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The Molecular Epidemiology and Antimicrobial Resistance of *Neisseria gonorrhoeae* in Australia: A Nationwide Cross-Sectional Study, 2012

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Background. Antimicrobial resistance (AMR) by *Neisseria gonorrhoeae* is considered a serious global threat.

Methods. In this nationwide study, we used MassARRAY iPLEX genotyping technology to examine the epidemiology of *N. gonorrhoeae* and associated AMR in the Australian population. All available *N. gonorrhoeae* isolates (n = 2452) received from Australian reference laboratories from January to June 2012 were included in the study. Genotypic data were combined with phenotypic AMR information to define strain types.

Results. A total of 270 distinct strain types were observed. The 40 most common strain types accounted for over 80% of isolates, and the 10 most common strain types accounted for almost half of all isolates. The high male to female ratios (>94% male) suggested that at least 22 of the top 40 strain types were primarily circulating within networks of men who have sex with men (MSM). Particular strain types were also concentrated among females: two strain types accounted for 37.5% of all isolates from females. Isolates harbouring the mosaic penicillin binding protein 2 (PBP2)—considered a key mechanism for cephalosporin resistance—comprised 8.9% of all *N. gonorrhoeae* isolates and were primarily observed in males (95%).

Conclusions. This large scale epidemiological investigation demonstrated that *N. gonorrhoeae* infections are dominated by relatively few strain types. The commonest strain types were concentrated in MSM in urban areas and Indigenous heterosexuals in remote areas, and we were able to confirm a resurgent epidemic in heterosexual networks in urban areas. The prevalence of mosaic PBP2 harboring *N. gonorrhoeae* strains highlight the ability for new *N. gonorrhoeae* strains to spread and become established across populations.

Keywords. gonorrhea; typing; resistance; molecular; surveillance.

The ability of *Neisseria gonorrhoeae* to develop and disseminate antimicrobial resistance (AMR) is well recognized. Resistant *N. gonorrhoeae* is now considered a serious global threat by both the US Centers for Disease Control and the World Health Organization [1, 2]. A notable example of the global dissemination of *N. gonorrhoeae* resistance was the appearance and subsequent spread of quinolone-resistant *N. gonorrhoeae* in the early 1990s, leading to ciprofloxacin being removed from first-line treatment in most countries over the following decade [1, 2].

More recently, gonococci harboring a mosaic penicillin binding protein 2 (PBP2), considered an important mechanism of resistance to cephalosporins, have been reported globally [3–7]. These mosaic PBP2 strains have largely been responsible for the discontinuation of cefixime as the treatment of choice in many settings [8–12]. In addition, these mosaic strains have also raised concerns over the future of ceftriaxone treatment, with sporadic variants of mosaic PBP2 strains exhibiting in vitro resistance to ceftriaxone reported in Japan, Europe, and Australia respectively [4, 13, 14]. Combination antibiotic therapy with ceftriaxone and azithromycin is now recommended for treatment of gonorrhea in many regions to limit further development and spread of *N. gonorrhoeae* AMR [15].

In Australia, the issue of *N. gonorrhoeae* AMR reached a new level of significance with the appearance of the ceftriaxone-resistant A8806 strain in late 2013 [14]. Although ongoing

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surveillance data have shown no further evidence of this strain in Australia, there is concern over increasing levels of azithromycin resistance in Australia, including the recent observation of *N. gonorrhoeae* strains with high-level azithromycin resistance [16]. Prompted by these concerns, we sought to better understand the genetics and distribution of gonococcal AMR in Australia. A key question we sought to examine was whether there remained a potential for new resistant strains to rapidly spread, as observed for the ciprofloxacin resistant strains more than 2 decades earlier.

MATERIALS AND METHODS

Overview

We utilized high-throughput Agena Bioscience single nucleotide polymorphism (SNP) iPLEX genotyping technology to characterize all available *N. gonorrhoeae* isolated in Australia from the first half of 2012. The genotypic profile for each isolate was then combined with the phenotypic AMR profile from the Australian Gonococcal Surveillance Programme (AGSP) to define *N. gonorrhoeae* strain types. Strain types were then compared with basic demographic information, including patient sex and geographical location.

Study Population

Laboratories in each state of Australia provide laboratory analysis of gonococcal isolates as part of the AGSP. Each year, in excess of 4000 *N. gonorrhoeae* clinical isolates are tested. This equates to almost 1 in 3 of the approximately 15 000 *N. gonorrhoeae* infections notified in Australia each year, and on a per-capita basis is the most comprehensive bacterial-culture based surveillance program globally. A total of 2452 *N. gonorrhoeae* isolates were received for this study from 1 January to 30 June of 2012 [17] (Table 1). The isolates were estimated to comprise 98% of all gonococci isolated by culture and 34.4% of the total 7128 gonorrhea cases (including NAAT-positive samples) notified in Australia in this period [18]. Isolates were from the Australian jurisdictions of New South Wales (NSW), the Northern Territory (NT), Queensland (QLD), South Australia (SA), Victoria (VIC), and Western Australia (WA). Two Australian jurisdictions, the Australian Capital Territory (ACT) and Tasmania, did not provide isolates for the study. Phenotypic AMR profiles were obtained via the AGSP [19].

Strain-type Assignment

Isolates were genotyped using 2 iPLEX methods: (a) the iPLEX-MLST — a typing method we have previously described, targeting 14 informative SNPs on *N. gonorrhoeae* house-keeping genes, for high-throughput multi-locus sequence typing (MLST) [20]; and (b) the iPLEX-AMR—a method targeting 11 chromosomal mutations reported to contribute to *N. gonorrhoeae* AMR, comprising: 23S rRNA A2059G [21], 23S rRNA C2611T [21 22], GyrA S91F [23–26], GyrA D95G/A [23–26], PBP1 L421P [27], mtrR adenine-deletion [28], mtrR thymine-insertion [22], MtrR G45D [28], PBP2 345A insertion [29, 30], mosaic PBP2 [31], and PBP2 A501T/V [32]. In brief, the iPLEX-AMR method was performed as per the iPLEX-MLST assay [20] except that the oligonucleotides in Supplementary Tables 1 and 2 were used. The combined results from both iPLEX methods were used to assign a genotype. The genotypic data were then combined with the AGSP phenotypic AMR data to assign a strain type. The 397 QLD isolates were previously genotyped by iPLEX-MLST as part of the initial iPLEX-MLST validation [20] and were further analyzed here.

Statistical Analyses

Statistical analyses were conducted using descriptive statistics and visual-summary representations (R statistical software; R.3.1.3).

Ethical Approvals

The study was approved by the Human Research Ethics Committee (HREC) of Northern Territory Department of Health

Table 1. Summary of Isolates Genotyped by the iPLEX-MLST and iPLEX-AMR Assays

Region	No. of Isolates Genotyped[a]				No. of Notified Gonorrhoea Cases[b]	Proportion of Notified Cases That Were Genotyped
	Sex					
	Female	Male	Unspecified	Total		
NSW	99	663	0	762	2030	37.5%
NT	34	56	2	92	916	10.0%
QLD	89	233	5	327	1441	22.7%
SA	18	128	0	146	289	50.5%
VIC	70	548	8	626	1307	47.9%
WA	86	179	0	265	1099	24.1%
ACT[c]	0[c]	0[c]	0[c]	0[c]	35	0.0%
Tasmania[c]	0[c]	0[c]	0[c]	0[c]	11	0.0%
Total	396	1807	15	2218	7128	31.1%

Abbreviations: ACT, Australian Capital Territory; NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; VIC, Victoria; WA, Western Australia.

[a] Isolates successfully genotyped by both the iPLEX-MLST and iPLEX-AMR assays.

[b] These data include both *N. gonorrhoeae* culture-based and NAAT testing data.

[c] No isolates from the ACT or Tasmania were included in the study.

and Menzies School of Health; the Central Australian HREC; the South Eastern Sydney Local Health District HREC, the Queensland Children's Health Queensland HREC and The University of Queensland HREC.

RESULTS

Overview

Genotyping was successful (ie, full SNP profiles obtained by both iPLEX-MLST and iPLEX-AMR) for 2218/2452 (90.4%) of *N. gonorrhoeae* isolates. These comprised 1807 males, 396 females, and 15 patients for whom the sex was unspecified (Table 1). The remaining 237 isolates that failed to provide genotypic data were excluded from further analysis; the basis for these failures was not investigated, however based on a previous study is likely due to variations in primer targets [20, 33]. For the 2218 isolates with complete genotypic profiles, there were 107 and 46 unique profiles observed for the iPLEX-MLST and iPLEX-AMR assays, respectively. The combination of both iPLEX data sets resulted in 185 distinct genotypes (G1 to G185). When genotype was further combined with AMR phenotype, 270 unique strain types (S1 to S270) were observed. The determination of genotype and strain type for the 40 most common strain types is summarized in Supplementary Table 3.

The Most Common Strain Types

The frequency and cumulative frequency of the most common strain types are summarized in Figure 1. The proportion of isolates represented by individual strain types ranged from 0.05% (1/2218 isolates, S132 to S270) to 10.5% (233/2218, S01). The 40 most common strain types accounted for 80% of all *N. gonorrhoeae* isolates (1784/2,218; 80.4%) and the 10 most common strain types for approximately half (1087/2,218; 49.0%) of all *N. gonorrhoeae* isolates.

Total numbers of isolates for the 40 most common strain types by jurisdiction and gender are provided in Figure 2 and the extent to which the top 40 strain types were shared between jurisdiction is summarized in Supplementary Table 4. These show that 32 of the top 40 strain types were found in 2 or more jurisdictions. Eight strains types were found in all jurisdictions except the NT, and 19 of the top 40 genotypes were shared between the eastern states of NSW, VIC, and QLD. Overall, the ratio of males to females differed noticeably between certain strain types (Figure 3) and suggested that at least 22 of the top 40 strain types circulated mainly among MSM (S02, S04 to S10, S13, S16, S17, S23, S26, S27, S28, S32, S34, S35, S37, S39, S40; 94.4% to 100% of isolates were from males). Isolates from females were concentrated among particular strain types, including S01, S03, S11, S12, S14, S15, S18, S19, S20, S21, S22, S24, S29, S30, S31, S33, S36, and S38 for which females contributed 11.0% to 60.7% (mean 34.7%) of isolates. The combined isolate numbers of samples from females for strain types S01 and S03 (89 + 60 = 149 isolates) comprised 37.6% of the total number of isolates (149/ 396) from females in the study.

Further details for the 10 most common strain types are provided in Figure 4 and Supplementary Figure 1: the proportion of total cases in each jurisdiction caused by these strain types; and a heat map representing the isolate percentages of these

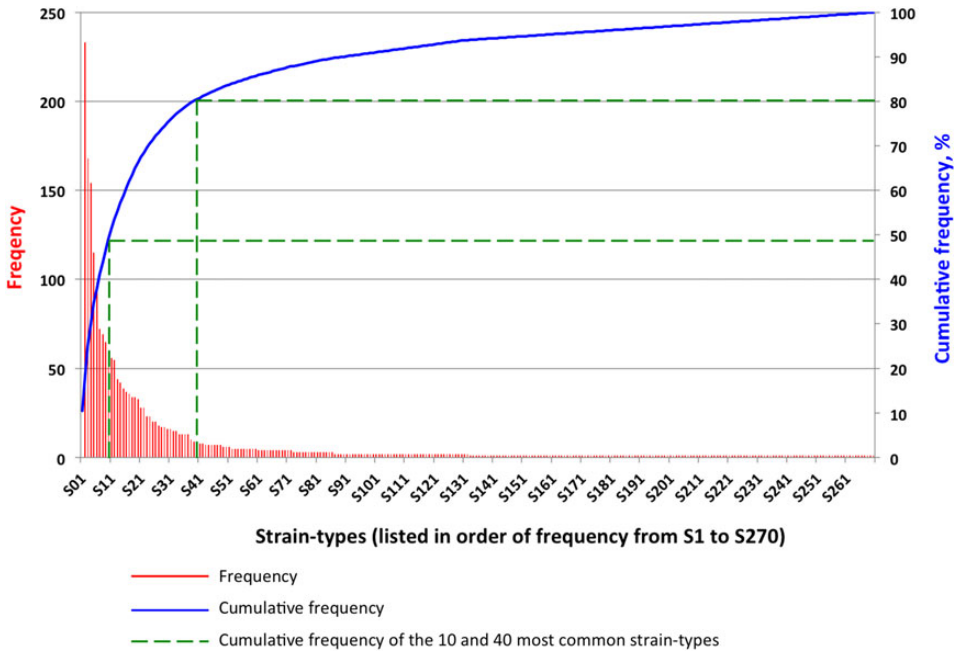

Figure 1. Frequency and cumulative frequency of 270 *N. gonorrhoeae* strain types.

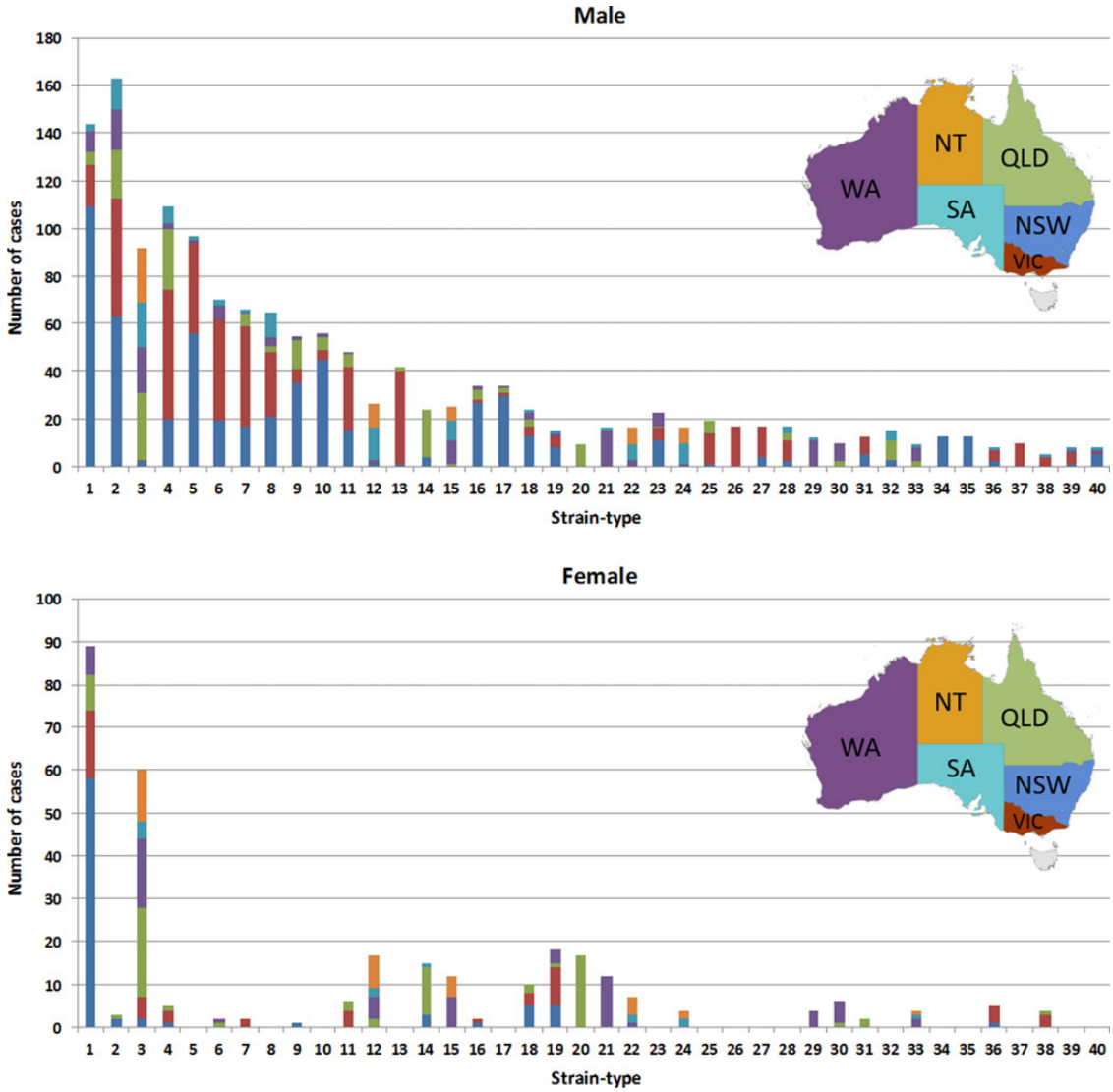

Figure 2. Total isolate numbers of the 40 most common *N. gonorrhoeae* strain types by jurisdiction and sex. (A different color is used to indicate each jurisdiction). Abbreviations: NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; VIC, Victoria; WA, Western Australia.

strain types by jurisdiction, sex, and AMR profile, respectively. The 3 most common strain types (S01, S02, and S03) illustrate contrasting patterns. S02 was highly concentrated in men (presumed to be MSM) in the jurisdictions with the largest urban centers, whereas S03 demonstrates a heterosexual pattern and is concentrated in the jurisdictions that provide most *N. gonorrhoeae* notifications from Indigenous people (Qld, WA, SA and particularly NT [34]). By contrast, S01 had a heterosexual distribution and was concentrated in jurisdictions with large urban centers (particularly NSW).

Supplementary Figure 2 provides a summary of the 10 most common strain types in each jurisdiction. It indicates that many of the top-10 strain types were shared between the states; for example, strain types S01, S02, S04, S05, S06 and S08 comprised 6 of the top-10 strain types in both NSW and VIC (jurisdictions with the largest urban centers), and S03 was the most common strain type in Qld, WA, SA, and the NT.

AMR Distribution

Nineteen of the top 40 strain types were sensitive to all antibiotics tested (Supplementary Table 3; see also Supplementary Figure 1 which shows the top-10). Other strain types provided examples of resistant clones, including those exhibiting resistance to penicillin (eg, S04, S05, and S07; 5.2%, 4.4%, and 3.1% of all isolates respectively; Supplementary Table 3 and Figure 1) and resistance to ciprofloxacin (eg, S06, S07, and S10; 3.2%, 3.1%, and 2.5% of all isolates, respectively; Supplementary Table 3 and Figure 1).

Mosaic PBP2

Strain types harboring a mosaic PBP2 protein (including S07, S10, and S11, Supplementary Table 3) comprised 8.9% (198/

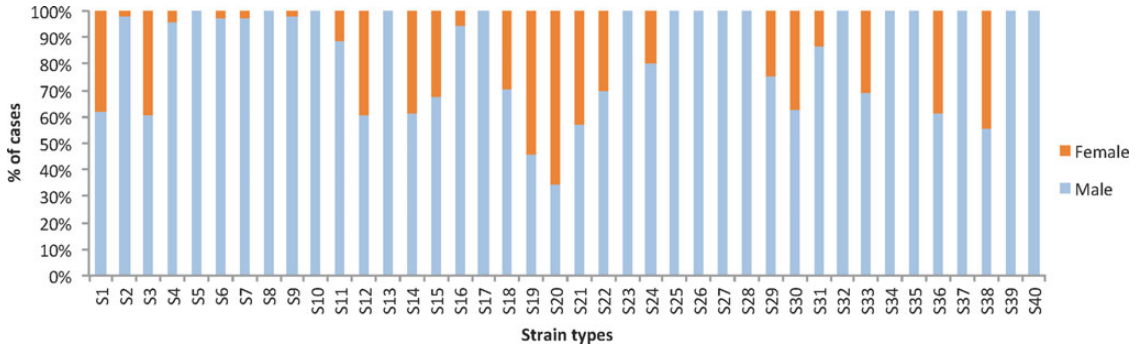

Figure 3. Male to female ratio for each of the top 40 stain types.

2218) of all isolates. These were overwhelmingly found in males, presumably MSM (188/198; 94.9%). Of note, only 30.5% of the mosaic PBP2 isolates phenotypically exhibited decreased susceptibility to ceftriaxone (minimum inhibitory concentrations [MICs] of 0.06 to 0.125 mg/L; eg, S11, Supplementary Table 3). The remaining mosaic PBP2 isolates (including S07 and S10; Supplementary Table 3) exhibited MICs of ≤0.008 mg/L (3.9% of isolates), 0.016 mg/L (17.8%) and 0.03 mg/L (47.8%).

Azithromycin Resistance and 23S rRNA Mutations

A total of 47 isolates (2.1% of the 2218 isolates; 37 male, 10 female) harbored the C2611T 23S rRNA mutation associated with *N. gonorrhoeae* resistance to azithromycin. Of interest was that almost 50% (23 of 47) of isolates were phenotypically susceptible to azithromycin (MICs of 0.125 mg/L [n = 2 isolates], 0.25 mg/L [n = 16] and 0.5 mg/L [n = 5]) and possessed mixtures of both wild type and C2611T alleles on the 23S rRNA genes (see eg, strain type S31; Supplementary Table 3); *N. gonorrhoeae* has 4 copies of the 23S rRNA gene, is able to harbor mixtures of both wild type and mutant alleles among these 4 copies, and resistance is influenced by the number of mutant alleles [22].

DISCUSSION

To our knowledge this study represents the largest national cross-sectional nationwide molecular epidemiology study of *N. gonorrhoeae* conducted to date and provides new insights into *N. gonorrhoeae* epidemiology that were not evident using current surveillance approaches, particularly those relying solely on AMR phenotype. These data demonstrate that, although large numbers of different gonococcal strains may be circulating within a population at any given time, *N. gonorrhoeae* infections on a population level are dominated by a relatively small

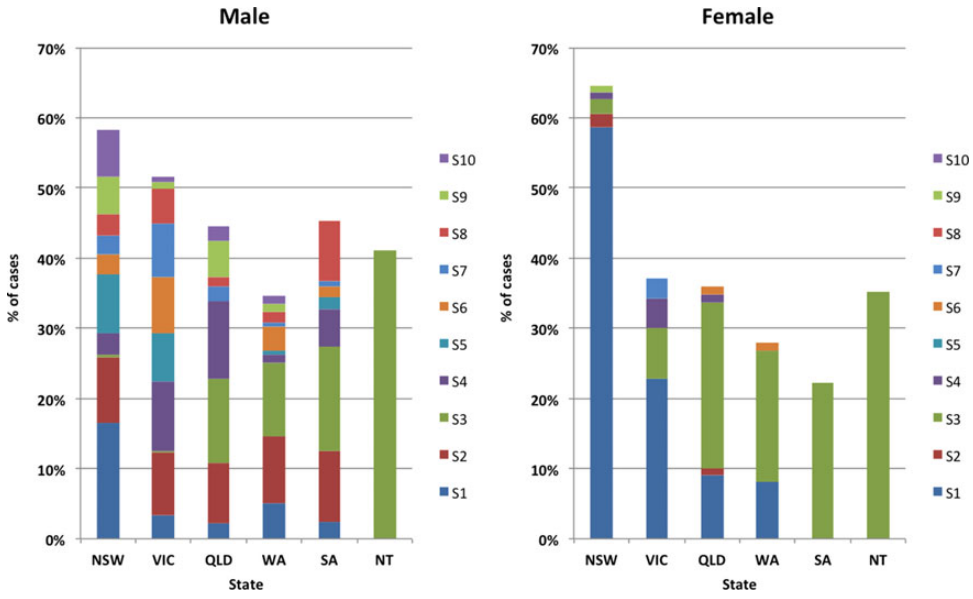

Figure 4. Proportion of total cases in each state caused by the 10 most common *N. gonorrhoeae* strain types in Australia. Abbreviations: NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; VIC, Victoria; WA, Western Australia.

number of strains. Although 270 different strain types were observed the 40 most common and 10 most common strain types accounted for 80% and almost half of all *N. gonorrhoeae* isolates, respectively. Also, the data provide further evidence of different strain types to predominate in distinct homosexual and heterosexual networks (based on male to female ratios), the ability of certain *N. gonorrhoeae* strains to disseminate widely and moreover the ability of molecular methods to identify new and emerging *N. gonorrhoeae* trends.

The 3 most common strain types S01, S02, and S03 comprised approximately 25% of all *N. gonorrhoeae* isolates. The observations associated with strain types S02 and S03 were consistent with recognized *N. gonorrhoeae* infection patterns in Australia where there are 2 distinct concentrations of *N. gonorrhoeae* infections, one in urban MSM and the other in young heterosexual people living in remote Indigenous communities [34, 35], and these are largely reflected by strain types S02 (found principally in men in eastern Australian states, where there are larger populations of MSM) and S03 (in both genders in western and northern regions where most remote Indigenous communities are located). The geographic distribution of S01 isolates, which was the most common strain type overall and the most common in NSW (Supplementary Figure 2), was similar to S02 isolates. However, unlike S02, which was almost exclusively found in MSM, S01 appeared to be principally in heterosexual networks based on the male to female ratio (61.8% M: 39.2% F; see also Figure 3). These data confirm a third epidemic emerging in heterosexuals in urban areas in eastern Australia and is consistent with increasing isolation rates and notifications of *N. gonorrhoeae* among females in NSW, which had effectively doubled in the period from 2010 to 2012 [36–39]. This resurgence of *N. gonorrhoeae* among urban heterosexuals had been suspected in recent years [34], but there was uncertainty because over the same period duplex NAAT testing for chlamydia and *N. gonorrhoeae* had become routine in Australian clinical laboratories [40] raising the possibility of a testing artifact or even false positive *N. gonorrhoeae* test results [41]. As recently as 2007, *N. gonorrhoeae* was described as rare in urban heterosexuals and was often associated with importation [42]. The fact that S01 was so common in urban heterosexuals by 2012 indicates sustained endemic transmission rather than ongoing importation which would not result in any common strain types.

In terms of *N. gonorrhoeae* AMR surveillance, it is of interest to note that the above 3 strain types were sensitive to all antibiotics tested and in fact had few genetic resistance mutations (Supplementary Table 3). Through our AGSP culture-based surveillance we had previously recognized the large proportions of sensitive strains circulating in our population but their genetic relatedness was undetermined. Accordingly, these data highlight the important role that molecular typing can play in teasing out phenotypic data, which typically provide only limited discriminatory power, and thereby enhancing our ability to identify underlying and previously unrecognized *N. gonorrhoeae* trends. Moreover, these molecular typing data could help inform public health responses. A previous Australian study [43] has shown how the combined use of phenotypic and genotypic data can be used to target an intervention against a particular *N. gonorrhoeae* strain; in that study the measures that were implemented, including enhanced contact tracing, were successful in containing an outbreak of gonorrhea that occurred mainly among heterosexual men. It is therefore possible that a similar intervention focused on populations associated with strain types S01 and S03 (which comprised almost 40% of all female infections) could have proved useful in reducing *N. gonorrhoeae* infection and related sequelae particularly among females.

The data also highlight the important influence that clonal expansion of particular *N. gonorrhoeae* strains has upon rates of AMR. Stain types S04, S05, and S07 provide a good example of this; all were resistant to penicillin and together comprised 12.7% of all isolates. AGSP data from 2012 showed that rates of *N. gonorrhoeae* penicillin resistance were 32.1% [38]. Hence, these 3 strain types together accounted for a significant proportion (almost 40%) of all *N. gonorrhoeae* penicillin resistance in the country. Isolates possessing the mosaic PBP2 provide a more recent example of clonal expansion. Our earlier data suggested that mosaic PBP2-harboring *N. gonorrhoeae* strains first appeared in the Australian population around 2002 [44], not long after they were first described in Japan [31]. These new molecular data now show that mosaic PBP2-harbouring isolates have since become well established in the population, comprising 8.9% of all *N. gonorrhoeae* isolates. Although mosaic PBP2-harboring strain types were found primarily in male patients, 5.1% were also identified in female patients, suggesting bridging from MSM into heterosexual networks via bisexual men. These data are consistent with a recent study of mosaic PBP2 isolates in the United States regarding network bridging [45]. For AMR surveillance, it is also of interest to note that we observed 23 isolates that were phenotypically susceptible to azithromycin yet possessed mixtures of both wild type and C2611T 23S rRNA allelles. In addition, only 30.5% of our mosaic PBP2 isolates were actually classified as exhibiting decreased susceptibility to ceftriaxone. These data highlight possible limitations of relying solely on phenotypic classifications and raise new questions over whether surveillance should also involve screening for genetic potential for AMR. Further studies are needed to answer these questions, including whether genetic potential for AMR in gonococci is important in influencing treatment outcomes.

This study had several limitations:(1) In the absence of any behavioral data, mode of transmission (homosexual or heterosexual) had to be inferred from M:F ratios of *N. gonorrhoeae* isolates; (2) In the absence of precise geographical data such as postcode or Indigenous status, the relative distribution of

N. gonorrhoeae strain types in Indigenous and non-Indigenous populations also had to be inferred from notification data [33]; (3) We also did not have individual data for anatomic site of infection and therefore could not compare strain types between sites; (4) There may be some bias in the isolate collection given isolation rates were typically higher in some jurisdictions compared to others, and also that two jurisdictions (the ACT and Tasmania; albeit having the lowest number of cases) did not provide isolates for the study (see Table 1); (5) The isolate panel is now 4 years old and so changes in strain-type patterns may have occurred; and (6) Strain types may have been underestimated or missed given 237 isolates were unable to be typed by the iPLEX methods.

In summary, the results of this study show that *N. gonorrhoeae* infections in Australia were dominated by relatively few strain types. The distribution of the most common strain type (S01) enabled us to confirm a resurgent heterosexually transmitted *N. gonorrhoeae* epidemic in Australia's largest cities. The success of a relatively small number of strain types, including the relatively recent expansion of mosaic PBP2 strains, is alarming and further highlights an existing theoretical potential for new (potentially extensively drug resistant) strains to readily spread and become established in our country. Hence, a repeat of the (quinolone) resistant-strain incursion of the 1990s [46] remains conceivable. Overall, these data highlight the limitations of relying solely on phenotypic-based surveillance to detect the presence of emerging or proliferating strains, or to identify the genetic potential for AMR. It should be noted that we do not see these data as devaluing the critical role of phenotypic-based AMR surveillance, including the important ongoing work of the AGSP. Rather, it is our opinion that molecular typing tools must be used in parallel with phenotypic surveillance to better map the epidemiology of *N. gonorrhoeae*, to detect emerging AMR at the point where public health interventions may be effective and ultimately to inform targeted public health responses.

Supplementary Data

Supplementary materials are available at http://cid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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