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From shoe-string to reference laboratory - testing for Hepatitis C virus antiviral drug resistance at Westmead

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Australia is in the midst of a treatment revolution for people living with chronic hepatitis C virus (HCV) infection. The new therapies mean that HCV elimination is a realistic possibility in the next decade. However, an important and unexplored thorn in the side of this plan is the potential for emergence and persistence of antiviral drug resistance in those who fail treatment. Testing for HCV resistance is recommended for patients failing antiviral therapy, but until recently there was nowhere in Australia offering this service. In late 2016 we established HCV resistance testing at Westmead, the first lab in Australia to do so, and have offered it as a free service since the beginning of 2017. To date we have tested blood from over 600 patients around Australia and confirmed high rates of resistance in patients failing treatment. In this article we summarise preliminary results from the first 18 months of testing (484 patients). In 2019, HCV resistance testing at Westmead has progressed from the research lab to the diagnostic lab, with Westmead now recognised as the NSW reference laboratory.

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Introduction

Chronic hepatitis C is the leading cause of cirrhosis, liver cancer and liver transplantation in Australia, with annual health care costs of ~ \$250 million [1]. Until recently, treatment involved 6-12 months of interferon, which cured fewer than 50% of people and caused significant side effects. Since March 2016, interferon-free, highly effective oral direct acting antiviral (DAA) therapies have revolutionised HCV treatment, curing 85-95% of people in 8-24 weeks, with minimal side effects [2]. Global elimination of hepatitis C is now being discussed as an achievable goal and the WHO has set targets of an 80% reduction in HCV incidence by 2030, and a 65% reduction in HCV-related deaths [3].

In March 2016, Australia became the first country to offer universal access to DAAs for all people living with hepatitis C, with no restrictions for fibrosis stage or high-risk behaviours. The Australian Government negotiated a unique volume-based agreement to pay A\$1 billion dollars over five years for unlimited access to HCV drugs [4], the so-called “Netflix Model” [5]. Prescribing by community health care providers was also encouraged, to facilitate treatment scale-up and provide access for marginalised populations. This led to a massive increase of DAA prescribing, with over 30,000 people treated in the first 12 months and 70,000 by the end of 2018, approximately 33 % of all people with chronic hepatitis C [6]. Eliminating hepatitis C as a public health problem in Australia within the next decade requires the continued rapid scale-up of treatment among those most at risk of transmission, the 45,000 people with chronic hepatitis C who recently injected drugs. This approach will be essential to achieve HCV elimination, but at the same time increase the likelihood of poor adherence among patient groups who are not usually included in clinical trials, including trials in drug and alcohol settings.

One potential barrier to HCV elimination is the emergence of drug resistance. Because HCV is an RNA virus, and RNA polymerase is highly error-prone, HCV mutates rapidly and exists as a quasispecies within an infected host, allowing the virus to become rapidly resistant to antiviral drugs. Resistance associated substitutions (RASs) occur in all regions of the virus genome targeted by current DAAs, i.e. NS3, NS5A and NS5B. Although the prevalence of RASs is relatively low among untreated people [7], in clinical trials the majority of people who fail DAA therapy develop resistant virus [8, 9]. NS5A RASs are the most clinically important as they have been shown to reduce cure rates, particularly among difficult to treat patients such as those with genotype 3 infection and/or cirrhosis [10, 11]. Importantly, NS5A RASs have minimal impact on viral fitness, so persist for many years, perhaps forever [11].

Testing for HCV resistance is recommended in Australian and international guidelines for patients who fail DAA therapy, to inform rational retreatment [12-14]. Treating with the same agents again is likely to fail, leading to accumulation of further resistance mutations. Emergence of antiviral resistance could undermine Australian and global elimination efforts, so monitoring for resistance during treatment scale-up is crucial. There is remarkably little real-world data on HCV resistance among patients failing modern DAA regimens, data that are crucial to understanding the clinical importance of HCV RASs for HCV elimination [15]. Australia’s early adoption of an open-access model makes it an ideal location to study the emergence of HCV resistance during treatment scale-up.

Method

In 2016 we developed an in-house assay to test for HCV resistance. RNA was extracted from frozen plasma samples and key regions of NS3, NS5A and NS5B were amplified with a two-step PCR, using primers and protocols derived from our recent systematic review [16-18]. Amplified DNA was purified and submitted for Sanger sequencing to the Australian Genome Research Facility (AGRF). Sequences were analysed for the presence of NS5A RASs using ReCALL (<http://pssm.cfenet.ubc.ca/>) [19] and NS3 or NS5B RASs using Geno2Pheno [hcv] [20]. Clinically significant RASs were identified based on consensus reports and international guidelines [10, 11, 14].

Results

Between 01 January 2017 and 30 June 2018) we analysed blood samples from 484 patients failing DAA treatment, from all states and territories in Australia. Of these 484 patients, 386 were male and 98 female, with the average age of males being 54 years and females 53.9 years (not significant). Rates of cirrhosis were similar in males (44%) and females (40%).

As shown in Figure 1A (page 3), of the 484 patients, 129 (27%) were infected with HCV genotype 1a, 31 (6%) with genotype 1b, 23 (5%) with genotype 2, 282 (58%) with genotype 3, eight (2%) with genotype 4 and 11 (2%) with genotype 6. As shown in Fig 1B, the majority of patients displayed one or more RAS: 77% (99/129) for genotype 1a, 71% (22/31) for genotype 1b, 26% (6/23) for genotype 2, 86% (231/268) for genotype 3a, 100% (14/14) for genotype 3b/k, 88% (7/8) for genotype 4 and 36% (4/11) for gt6.

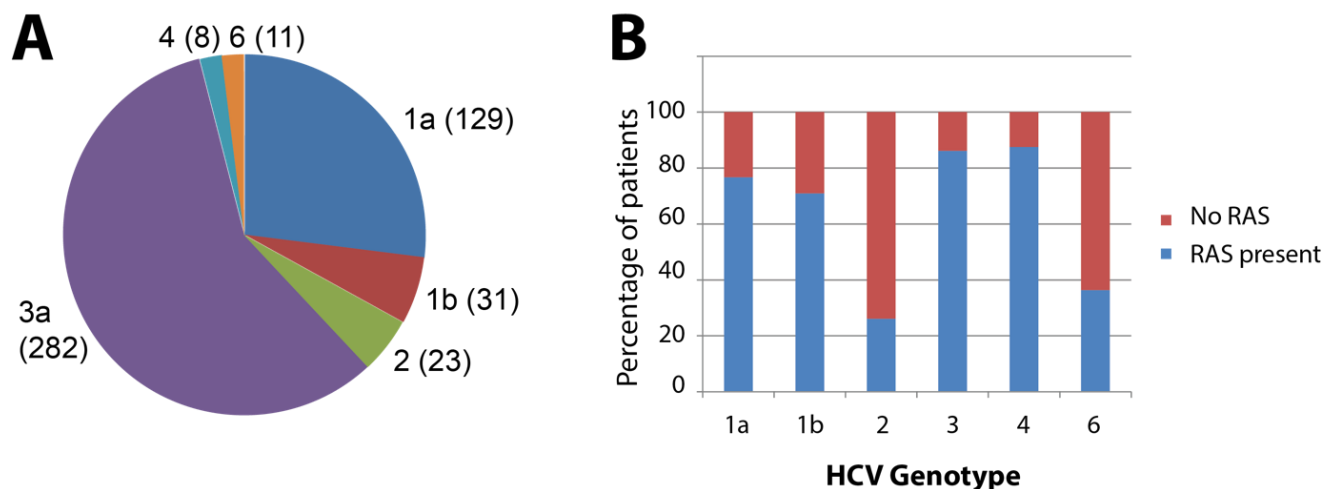


Figure 1. A) Genotype distribution of HCV isolates from patients failing DAA therapy (absolute number for each genotype). B) Proportion of isolates from each genotype with detectable RASs.

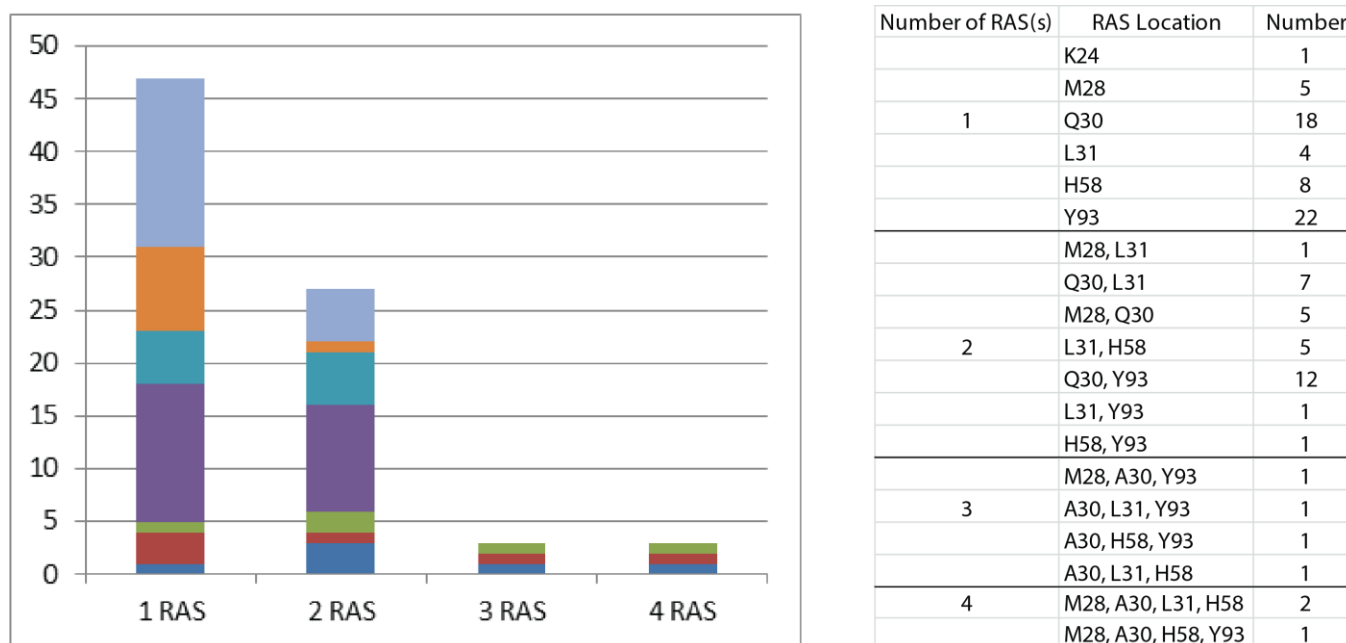


Figure 2. Distribution of RASs in patients with HCV genotype 1a who failed DAA therapy, including sites of substitutions.

As outlined above, RASs in NS5A are the most clinically relevant, as they impair response to re-treatment with a DAA regimen containing a NS5A inhibitor [21]. Further, they have minimal effect on viral fitness, so persist for months to years after DAA failure, providing potential for transmission of drug resistant virus [11, 22, 23]. For the purpose of this study, we have restricted the main analysis of NS5A RASs to patients with genotypes 1a and 3a, as this provides a sufficient sample size for analysis of RAS frequency.

As shown in Figures 2 and 3, there was a significant divergence in the patterns of RASs between genotypes 1 and 3. For patients with HCV genotype 1a who failed DAA therapy, 99/129 (77%) had detectable RASs, at residues K24, M28, Q30, L31, H58 or Y93. Fifty eight patients had a RAS at a single residue, 32 patients had RASs at two different residues, four patients had RASs at three residues and three had RASs at four residues (Fig 2).

For patients with HCV genotype 3a who failed DAA therapy, 231/268 (86%) had detectable RASs. Of these 231, 209 had a RAS on a single residue, 20 had RASs on two separate residues, and two had RASs on three separate residues (Fig 3).

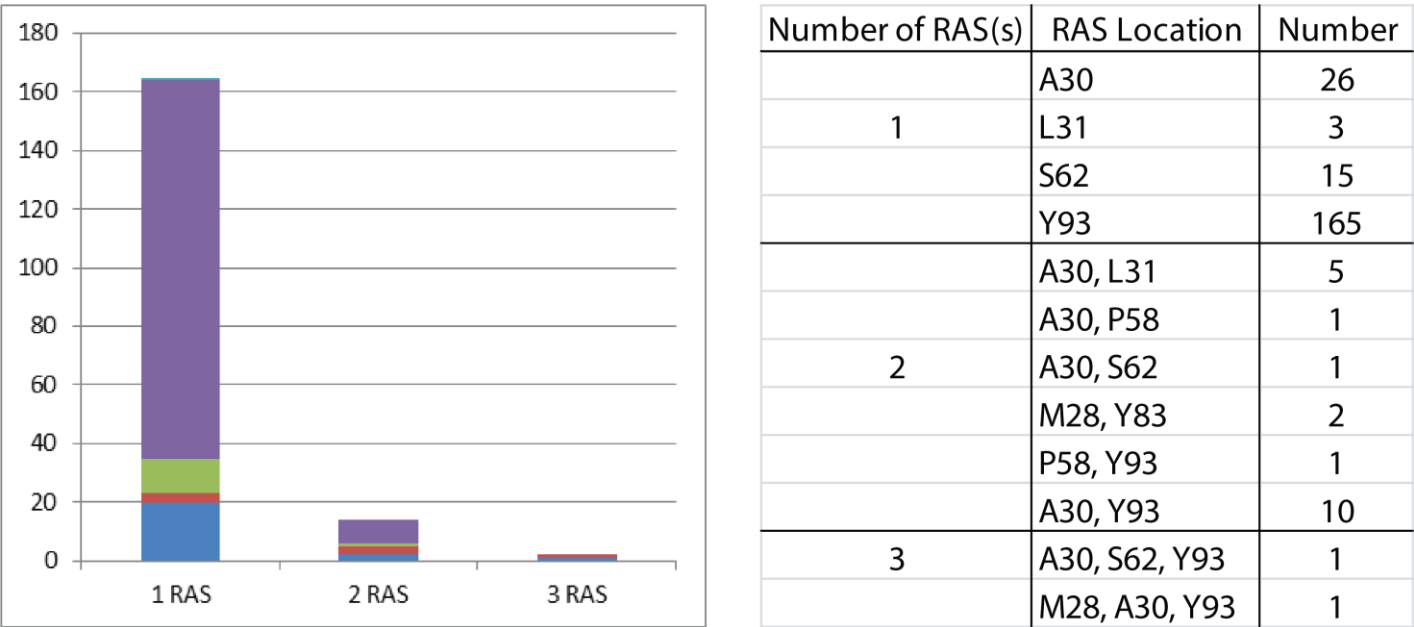


Figure 3. Distribution of RASs in patients with HCV genotype 3 who failed DAA therapy, including sites of substitutions

Concerning RASs against other classes of DAAs, we identified NS3 RASs in 8.8% (36/411) of patients for whom NS3 sequence data was available. Of these 36 patients, 17 had failed a NS3-containing regiment (seven failed paretaprevir/ritonavir/ombitasvir/dasabuvir (ProD), ten failed elbasvir/grazoprevir). The prevalence of NS3 RASs was 58% (7/12) among patients failing ProD and 56% (10/18) for patients failing elbasvir/grazoprevir, compared to 5% (19/381) for patients failing DAA regimens that did not contain a NS3 inhibitor.

Concerning NS5B RASs, among the 13 patients treated with ProD, three had detectable RASs in NS5B. The first had 556G and 561I RASs, the second had a single RAS (556G), while the third patient had 444D and 553x RASs. One patient treated with a Sofosbuvir containing regimen displayed a RAS in NS5B (282T). This clinically significant RAS was detected 15 weeks after completing treatment with sofosbuvir + daclatasvir, but was no longer detectable by Sanger sequencing 12 weeks later.

Discussion

This study provides valuable real-world data on the prevalence of hepatitis C resistance among people who fail DAA therapy. Overall it confirms high rates of resistance against NS5A inhibitors, with NS5A RASs detected in 77% of patients with genotype 1 and 86% of patients with genotype 3. NS5A RASs are the most clinically important, as they reduce response to treatment with commonly prescribed DAA regimens, including ledipasvir/sofosbuvir, daclatasvir + sofosbuvir, and elbasvir/grazoprevir [11]. Importantly, NS5A RASs have minimal impact on viral fitness, so persist for many years, perhaps forever [11]. A prominent finding was the divergence between genotypes 1 and 3 in the distribution of NS5A RASs. Patients with genotype 1a infection failing DAA therapy had a heterogeneous range of NS5A RASs, which were more evenly distributed than in patients with genotype 3a, in whom a single RAS (Y93H) predominated. This very high prevalence of Y93H among treatment failures is clinically important, as Y93H confers high levels of resistance against most NS5A inhibitors, with a 3,500-fold reduction in half maximal effective concentration (EC50) for daclatasvir and over 700-fold reduction in EC50 for the new, pan-genotype NS5A inhibitor velpatasvir [24].

In addition to NS5A RASs, we analysed the identity and frequency of NS3 and NS5B RASs. DAA regimens containing a NS3 inhibitor provide selective pressure for emergence of NS3 RASs. Consistent with this, in our cohort there was an increased prevalence of NS3 RASs among patients failing DAA therapy that included a NS3 inhibitor, with multiple NS3 RASs frequently seen. NS3 RASs usually disappear within weeks to months due to reduced viral fitness [11]. Of concern, there was the high prevalence of multi-class drug resistance among patients exposed to both NS3 and NS5A inhibitors, consistent with findings from registration trials and other real-world studies [25-27].

Regarding NS5B resistance, a single genotype 3 patient had the high level sofosbuvir RAS (S282T). This was detected 15 weeks after completing sofosbuvir/daclatasvir, but not 12 weeks later, consistent with the known impact of S282T on viral fitness [11]. However, compensatory mutations can restore viral fitness in the context of S282T, providing potential for transmission of sofosbuvir resistance in high risk settings. This has been demonstrated *in vitro* for genotypes 3 and 6 [28, 29], including a replication competent virus that is resistant to all three classes of pan-genotype DAAs, pibrentasvir (NS3), velpatasvir (NS5A) and sofosbuvir (NS5B) [28]. Chronic infection with sofosbuvir resistant virus (S282T) can also occur in humans, as confirmed in a recent case report of a high-risk patient with genotype 4d [30]. This highlights the potential for

increasing prevalence of clinical sofosbuvir resistance among patients failing DAAs, with risk of ongoing transmission in high risk groups.

In summary, in this large real-world study of people with chronic hepatitis C who have failed interferon-free DAA therapy we confirm a high prevalence of NS5A RASs, with a high prevalence of NS3 RASs among patients exposed to NS3 inhibitors and high rates of multi-class drug resistance. Elimination of hepatitis C requires widespread treatment scale-up and open access to DAAs, strategies that also increase the risk of emergence and transmission of drug-resistant virus among high-risk groups. Ongoing surveillance of HCV resistance rates in the community is essential, to ensure that emerging drug resistance does not undermine elimination efforts.

To facilitate ongoing clinical testing and surveillance for hepatitis C resistance, we have recently installed an automated workflow in the Westmead diagnostic lab that performs next-generation HCV sequencing from clinical samples [31]. The VELA Sentosa[®] platform is approved for HCV and HIV resistance testing by the Australian Therapeutic Goods Administration (TGA), allowing laboratory accreditation and clinical testing under a sustainable funding model. It uses Ion Torrent technology to perform next generation sequencing and has integrated bioinformatics analysis, allowing a streamlined and customisable workflow in a large diagnostic lab. As an accredited reference laboratory, we will use this platform to offer clinical resistance testing for patients with hepatitis C who fail antiviral therapy, as well as for other high-risk patients who may benefit from resistance testing at baseline.

In addition to clinical reporting, we will continue to monitor HCV resistance at the population level, in collaboration with Public Health units. This will allow surveillance for increasing antiviral resistance in the community, particularly as treatment is rolled out to high-risk communities, including people who currently inject drugs and people in prisons. At the global level, we will continue to contribute to international collaborations on HCV resistance, including the SHARED database. Global elimination of hepatitis C will require widespread treatment scale-up and open access to DAAs, strategies that also increase the risk of emergence and transmission of drug-resistant virus among high-risk groups. Thus, surveillance for increasing antiviral resistance during treatment scale-up is essential, to maintain the efficacy of current DAA regimens.

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Staff Profile

DR CONNIE LAM

Connie earned her PhD from UNSW in 2014, studying how *Bordetella pertussis* evolution has been affected by widespread vaccination. In late 2014, she joined CIDM-PH briefly as a postdoctoral fellow continuing her work on *B. pertussis*, before taking on an American Society for Microbiology fellowship to the US Centers for Disease Control and Prevention.

While she was in Atlanta, Connie worked on *B. pertussis* genomics and transcriptomics as well as helping the US National Tuberculosis Reference Laboratory set up their next generation sequencing service to detect drug resistant tuberculosis. In 2018, Connie joined the National Mycobacterium Reference Laboratory in Singapore, working in all aspects of tuberculosis and non-tuberculous mycobacteria.

Connie rejoined CIDM-PH in September 2019 as a postdoctoral research fellow- this time working on the genomics and molecular epidemiology of *M. tuberculosis*, with a particular focus on drug resistance. Her research interests are in the evolution and molecular epidemiology of bacterial pathogens, and also the molecular determinants of drug resistance.



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UPCOMING EVENTS

16 October 2019:

‘Raising the Bar’

Join Dr Cameron Webb (NSW Health Pathology & University of Sydney) for his talk titled "Climate change and the rise of mosquitoes" at Nick and Nora's, Level 26/45 Macquarie St, Parramatta, Sydney

Free ticket, registration essential:
<https://www.rtbevent.com/cameron-webb>

22 November 2019:

CIDM-PH Colloquium

Westmead Hospital, Sydney

<https://cidmphcolloquium2019.eventbrite.com.au>

Event Enquiries:

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2 December 2019:

MBI Colloquium

Veterinary Science Conference Centre,
University of Sydney

[Register](#)

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