Utility of whole genome sequencing for multidrug resistant Mycobacterium tuberculosis isolates in a reference TB laboratory in New Zealand

Indira Basu, James E Bower, Sally A Roberts, Gillian Henderson, Htin Lin Aung, Gregory Cook, Odette Lowe, Sandie Newton

ABSTRACT

New Zealand has a low burden of multi-drug resistant TB (MDR-TB), but with increased mobility within the population, rapid detection and treatment of MDR-TB is a priority from the public health point of view. Mycobacterium Reference Laboratory in LabPLUS, Auckland City Hospital receives referred Mycobacterium tuberculosis complex (MTBC) isolates from all over New Zealand for second-line drug susceptibility testing (DST) and 24-loci MIRU VNTR genotyping. Between 2002 and 2013, 38 multidrug resistant Mycobacterium tuberculosis (MDR-TB) isolates were recorded by culture-based DST. A retrospective study revealed that in 12 of these 38 MDR-TB isolates (28%) there was a discrepancy between the genotypic and the phenotypic results. In order to address this, whole genome sequencing (WGS) was performed on the discrepant MDR-TB isolates. Reported here are the additional information on the drug resistant markers from WGS, which shed light on the discordance between results from the culture-based DST and the molecular diagnostic tests. These results underscore the utility of WGS in a reference mycobacterium laboratory in New Zealand to supplement other molecular tests and to assist in a rapid but accurate diagnosis and appropriate management of MDR-TB.

Mycobacterium tuberculosis (Mtbc), the causative agent of tuberculosis (TB) kills 1.4 million people annually according to WHO. Increase in multi-drug resistant TB (MDR-TB) defined by resistance to the first line anti-tuberculosis agents, rifampicin (RIF) and isoniazid (INH), has added to the global concern with increased travel and globalisation. New Zealand has a low burden of MDR-TB at <7.0 cases per 100,000 population. However, a significant proportion of cases, 76%, are born outside of New Zealand, or are currently residing with, or have recently resided with a person born outside of New Zealand. In 2014, cases born in the Southern and Central Asia region had the highest notification rates followed by South-East Asia. A number of countries from these regions, such as India, China, Philippines and Indonesia are considered to be high TB burden countries. Of concern is the emergence of multi-drug resistance TB with China, India and the Russian Federation accounting for 47% of all MDR-TB cases globally. Treatment for MDR-TB is longer, and requires more expensive and more toxic drugs.

For these reasons it is important to be able to rapidly identify cases infected with MDR-TB strains to ensure that the case is treated appropriately and that the risk of further transmission to others is minimised.

The timely diagnosis and administration of the appropriate anti-tuberculous drug...
regimen depends to a large extent on the laboratory confirmation of MDR-TB. However, phenotypic drug susceptibility testing (DST) is time consuming, technically challenging and the laboratory undertaking DST requires a level of physical containment not routinely available in most diagnostic laboratories nationally. Slow growing or mixed cultures further add to the delay. For this reason there has been a shift away from culture-based DST methods, so-called phenotypic methods, to molecular or genotypic methods that detect mutations in genes associated with resistance. In the recent years, implementation of molecular tests worldwide has dramatically reduced the time to TB diagnosis. The rapid front-line diagnostic test for detection of M. tuberculosis and rifampicin resistance (a surrogate marker for MDR-TB) from clinical specimen is performed in an automated real-time PCR assay, with an integrated semiautomated device for DNA extraction called Cepheid Xpert® MTB/RIF test (Cepheid, Sunnyvale, CA, US). It was endorsed by WHO for TB testing in 2010. Molecular line-probe assays (LPA) for detection of MDR-TB, eg, Genotype MTBDRplus (Hain Lifescience, Nehren, Germany) detects active TB and resistance-conferring mutations for RIF, and INH with modest accuracy. The LPAs are still dependent on laboratory expertise. Both these commercial molecular diagnostic tests for detection of Mtb and first-line drug resistance target the mutations in a hotspot 81bp region of rpoB gene, referred to as the rifampicin-resistance determining region (RRDR), which can be found in 96% of RIF-resistant MTb strains worldwide. Furthermore, Genotype MTBDRplus targets most common mutations in inhA or katG genes which are supposed to be responsible for INH-resistance. It has been shown in literature that these two molecular tests show excellent specificity but lower sensitivity for these first-line antibiotics.

The sensitivity and the specificity of these rapid commercial molecular diagnostic tests for RIF and INH resistance are not 100%. Hence there is a risk if treatment of the patients is commenced with second-line drugs based on RIF-resistant and INH-resistant results from these rapid molecular diagnostic tests alone. Such a situation might permit further spread of drug-resistant strains and also promote the selection of strains with even greater resistance. Thus any discrepancy in result between molecular resistance testing and phenotypic testing is of importance to clinicians treating a patient.

More recently these limitations in the commercial molecular diagnostic tests are being overcome by shifting from targeting of individual genes for resistance mutation towards sequencing the entire bacterial genome allowing for the detection of mutations in multiple genes all at once. The technology is widely known as the whole genome sequencing, which is performed on next-generation sequencing platforms, the Illumina Mi-Seq (Illumina, Inc., Hayward, CA) being one of them. But this technology needs highly skilled scientists.

In response to an expected increase in the number of cases of MDR-TB in New Zealand we sought to increase our capability to diagnose rapidly and accurately cases of MDR-TB. We report here our experience with whole genome sequencing of selected New Zealand MDR-TB isolates from 2002 till 2013.

Method

The Mycobacterium Reference Laboratory in the Microbiology Department, LabPLUS, Auckland City Hospital performs first-line DST for all Mtb isolates from cases in the Northern District Health Board (DHB) Region as well as the second-line antituberculous drug susceptibility testing for MDR-TB isolates for all of New Zealand. Molecular Microbiology section routinely performs different molecular tests for genotypic drug resistance profile to provide faster result on suspected MDR-TB isolates as well as mycobacterial interspersed repeat unit-variable number of tandem repeat (MIRU-VNTR) 24-loci genotyping of all New Zealand Mtb isolates to assist the Public Health in an outbreak investigation and for epidemiological purposes. The results of MIRU-VNTR 24-loci genotyping for MDR-TB isolates in this study are mostly unique strains of Mtb (Table 1).

Selection of isolates for the study

From 2002 till 2013, 38 MDR-TB were isolated in New Zealand (Figure 1). Cepheid Xpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, US), and Genotype MTBDRplus test
(Hains Lifescience, Nehren, GmbH) were used for routine clinical diagnostics from 2010. About 44% of these MDR-TB cultures were isolated and cultured prior to introduction of these molecular tests and hence were retrospectively tested on these two platforms. Results showed discordance in interpretation of the resistance to first-line drugs between the phenotypic culture-based DST and genotypic molecular results for 12 MDR-TB isolates. These 12 isolates were selected for whole genome sequencing. The discrepancies are documented in Table 1 and explained in detail later in the Results and Discussion section.

Phenotypic drug susceptibility testing

Culture-based DST is performed routinely on all MTBC isolates using the MGIT as described previously. Critical concentrations of antibiotics were in accordance with World Health Organization guidelines for MGIT™ BACTEC™ 960. Using culture-based DST result, 38 laboratory confirmed cases of MDR-TB isolates were recorded in the time period spanning 2002 till 2013. (Figure 1).

Molecular tests

Cepheid Xpert® MTB/RIF test (Cepheid, Sunnyvale, CA, US) and Genotype MTBDRplus (Hains Lifescience, Nehren, GmbH) commercial tests for MDR-TB are used routinely on clinical specimens when they fit the pre-test probability criteria as described earlier. Briefly, the criteria used to determine whether the cultured isolate had a high pre-test probability of being MDR-TB are as follows: the specimen was from a patient from an area of high TB incidence, or from a contact of a confirmed TB patient or from a relapsed or a treatment failure case.

Rifampicin (RIF) resistance when detected by Cepheid Xpert® MTB/RIF test and/or Genotype MTBDRplus was further confirmed by in-house rpoB gene sequencing of the rifampicin-resistance determining region (RRDR) of rpoB gene as previously described. This confirmation of mutation in the rpoB gene by sequencing was routinely performed after an earlier study from our laboratory revealed the possibility of false positive RIF-resistance result from Cepheid Xpert® MTB/RIF test.

Whole genome sequencing

The genomic DNA extraction of these 12 MDR-TB isolates chosen for whole genome sequencing (WGS) were performed on pure Mtb grown in the BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960 system (Becton Dickinson, Sparks, MD, US). Briefly, 1.5ml of MGIT broth was vortexed with glass beads for 30s in a 2ml sterile micro-centrifuge tube, and centrifuged at 13,000Xg for 10 min. The supernatant was discarded and 300μl of Tris buffer was added to the pellet. After further vortexing for 30s, the

Figure 1: Number of MDR-TB patients in New Zealand confirmed by laboratory from 2002–2013.
microcentrifuge tube was heated at 95°C for 15 min in a heating block. The isolates were then treated with lysozyme and proteinase K/RNase A for lysis of the cells and removal of protein/RNA, respectively. MagNA Pure LC semi-automated extraction platform (Roche Molecular Diagnostics, Branchburg, NJ, US) was used for extraction of genomic DNA following the manufacturer's protocol. Nextera XT kit (Illumina, Inc., Hayward, CA) was used for library preparation for WGS. Nanopore and Qubit instruments (Thermo Fisher Scientific) were used for the quantification of the library. Sequencing was performed on the Illumina MiSeq (Illumina, Inc., Hayward, CA) using paired-end 250-bp reads. After sequencing, for analysis of the data, the reads were mapped against the *Mycobacterium tuberculosis* H37RV reference sequence (NC_000962.3) using Burrows-Wheeler transform (BWA) software. Single-nucleotide polymorphism (SNP) analysis was performed using Genome Analysis Toolkit (GATK) pipeline to identify mutations associated with resistance and the effects of the SNP were predicted using a program for annotating and predicting the effects of single nucleotide polymorphisms called SNPeff. The accession number for these isolates are SAMN06472852, SAMN06472853, SAMN06472854, SAMN06472855, SAMN06472856, SAMN06472857, SAMN06472858, SAMN06472859, SAMN06472860, SAMN06472861, SAMN06472862, SAMN06472863 in the GeneBank.

Results and discussion

Retrospective testing of the NZ MDR-TB isolates with Cepheid Xpert® MTB/RIF test (Cepheid, Sunnyvale, CA, US) and Genotype MTBDRplus (Hains Lifescience, Nehren, GmbH) failed to identify 12 of the isolates as MDR-TB (28%) as documented in Table 1. The darker cells in each row of Table 1 highlight the phenotypic and genotypic discrepancy.

### Table 1: Discrepancy between the phenotypic and the genotypic drug resistance profiles of the 12 multi-drug resistant *M. tuberculosis* culture isolates, New Zealand, 2003–2013.

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Year of isolation</th>
<th>Site of infection</th>
<th>MIRU-VNTR 24-loci typing result</th>
<th>Phenotypic resistance to RIF</th>
<th>Genotypic resistance to RIF: rpoB gene mutation</th>
<th>Phenotypic resistance to INH</th>
<th>Genotypic resistance to INH: inhA gene mutation</th>
<th>Genotypic resistance to INH: katG gene mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2005</td>
<td>Sputum</td>
<td>Clustered</td>
<td>resistant</td>
<td>NMD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>High level</td>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
<td>S315T</td>
</tr>
<tr>
<td>2</td>
<td>2006</td>
<td>Sputum</td>
<td>Clustered</td>
<td>resistant</td>
<td>NMD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>High level</td>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
<td>S315T</td>
</tr>
<tr>
<td>3</td>
<td>2013</td>
<td>Sputum</td>
<td>Unique</td>
<td>resistant</td>
<td>NMD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>High level</td>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
<td>S315T</td>
</tr>
<tr>
<td>4</td>
<td>2013</td>
<td>Pelvic aspirate</td>
<td>Unique</td>
<td>resistant</td>
<td>S531L</td>
<td>High level</td>
<td>C-15T&lt;sup&gt;e&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>2012</td>
<td>Sputum</td>
<td>Unique</td>
<td>resistant</td>
<td>S531L</td>
<td>High level</td>
<td>C-15T&lt;sup&gt;e&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>2010</td>
<td>Bronchial Washings</td>
<td>Unique</td>
<td>resistant</td>
<td>S531L</td>
<td>High level</td>
<td>C-15T&lt;sup&gt;e&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>2009</td>
<td>Right Cervical LN</td>
<td>Unique</td>
<td>resistant</td>
<td>S531L</td>
<td>High level</td>
<td>C-15T&lt;sup&gt;e&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>2009</td>
<td>Sputum</td>
<td>Clustered</td>
<td>resistant</td>
<td>D516Y</td>
<td>High level</td>
<td>C-15T&lt;sup&gt;e&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>2010</td>
<td>Sputum</td>
<td>Unique</td>
<td>resistant</td>
<td>H526Y</td>
<td>High level</td>
<td>NMD&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2009</td>
<td>Sputum</td>
<td>Unique</td>
<td>resistant</td>
<td>S531L</td>
<td>High level</td>
<td>NMD&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>2007</td>
<td>Sputum</td>
<td>Clustered</td>
<td>resistant</td>
<td>S531L</td>
<td>High level</td>
<td>NMD&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2005</td>
<td>Sputum</td>
<td>Unique</td>
<td>resistant</td>
<td>S531L</td>
<td>High level</td>
<td>NMD&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The cells with phenotypic and genotypic discrepancies are in darker shades.

<sup>b</sup>Result from Xpert® MTB/RIF and Genotype MTBDR Plus with confirmation by RDRD rpoB sequencing.

<sup>c</sup>Result from Genotype MTBDR Plus test.

<sup>d</sup>NMD—No mutation detected.

<sup>e</sup>WT—Wild-type referring to no mutation in the gene conferring resistance.
Rifampicin resistance

The first 3 isolates (Table 1; isolate numbers 1, 2 and 3) did not show any RIF-resistance using Cepheid Xpert® MTB/RIF test (Cepheid, Sunnyvale, CA, US) and Genotype MTBDRplus (Hains Lifescience, Nehren, GmbH). As mentioned in the introduction, these tests target the mutations located in the 81bp rifampicin-resistance determining region (RRDR) of the rpoB gene, which can be found in 96% of RIF-resistant Mtb strains worldwide. As these two molecular diagnostic tests only interrogate the most frequent mutations responsible for RIF resistance, they have their limitations.

WGS revealed that these three phenotypically and genotypically discrepant MDR-TB isolates (Table 2; isolate number 1, 2 and 3 from 2005, 2006 and 2013 respectively) showed a mutation at position G508T (M. tuberculosis numbering) of the rpoB gene resulting in amino acid change, V170F (Table 2). This mutation has been previously described in the literature to be associated with rifampicin resistant strains. Presence of this uncommon mutation outside the 81bp rpoB gene RRDR explains why Cepheid Xpert® MTB/RIF and Genotype MTBDRplus did not detect these rpoB gene mutations. Hence these tests with their limitations have the potential to give a rapid yet false negative result when used prospectively.

Isoniazid resistance

The next five discrepant MDR-TB isolates (Table 1; isolate numbers 4–8) were resistant to isoniazid (INH) both at a concentration of 0.1ug/ml (low-level) and 0.4ug/ml (high-level). Resistance to INH at a concentration of 0.1ug/ml (low-level) has been shown to be conferred by a mutation in the promoter region of the inhA gene at -C15T while resistance to INH at 0.4ug/ml (high-level) is conferred by mutation in the katG gene. Both these mutations can be detected by the Genotype MTBDRplus test. Even though these five discrepant MDR-TB isolates (Table 1; isolate numbers 4–8) were phenotypically resistant to isoniazid (INH) both at a concentration of 0.1ug/ml (low-level) and 0.4ug/ml (high-level), they only exhibited mutation at C-15T, which has been shown to correspond to low-level INH resistance at 0.1ug/ml.

Table 2: Results from whole genome sequencing addressing the discrepancy between phenotypic and genotypic drug resistance results for first line drugs—rifampicin (RIF) and isoniazid (INH).

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Year of isolation</th>
<th>Site of infection</th>
<th>rpoB mutation</th>
<th>katG mutation</th>
<th>inhA mutation</th>
<th>Other genes for INH resistance (mutation)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2005</td>
<td>sputum</td>
<td>V170F</td>
<td>S315T</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2006</td>
<td>sputum</td>
<td>V170F</td>
<td>S315T</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2013</td>
<td>sputum</td>
<td>V170F</td>
<td>S315T</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2013</td>
<td>pelvic aspirate</td>
<td>S531L, A286V</td>
<td>NMD</td>
<td>C-15T, I21V</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2012</td>
<td>sputum</td>
<td>S531L</td>
<td>NMD</td>
<td>C-15T, I194T</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2010</td>
<td>BW</td>
<td>S531L</td>
<td>NMD</td>
<td>C-15T, I21V</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2009</td>
<td>R. cervical LN</td>
<td>S531L, I491V</td>
<td>NMD</td>
<td>C-15T, I21V</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2009</td>
<td>sputum</td>
<td>D516Y</td>
<td>D419Y</td>
<td>C-15T</td>
<td>accD6 (D229G)</td>
</tr>
<tr>
<td>9</td>
<td>2010</td>
<td>sputum</td>
<td>H526Y</td>
<td>NMD</td>
<td>NMD</td>
<td>accD6 (D229G)</td>
</tr>
<tr>
<td>10</td>
<td>2009</td>
<td>sputum</td>
<td>S531L, T347A</td>
<td>NMD</td>
<td>NMD</td>
<td>accD6 (D229G)</td>
</tr>
<tr>
<td>11</td>
<td>2007</td>
<td>sputum</td>
<td>S531L</td>
<td>NMD</td>
<td>NMD</td>
<td>ndh (V18A)</td>
</tr>
<tr>
<td>12</td>
<td>2005</td>
<td>sputum</td>
<td>S531L, T52P</td>
<td>NMD</td>
<td>NMD</td>
<td>accD6 (D229G)</td>
</tr>
</tbody>
</table>

aResult obtained from WGS data are in bold.
bResult obtained from Genotype MTBDRplus tests.
cResult obtained from Xpert MTB/RIF and Genotype MTBDRplus tests confirmed by RRDR rpoB sequencing.
dNMD—No mutation detected.
WGS showed additional mutations in \textit{inhA} gene at I21V for three of these discrepant isolates (Table 2; isolate numbers 4, 6 and 7). This mutation has been previously reported to correspond to a high-level INH resistance at 0.4ug/ml where they have been present in addition to C-15T.\textsuperscript{17} For the isolate 5, WGS showed a different mutation, I194T in the \textit{inhA} gene, which also has been associated previously with high-level INH resistance in MDR-TB isolate from Portugal in addition to the C-15T.\textsuperscript{18} Isolate 8 had a mutation in D419Y of the \textit{katG} gene which has been linked to high-level INH resistance in Brazilian isolates.\textsuperscript{19} These additional mutations in \textit{inhA} gene and \textit{katG} gene explain the high-level phenotypic resistance to INH for these five discrepant MDR-TB isolates (numbers 4–8), but these additional uncommon mutations were not detected by Genotype MTBDR\textit{plus} test.

The last four discrepant MDR-TB isolates (Table 1; isolates 9–12) were phenotypically resistant to INH using culture-based DST but did not show any mutation in \textit{inhA} or \textit{katG} genes, which are commonly implicated in most of INH-resistance\textsuperscript{5} and hence are not detected by Genotype MTBDR\textit{plus}. WGS also did not detect any mutation in the \textit{inhA} gene or the \textit{katG} gene that has been associated with INH resistance for these four discrepant MDR-TB isolates (Table 1; isolates 9–12). Further interrogation of the WGS data for other putative genes implicated in INH resistance showed a mutation in the acetyl-CoA carboxylase gene, \textit{accD6} in isolates 9, 10 and 12 (Table 2). This mutation in \textit{accD6} gene at D229G has been reported in literature to be associated with INH-resistance and present in MDR-TB strains.\textsuperscript{20} Isolate 11 had a mutation in NADH dehydrogenase gene, \textit{ndh} resulting in the amino acid change V18A that has been reported to be associated with an isoniazid-resistant Mtb isolate from Brazil.\textsuperscript{21}

This is a rapidly changing area; over the last 10 years rapid molecular assays designed to detect MTb, resistance to rifampicin as well as resistance to other first- and second-line anti-tuberculous medications directly from clinical specimens or cultures have become available. For the majority of Mtb cases in New Zealand, this capability is not required. However, with an increasing MDR-TB burden globally it is important that, despite being a low TB burden country, we can rapidly diagnose such cases. WGS provide useful information over and above that obtained from the current widely used commercial molecular diagnostic tests and help address the discrepancy between phenotypic and genotypic results. It will benefit clinical decision-making by supporting the choice of an appropriate and accurate drug regime for MDR-TB cases in a timely manner. With decreasing cost of WGS and the increasing ease of data handling with newer pipelines, WGS results hold the potential to be available ahead of the culture-based DST.\textsuperscript{22}

Not only can WGS provide information about drug susceptibility but it will replace the current molecular typing method since it has been shown that result from WGS is superior to MIRU-VNTR 24-loci typing for assisting Public Health in contact tracing.\textsuperscript{22} WGS provides a higher level of discrimination between isolates than this method because it looks at the entire genome and not just a standard set of ‘housekeeping’ genes. This has the potential to reduce unnecessary public health contact traces from occurring. Currently cases may belong to the same MIRU-VNTR but have no epidemiological links. These cases in the MIRU-VNTR cluster may be found to be unrelated by WGS reducing the need for contact tracing.

Other high income countries are moving away from culture-based TB diagnostics and shifting to WGS for identification of \textit{Mycobacterium} species, identification of drug resistance and molecular strain typing.\textsuperscript{22} New Zealand is in a position to be able to follow this approach. The DHB diagnostic laboratories have well-established referral pathways that would support the establishment of a single provider for TB molecular diagnostics.

Hence, WGS promises to increase the sensitivity of the molecular diagnostic result for timely and effective management of MDR-TB and would eventually help develop an algorithm that would reduce the potential for misdiagnosis of MDR-TB prospectively when used in combination with the currently used molecular methods. This study underscores the utility of WGS to supplement the current molecular tests in a reference Mycobacteriology laboratory setting in New Zealand.
Competing interests:
Nil.

Acknowledgements:
We would like to thank Mr Ross Vaughan for his contribution. We would also like to acknowledge the Asser Trust Grant for funding the whole genome sequencing project and the services of New Zealand Genomic Limited (project numbers NZGL01133 and NZGL01739) for the whole genome sequencing especially Lorraine Berry for the library preparation and sequencing and Gregory Gimenez for help with the Bioinformatics.

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