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Editor's Choice

ORIGINAL ARTICLE

An outbreak of multiple genotypes of *Listeria monocytogenes* in New Zealand linked to contaminated ready-to-eat meats—a retrospective analysis using whole-genome sequencing

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Significance and Impact of the Study: This study describes a listeriosis outbreak associated with a hospital in New Zealand and attributed to contaminated ready-to-eat (RTE) meat supplied to the hospital by a single producer. Retrospective whole-genome sequence analysis of outbreak isolates was found to provide a greater degree of discrimination between isolates compared to pulsed-field gel electrophoresis and supported the conclusions made at the time of the outbreak. The multiple genotypes identified from clinical cases and the RTE meats obtained during the outbreak highlight the importance of epidemiological concordance alongside genotyping.

Keywords
epidemiology, food safety, genomics, genotyping, Listeria.

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Abstract

Four cases of listeriosis in a hospital (A) in New Zealand were identified in 2012. Pulsed-field gel electrophoresis (PFGE) used at the time identified four pulsotypes amongst the clinical isolates. Two of the pulsotypes matched to *Listeria monocytogenes* isolates obtained from ready-to-eat (RTE) meat samples from a RTE producer tested during a nationwide microbiological survey the month prior. The outbreak investigation confirmed that the RTE producer had supplied product to the hospital and additional testing confirmed the presence of *L. monocytogenes* in RTE meats from the hospital kitchen. Two further listeriosis cases presented in another hospital (B) with one clinical isolate identified as the same pulsotype as identified for one case in hospital A, but the epidemiology information concluded that the clinical cases from hospital B were not linked to the outbreak. Retrospective whole-genome sequencing confirmed that epidemiologically linked isolates belonging to three different genotypes for clinical cases from hospital A and RTE meats samples from the hospital kitchen differed by 0-1 core-genome locus or single nucleotide polymorphisms (SNP). The use of core-genome multilocus sequence typing and SNP analysis provided a greater degree of discrimination between isolates compared to PFGE.

Introduction

Listeriosis is a bacterial infection caused by *Listeria monocytogenes*. It is a rare disease in New Zealand (NZ) with a rate of approximately 0·4 per 100 000 population (21 cases in 2017) (Pattis *et al.* 2018). While the illness causes the third highest human health burden in this country (Cressey 2014), very few outbreaks occur. *L. monocytogenes* continues to be a major focus for the food industry internationally due to the severity of disease that it can cause amongst those at higher risk (i.e. immunocompromised individuals, the elderly and neonates) (McLauchlin *et al.* 2004; Buchanan *et al.* 2017). Foods of most concern for listeriosis are those in which *L. monocytogenes* can multiply. Ready-to-eat (RTE) foods present a significant risk for listeriosis as these products do not undergo further treatment

such as cooking before consumption and growth of *L. monocytogenes* can occur during refrigerated storage (Tompkin 2002; Kurpas *et al.* 2018).

This study describes an outbreak associated with RTE meat in NZ in 2012. At the time of the outbreak, traditional typing of *L. monocytogenes* had involved the use of an internationally standardized method for pulsed-field gel electrophoresis (PFGE) by PulseNet International (Martin *et al.* 2006). Since 2014, NZ has transitioned to the use of whole-genome sequencing (WGS), a method that has been employed to assist in listeriosis outbreak investigations internationally and is now being used in real-time surveillance in the United States and other countries (Jackson *et al.* 2016; Kwong *et al.* 2016; Van Walle *et al.* 2018; Besser *et al.* 2019).

There are two main analytical approaches to analyse WGS data and understand the genetic relationships between isolates of interest. Single-nucleotide polymorphism (SNP) analysis assesses a WGS base by base, while a multilocus sequence typing (MLST) approach involves a gene by gene analysis that can include core genes (cgMLST) or both core and accessory genes (whole-genome MLST) (Besser *et al.* 2019). The application of cgMLST analysis for *L. monocytogenes* has been demonstrated as a highly reproducible method with the ability to standardize nomenclature of alleles and types (Ruppitsch *et al.* 2015; Moura *et al.* 2016; Moura *et al.* 2017; Nadon *et al.* 2017; Pietzka *et al.* 2019). Depending on the discriminatory ability of cgMLST, additional typing procedures such as SNP analysis can also be applied. Although SNP analysis provides maximal discriminatory power, results are difficult to standardize and interpret (Pightling *et al.* 2018).

This study describes the investigation of an outbreak of listeriosis linked to RTE meats using PFGE, and retrospective WGS analysis (using cgMLST and SNP analysis) of the *L. monocytogenes* isolates associated with the outbreak.

Results and discussion

In June 2012, four cases of listeriosis, including two deaths, were reported in persons who were being treated as in- or outpatients at hospital A prior to infection. PFGE typing of the clinical isolates was performed. The four clinical isolates from hospital A (A1–A4) generated four pulsotypes (Fig. 1). Two pulsotypes (Asc0043a:Apa0026 and Asc0103:Apa0058) were indistinguishable from isolates obtained from RTE meats tested from a local producer (located in the same region as hospital A) during a national microbiological survey in May 2012 (Rivas *et al.* 2017). These pulsotypes were uncommon within the database at that time and neither pulsotype had been observed amongst human cases in NZ in the 10 years since the establishment of the database.

An investigation confirmed that the RTE meat producer identified during the PFGE analysis supplied RTE meats to hospital A. The product was used in the hospital cafeteria and inpatient meals. All 12 composite RTE meat samples (from a total of 62 unopened packages) were positive for *L. monocytogenes* by culture. PFGE identified four pulsotypes amongst the 12 *L. monocytogenes* isolates selected from the composite samples from the hospital kitchen, with 11 of the isolates resulting in a PFGE profile that was indistinguishable to three of the clinical cases (A1–A3) (Fig. 1). The pulsotype identified for the remaining clinical isolate (A4) was a pulsotype that was not observed amongst the other clinical or food isolates obtained during the investigation or from the microbiological survey.

In July 2012, two cases of listeriosis were confirmed from persons admitted to hospital B, located 400 km from hospital A. PFGE results showed that one of the clinical isolates was the same pulsotype (Asc0043a:Apa0026) as clinical case A1. However, the epidemiological investigation found that no RTE meats from the implicated producer were supplied into the region serviced by hospital B and there was no evidence that any recalled product was purchased or consumed by the patients. It was concluded that the clinical cases from hospital B were not linked to the outbreak in hospital A.

Retrospective WGS analysis of all of the *L. monocytogenes* isolates associated with the outbreak in both hospitals was performed. The lineage, sublineage, multilocus sequence type (ST) and cgMLST type (CT) were inferred from WGS data (Fig. 1). One clinical isolate from hospital A identified as CT3423 was observed to be highly related (one locus and two SNP differences) and epidemiologically linked to an isolate obtained from RTE meat product tested from the hospital kitchen.

All isolates previously identified as pulsotype Asc0043a:Apa0026 and Asc0103:Apa0058 were designated ST7 and ST9, respectively. However, when cgMLST was used, isolates within each of these two ST groups were further subdivided into two different CT types (Fig. 1). A separate SNP analyses for isolates within the ST7 and ST9 groups were performed, whereby a clinical isolate was used as a reference sequence (A1 and A3, respectively) for each group (Fig. 2). This approach provides the most accurate assessment of the SNP differences in an outbreak setting (Kwong *et al.* 2016; Dahl *et al.* 2017). Isolates within the ST9 group and identified as CT3406 consisted of epidemiologically linked isolates from a clinical case from hospital A and from RTE samples from the hospital kitchen and the microbiological survey. The isolates identified as CT3406 differed between 0 and 5 loci when analysed by

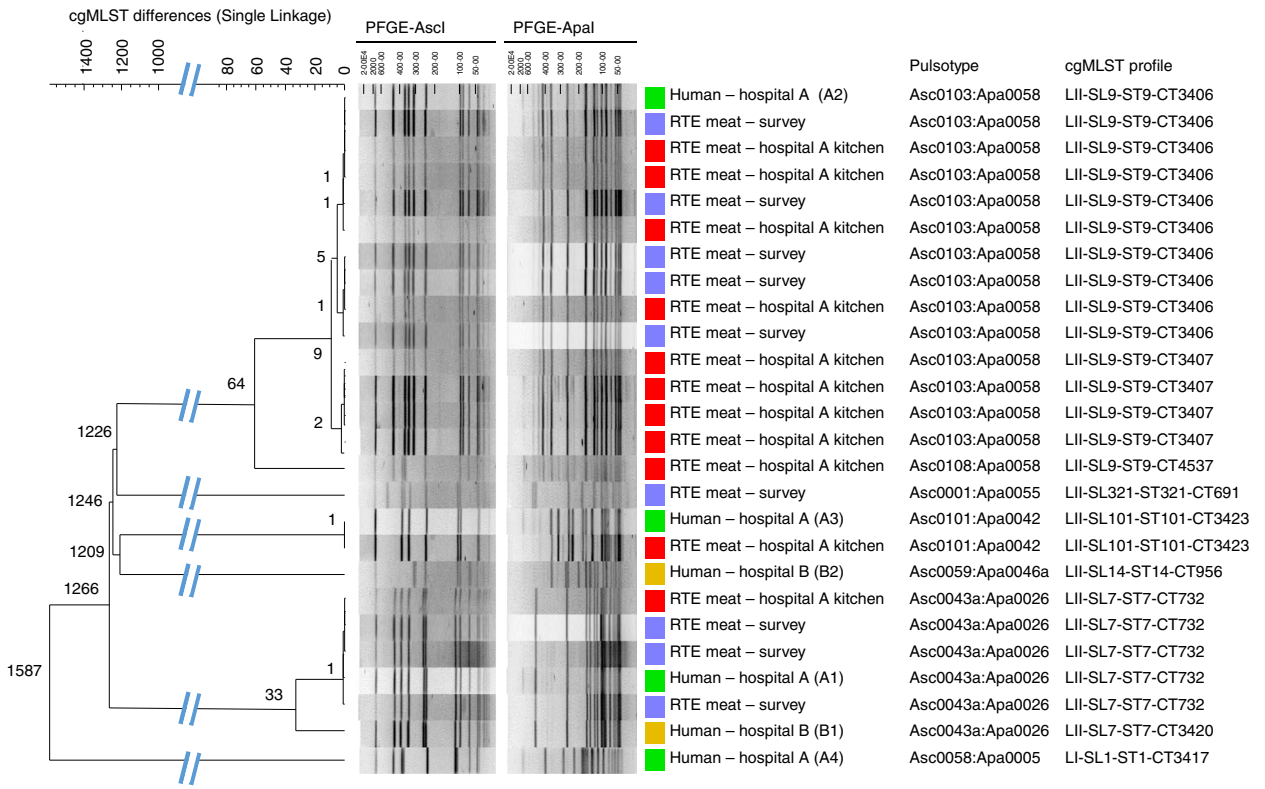

Figure 1 Dendrogram of core genes multilocus sequence typing (cgMLST) results for *Listeria monocytogenes* isolates implicated in the outbreak and relevant isolates from the microbiological survey. Single linkage was used to construct dendrogram within BioNumerics, and the number of loci differences between isolates is indicated on the branch nodes. The pulsed-field gel electrophoresis (PFGE) profiles using *Asc*I and *Apa*I enzymes, as well as the assigned pulsotype, are provided. The cgMLST profile for each isolate represents the lineage (L), sublineage (SL), ML sequence type (ST) and cgMLST type (CT). [Colour figure can be viewed at wileyonlinelibrary.com]

cgMLST (Fig. 1) and between 0 and 10 SNP differences using SNP analysis (Fig. 2a). The results observed in this study are similar to those reported for the largest outbreak of listeriosis to date (total of 1060 cases) that was attributed to a processed RTE meat in South Africa. That investigation also used the same cgMLST scheme (Moura *et al.* 2016) and reported that clinical isolates were highly related to nonclinical isolates with <10 SNP and four allele differences suggesting a high probability of epidemiological relatedness (Smith *et al.* 2019).

Internationally recognized thresholds for cgMLST allele or SNP analysis differences between genetically related isolates are still being established. Publications describing the use of WGS within listeriosis outbreaks globally have reported ≤12 alleles by whole-genome MLST or ranges of 0–50 SNPs as criteria to distinguish outbreak-related isolates from unrelated isolates (Ruppitsch *et al.* 2015; Jackson *et al.* 2016; Moura *et al.* 2016; Chen *et al.* 2017; Moura *et al.* 2017; Smith *et al.* 2019). However, there is some variation between studies regarding the classification of strains as closely linked (Jackson *et al.* 2016; Besser *et al.* 2019). It is important to note that studies have reported the use of different bioinformatic tools or parameters to identify alleles or SNPs or different regions of the genome that are targeted (Chen *et al.* 2016). For example, differences in cgMLST schemes (Ruppitsch *et al.* 2015; Moura *et al.* 2016) have been reported to impact on cluster detection (Van Walle *et al.* 2018). The allele difference cut-off of ≤7 defined by Moura *et al.* (2016) has been confirmed as useful for cluster detection for both cgMLST schemas. A more stringent cluster cut-off of ≤4 allele differences in combination with single-linkage clusters may be considered for identifying isolates with more compelling microbiological evidence of being part of the same outbreak (Van Walle *et al.* 2018).

In the current study, four isolates identified as CT3407 that differed to the CT3406 group of isolates by nine loci using cgMLST and 96 SNPs using SNP analysis (Fig. 2a). These CT3407 isolates were isolated from range of RTE meats from the kitchen of hospital A, that had also isolated *L. monocytogenes* from the CT3406 group. If a smaller scale sampling effort had only isolated CT3407 from RTE meats, and not CT3406, then uncertainty as to whether the supplier was the source of the contamination

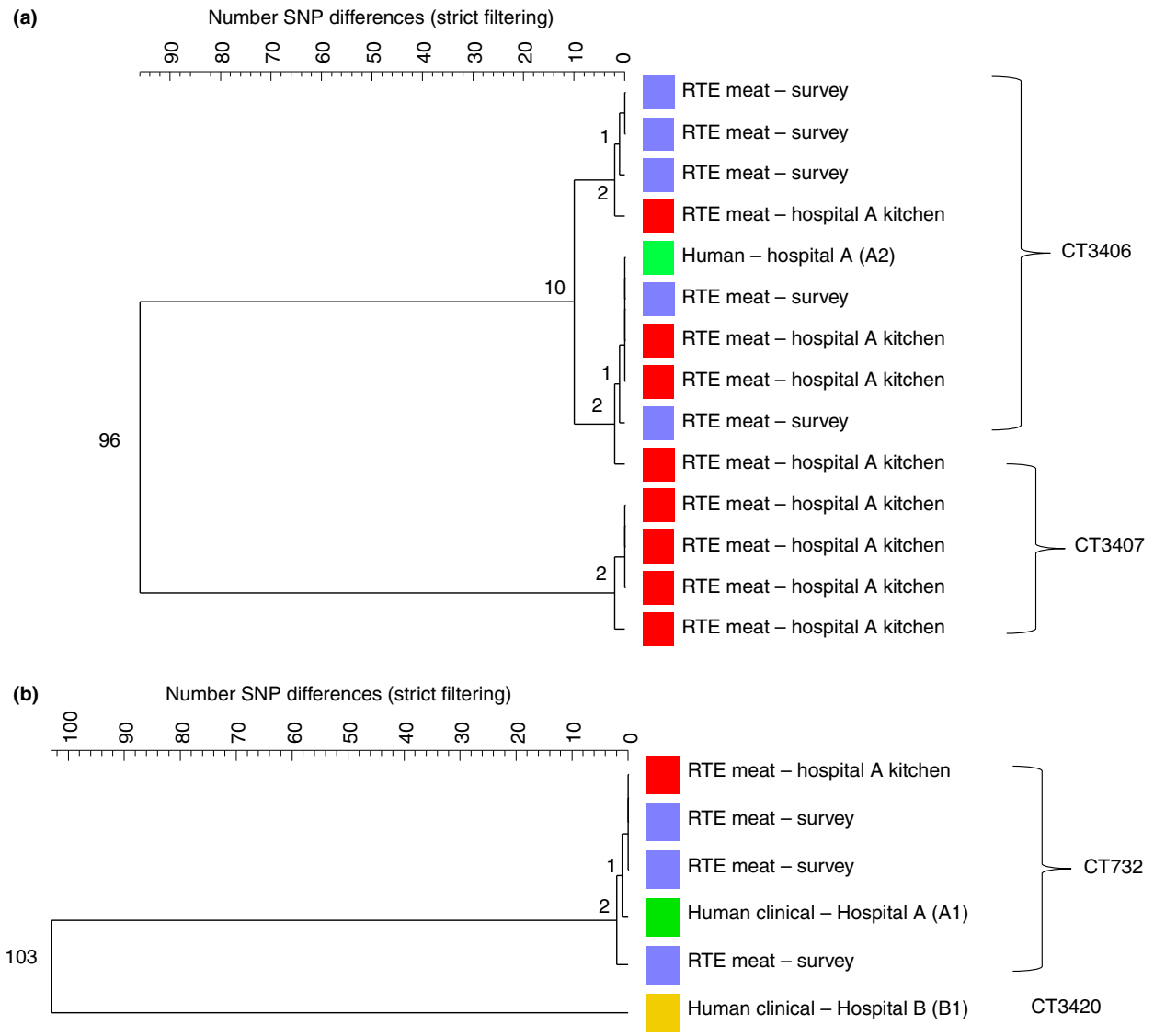

Figure 2 Dendrogram of whole-genome single nucleotide polymorphism (SNP) analysis of *Listeria monocytogenes* isolates associated with the outbreak and relevant isolates from the microbiological survey. Separate SNP analyses were performed for isolates identified as multilocus sequence type (ST) 9 and pulsotype Asc0103:Apa0058 (a) or ST7 and Asc0043a:Apa0026 (b). Single linkage with strict SNP filtering was used to construct dendrograms within BioNumerics, and the number of SNP differences between isolates is indicated on the branch nodes. The core-genome multilocus sequence type (CT) is indicated. [Colour figure can be viewed at wileyonlinelibrary.com]

may have arisen, due to the number of loci differences observed amongst these isolates. However, studies have indicated that a single cut-off cannot consistently predict whether isolates will be epidemiologically related and that WGS results should not be interpreted in isolation and other contextual information, such as epidemiological or product trace-back data, is needed to confirm a source (Jackson *et al.* 2016; Besser *et al.* 2019).

This latter point is also demonstrated for hospital B clinical isolate B1 which was indistinguishable by PFGE from the clinical isolate A1, and five isolates from RTE meats. WGS analysis demonstrated 33 cgMLST differences and 103 SNP analysis differences between these isolates. These levels of allele and SNP differences between clinical isolates B1 and A1 and the absence of any epidemiological connection suggest that the clinical isolate from hospital B was outside the epidemiologically relevant timeframe and therefore not associated with the outbreak in hospital A. Multiple WGS analyses, such as cgMLST and SNP analysis, were also observed to increase the confidence during the current outbreak investigation as previously reported (Chen *et al.* 2017).

The NZ outbreak also presented four unrelated genotypes amongst four cases from hospital A, and the presence of one other genotype amongst the RTE meats tested from the hospital was observed. Outbreaks involving more than one *L. monocytogenes* genotype have been reported previously (Laksanalamai *et al.* 2012; Garner and Kathariou 2016). Patients may be infected with more than one strain (Garner and Kathariou 2016), but the common practice in NZ of isolating and genotyping one isolate from each patient could potentially be missing some of the bigger picture. A number of factors can influence the diversity of isolates within an outbreak including the source of the food contamination, the type of food, the number and manner of *L. monocytogenes* introductions into a food processing environment, the size of the food processing facility and the environmental conditions and duration in which *L. monocytogenes* survives and grows before contaminating the food (Jackson *et al.* 2016).

In conclusion, retrospective WGS analysis of all of the *L. monocytogenes* isolates associated with the outbreak described here demonstrated that cgMLST and SNP analysis provided a greater degree of discrimination between isolates compared to PFGE. The multiple genotypes identified from clinical cases and RTE meats tested highlight that epidemiological concordance remains the key factor in outbreak investigations, for which genotyping can provide support or confirmation. Extensive testing of suspected food products driven by case epidemiology is recommended as the genomic characterization of multiple isolates can enhance outbreak investigations. The implementation of WGS analysis for routine *L. monocytogenes* surveillance in NZ will not only provide a greater discriminatory power than previously possible with PFGE, but also provide an expanding data set that can be further interrogated to help elucidate the evolution and population structure of *L. monocytogenes* in NZ.

Materials and methods

Isolation of *L. monocytogenes* isolation from foods

Samples of RTE foods, including a total of 62 unopened, prepackaged RTE meat products from the producer, were obtained from the hospital kitchen for *L. monocytogenes* testing. All products obtained during the outbreak investigation were tested using International Standards Organization Method 11290-1:1996/Amd1·2004 (International Organization for Standardization (ISO) 1996). A 25 g 'analytical unit' was sampled from each individual pack ('sample unit'). Analytical units were composited (up to a maximum of 5 × 25 g) for sample units of the same product type and production date (total of 17 composite samples). The confirmed *L. monocytogenes* isolates per product type and production date were selected for PFGE analysis.

Pulsed-field gel electrophoresis

All *L. monocytogenes* isolates obtained during the investigation, including human and food isolates, were typed using PFGE. Two restriction enzymes *Asc*I and *Apa*I were used as per standard protocols outlined by the Centre of Disease Control, Atlanta, PulseNet and analysed using procedures previously described (Rivas *et al.* 2017). All of the PFGE patterns obtained were compared to those contained in the PulseNet Aotearoa/New Zealand database and assigned a Asc:Apa pulsotype based on previously identified patterns. At the time of analysis (July, 2012), the database contained approximately 286 DNA profiles from human listeriosis cases since 1999, which consisted of 71 different PFGE patterns using *Asc*I enzyme and 44 different PFGE patterns using *Apa*I enzyme. The database contained very few isolates from sources other than human, with approximately 20 isolates from outbreak investigations and other microbiological surveys.

Whole-genome sequence analysis

Whole-genome sequencing and analysis were undertaken retrospectively. A total of 26 *L. monocytogenes* isolates associated with the outbreak and microbiological survey (Rivas *et al.* 2017) were selected and grown in 10 ml Tryptone Soya Broth at 37°C, for 18 h. One millilitre of culture was used for subsequent DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and final elutions were performed in 50 µl RNase-free water. DNA quality and concentration were assessed using NanoDrop (Thermo Fisher Scientific, Waltham, MA. USA), Qubit and PicoGreen (Quant-iT; Thermo Fisher Scientific). Sequencing libraries containing 1 ng of DNA were prepared using Nextera XT chemistry (Illumina, San Diego, CA, USA) for 250-bp pair-end sequencing run on an Illumina MiSeq sequencer, according to the manufacturer's recommendations (Illumina).

Sequence quality was evaluated on a per genome basis using BioNumerics Ver. 7·6.3 (Applied Maths, Austin, TX, USA). All genomes passed the basic quality metrics for raw sequence data from Illumina sequencers of average *Q*-score > 30 in both reads and at least 40 × average coverage with expected genome size for *L. monocytogenes* of approximately 3 Mb. The lineage, sublineage, multilocus ST and CT were inferred from WGS data and defined by using international nomenclature (http://bigsdb.pasteur.fr/listeria) (Moura *et al.* 2016). Within BioNumerics, phylogenetic cluster analysis of the isolates was investigated using cgMLST data (categorical data values) and

analysed using single-linkage algorithm. Whole-genome SNP analysis was also performed within BioNumerics using the strict SNP filtering option. A separate SNP analyses for isolates within the ST7 and ST9 groups were performed, where a clinical isolate (A1 and A3, respectively) was chosen as the reference sequence for each group (Kwong *et al.* 2016; Dahl *et al.* 2017). Within BioNumerics, phylogenetic cluster analysis of the isolates was investigated using cgMLST or SNP data (categorical data values) and analysed using single-linkage algorithm.

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Conflict of Interest

No conflict of interest declared.

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