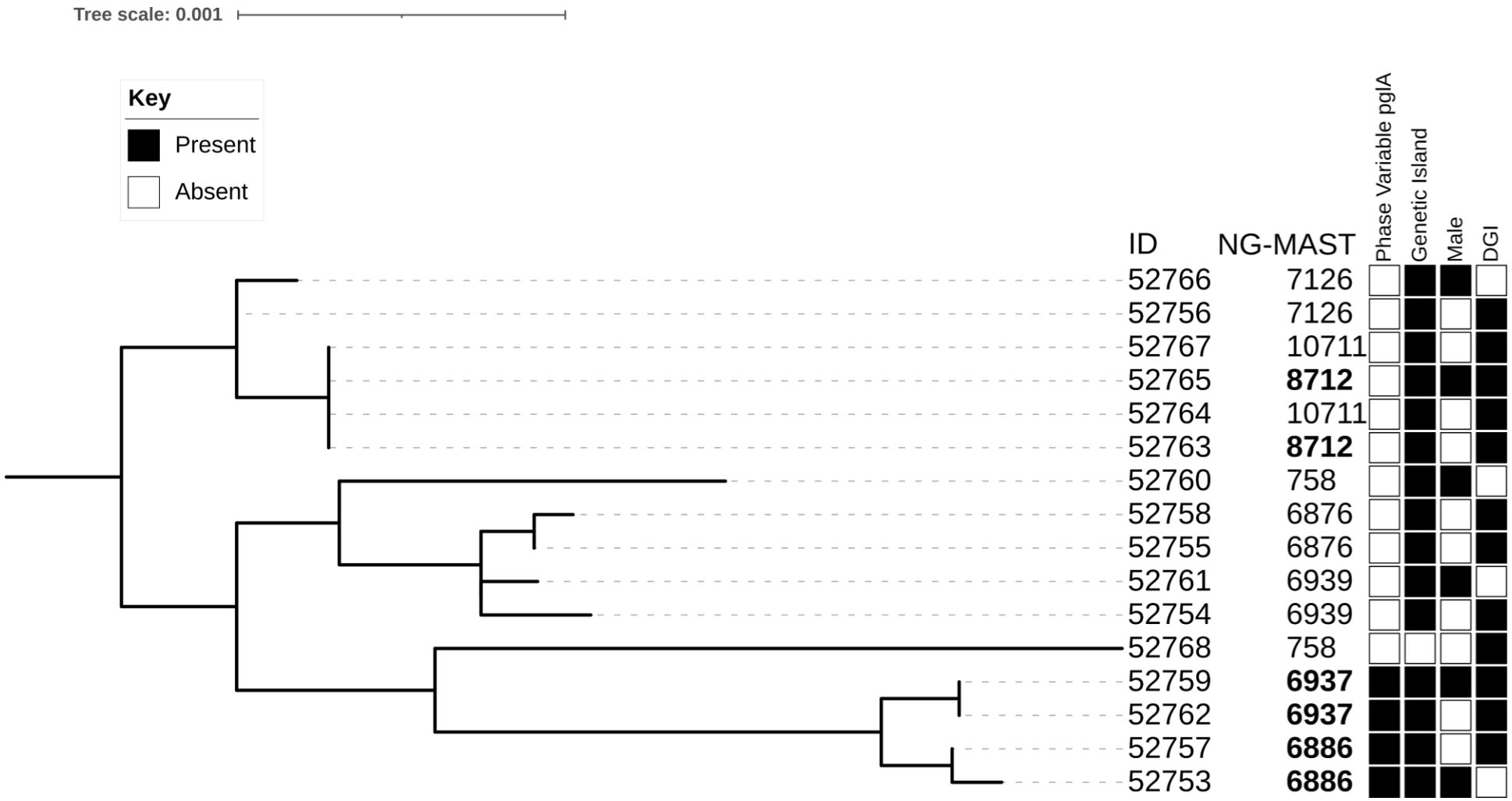
Figure legend

Figure 1: Core genome maximum likelihood phylogeny of 16 PIA strains of *N. 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].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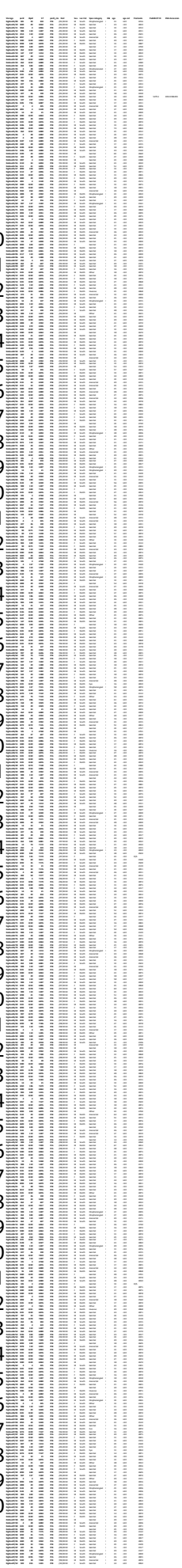

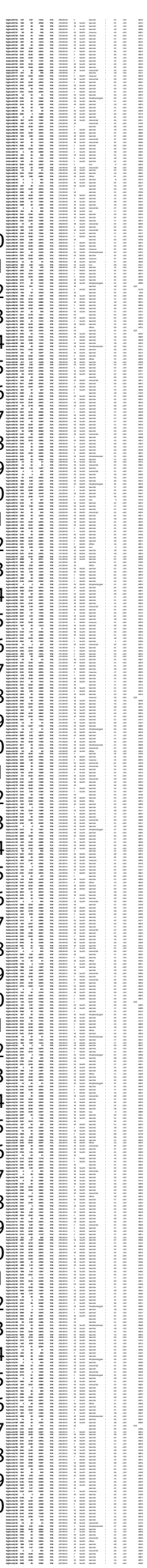

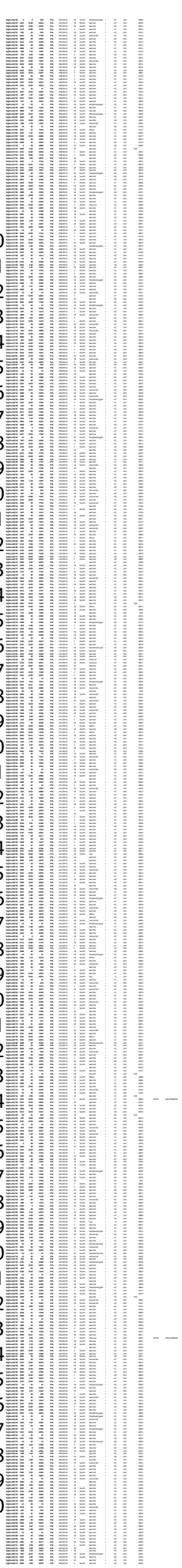

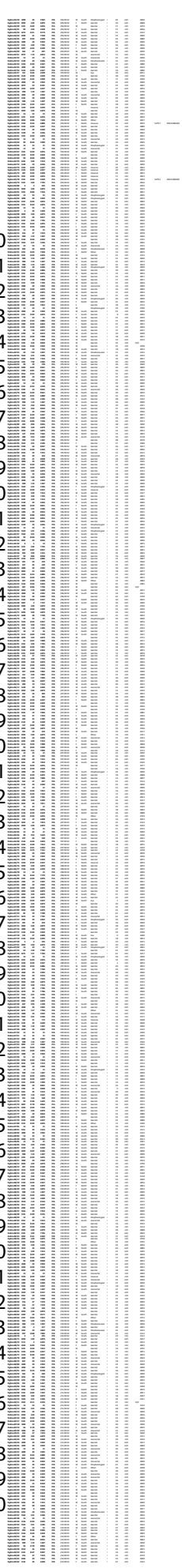

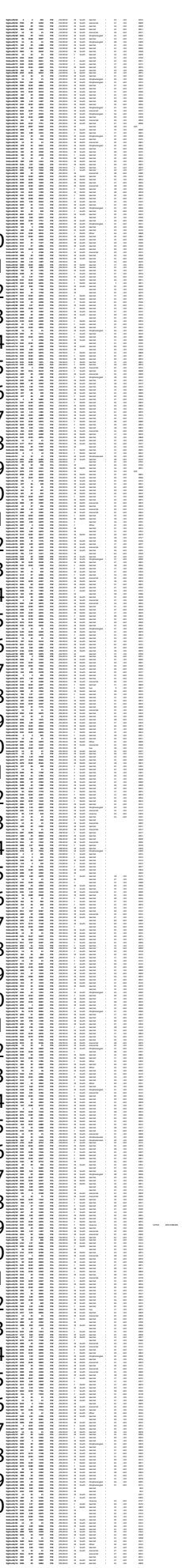

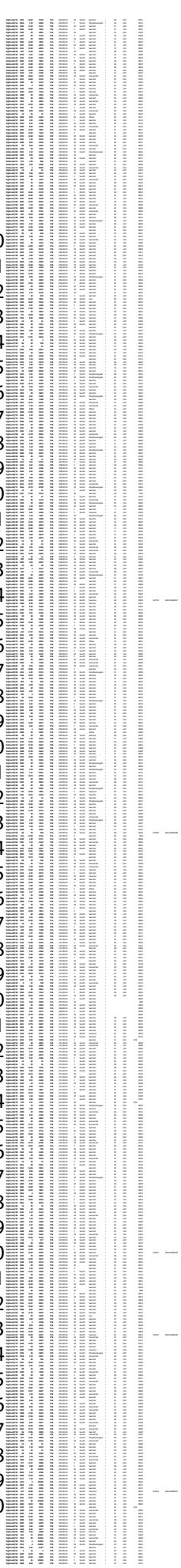

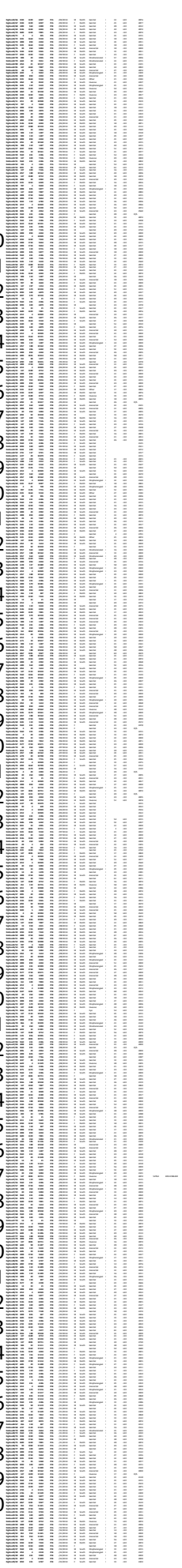

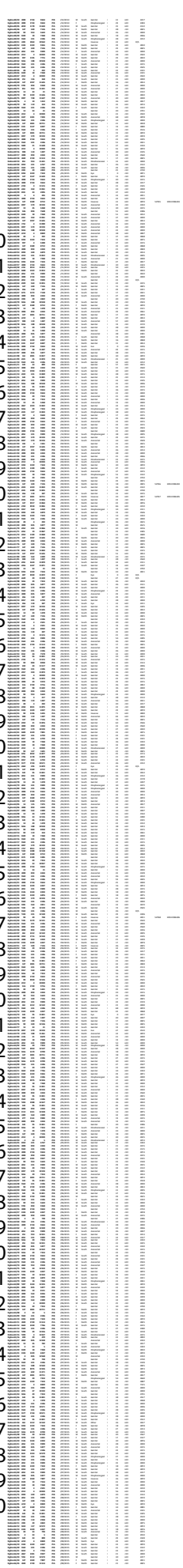

STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State specific objectives, including any prespecified hypotheses	3
Methods			
Study design	4	Present key elements of study design early in the paper	4-5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*4*
Bias	9	Describe any efforts to address potential sources of bias	6, 12
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5,6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	5
		(*b*) Describe any methods used to examine subgroups and interactions	5
		(*c*) Explain how missing data were addressed	NA
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	NA
		(*e*) Describe any sensitivity analyses	NA
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	4
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	4
		(b) Indicate number of participants with missing data for each variable of interest	4
Outcome data	15*	Report numbers of outcome events or summary measures	8-11
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make	6,8,10,11

		clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	10
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	10
Discussion			
Key results	18	Summarise key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12
Generalisability	21	Discuss the generalisability (external validity) of the study results	12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.