Food Microbiology 84 (2019) 103237

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Traditional *Salmonella* Typhimurium typing tools (phage typing and MLVA) are sufficient to resolve well-defined outbreak events only.

Helen Kathleen Crabb*, Joanne Lee Allen, Joanne Maree Devlin, Simon Matthew Firestone, Mark Stevenson, Colin Reginald Wilks, James Rudkin Gilkerson

Asia-Pacific Centre for Animal Health, Melbourne Veterinary School, Faculty of Veterinary and Agricultural Science, The University of Melbourne, Victoria, Australia, 3010

ARTICLE INFO

Keywords:
Salmonella
Cluster analysis
Epidemiology
Serotyping
MLVA
Source attribution

ABSTRACT

Between 1991 and 2014 the per capita notification rate of salmonellosis in Australia increased from 31.9 to 69.7 cases per 100,000 people. *Salmonella* Typhimurium accounted for nearly half the human cases until the end of 2014. In this study, we used cluster analysis tools to compare *S.* Typhimurium isolates from a chicken-meat study with those reported to the National Enteric Pathogen Surveillance System (NEPSS) from the coincident human and non-human populations. There was limited phage type diversity within all populations and a lack of specificity of MLVA profiling within phage types. The chicken-meat study isolates were not significantly clustered with the human cases and at least 7 non-human sources, based on typing profiles (PT/MLVA combination), could be implicated as a source of human cases during the same period. In the absence of a strong surveillance system representative of all putative sources, MLVA and phage typing alone or in combination are insufficient to identify the source of human cases.

1. Introduction

The 2010 global human health impact of all foodborne associated *Salmonella enterica* infections was estimated to be the equivalent of 6.43 million (95% UI: 3.1–13.2 million) disability adjusted life years (Kirk et al., 2015). Between 1991 and the end of 2014 the per capita notification rate of salmonellosis in Australia increased from 31.9 to 69.7 cases per 100,000 people (Department of Health and Ageing) with *Salmonella enterica* subsp. *enterica* serovar Typhimurium the most frequently identified cause of foodborne salmonellosis. The proportion of cases attributable to this serovar appeared to be slowly increasing, from 42% in 2010 to 48% in 2015 (OzFoodNet, 2010, 2015). In contrast, the rate of foodborne salmonellosis has either declined or remained static in most other developed countries (Table 1) (Department of Health and Ageing; European Food Safety Authority, 2014, 2015; FoodNet, 2015; The Institute of Environmental Science and Research Ltd, 2015; Wingstrand et al., 2015). In contrast to other major poultry producing countries, *Salmonella enterica* subsp. *enterica* serovar Enteritidis is not considered to be endemic in the Australian commercial poultry industry and most human cases are attributed to overseas travel (Biosecurity Australia, 2008). A recent introduction of a novel phage type (PT 7a) into the poultry industry is currently undergoing eradication (Communicable Diseases, 2019).

In 2011, 7.5% of human salmonellosis in Australia could be attributed to a source based on epidemiological investigation of clustered cases with the same or similar *Salmonella* phenotype and genotype. Where a single source was identified, eggs and poultry meat were frequently implicated. Of those identified outbreaks 53% (26/49) were attributed to eggs and 16% (8/49) to poultry meat. The remaining 92.5% of *Salmonella* associated cases of human gastroenteritis could not be attributed to a single food source (Green and Fitzsimmons, 2013; OzFoodNet, 2015). The characterization of *Salmonella* serovars by phenotyping and genotyping methods such as phage typing (PT), pulsefield gel electrophoresis (PFGE), multi-locus variable-number tandem-repeats analysis (MLVA), multi-locus sequence typing (MLST) and now whole genome sequencing (WGS), have become standard techniques for investigating the epidemiology of *Salmonella* spp. and associated foodborne outbreaks of human salmonellosis in Australia and elsewhere (Achtman et al., 2012; Leekitcharoenphon et al., 2014; Ross et al., 2011; Wuyts et al., 2013). The advantages and disadvantages of each technique have been reviewed previously and it is generally agreed that MLVA profiling is the most discriminatory method for clonal point

* Corresponding author.
E-mail addresses: Helen.Crabb@unimelb.edu.au (H.K. Crabb), jlallen@unimelb.edu.au (J.L. Allen), devlinj@unimelb.edu.au (J.M. Devlin), simon.firestone@unimelb.edu.au (S.M. Firestone), mark.stevenson1@unimelb.edu.au (M. Stevenson), colinrw@unimelb.edu.au (C.R. Wilks), jrgilk@unimelb.edu.au (J.R. Gilkerson).

https://doi.org/10.1016/j.fm.2019.06.001
Received 26 August 2018; Received in revised form 10 May 2019; Accepted 2 June 2019
Available online 05 June 2019

Table 1
Comparison of per capita (100,000) notifications of human salmonellosis (1990–2014)[a].

Country	Notifications per 100,000 people (% Salmonella Typhimurium)			
	1990's	2000	2010	2013–14
Australia[b]	31.9 (42)	32.2	54.0 (48)	69.7
USA[c]	14[g]	14	18	15
New Zealand[d]	_	48 (51)	26 (52)	21 (41)
Denmark[e]	82[h] (32)	43 (19)	29 (32)	20 (38)
EU[f]	_	35[i] (13)	22 (22)	20 (20)

[a] Rates are sourced from national surveillance reports.
[b] (Department of Health and Ageing).
[c] (FoodNet, 2015).
[d] (The Institute of Environmental Science and Research Ltd, 2010).
[e] (Wingstrand et al., 2015).
[f] (European Food Safety Authority, 2012, 2015). Between country comparisons are indicative only; Country and time comparisons of reported data is limited as methodologies for calculating incidence, sampling and reporting vary between countries and years.
[g] 1994.
[h] 1996.
[i] 2006.

source outbreak investigations, but its value in complex epidemiological investigations over protracted time periods or in general surveillance is not clear (Baggesen et al., 2010; Boxrud et al., 2007; Rabsch, 2007; Sabat et al., 2013).

In Australia there is no nationally coordinated active surveillance program for *Salmonella* in food or food animals (Animal Health Australia, 2017). Multiple sources of passively acquired data are collated to provide national surveillance information. Food businesses, such as poultry meat processors, must test food products in accordance with the food safety legislation as part of HACCP based third party audited quality assurance programs that are monitored by food safety regulators (Food Standards Australia New Zealand, 2012). Two national reference laboratories provide *Salmonella* serotyping and phage typing services and this data is collated into both the National Enteric Pathogen Surveillance System (NEPSS) for enteric diseases (Department of Health and Ageing, 2007) and the National Notifiable Diseases Surveillance System (Department of Health and Ageing). Investigation of notifiable foodborne disease events in humans is conducted by state health departments and members of OzFoodNet (Department of Health and Ageing, 2007).

2. Materials and methods

2.1. Chicken meat study

A prospective cohort study investigating the transmission of *Salmonella enterica* within a single vertically integrated poultry enterprise was conducted between January 2013 and September 2014 (Crabb et al., 2018a, 2018b). Poultry supplied from this enterprise comprise ~7% of Australian chicken meat production. Environmental samples from the parent and broiler generation, hatchery and processing sites (60% of all locations) within the enterprise were collected for the duration of the study. Thirty-six percent of all samples collected (1503/4219) were positive for salmonellae of which 65% of those serotyped were identified as *Salmonella enterica* subsp. *enterica* serovar Typhimurium. The subset of isolates selected for this current analysis comprised all *S.* Typhimurium isolates (n = 421) fully identified using the same phage typing and MLVA profiling methodology as isolates reported to the NEPSS. A full description of the integration is published elsewhere, but in brief the integration operates with separate sites for parent breeder production (rearing and egg production), the hatchery, broiler production and meat processing (Crabb et al., 2018a, 2018b). Information on the study design, sampling methods and sample processing are available online at dx.doi.org/0.17504/protocols.io.n6zdhf6, dx.doi.org/10.17504/protocols.io.n6pdhdn and dx.doi.org/10.17504/protocols.io.n6sdhee.

2.2. Human and non-human surveillance data

A de-identified extract of *S.* Typhimurium human and non-human (animal, food, or environment) records submitted to the NEPSS from January 2013 through September 2014, were obtained. A total of 3950 human and 558 non-human records were available for analysis. Human records include culture positive salmonellosis cases reported to NEPSS, while the non-human records include all *Salmonella* isolates from sources other than humans reported to NEPSS. Non-human sources include food, animal, and environmental samples from all possible sources across Australia. Metadata for each *S.* Typhimurium record was limited to date of isolation, phage type, MLVA profile and origin (human or non-human).

2.3. Statistical analysis

All statistical analyses were conducted in the R statistical package unless otherwise stated (R Core Team, 2016). Ecological diversity and cluster analysis was conducted using the R libraries "vegan" (Oksansen et al., 2017), "BiodiversityR" (Kindt and Coe, 2005), "FactoMineR" (Le et al., 2008), "factoextra" (Kassambara and Mundt, 2017) and "pvcluster" (Suzuki and Shimodaira, 2015).

2.4. Sample size estimates

The null hypothesis under investigation was that there was no difference in *S.* Typhimurium typing profiles (PT, MLVA or combined PT/MLVA) between population groups. To ensure the sample size within each group was sufficient to detect a difference between two populations with 95% confidence and 80% power, the source population variation for each typing method (PT, MLVA, PT/MLVA) was estimated using the Shannon-Weiner Index of diversity (H') (Magurran, 1998). This variation was used to estimate the sample size required to detect a true difference between populations using an ANOVA power calculation for multiple group comparison (Kindt and Coe, 2005). As more within-population (s^2 = 21.1) than between-population (s^2 = 2.0–3.0) variance was observed using PT/MLVA combinations it was estimated that at least 50 isolates per group were required to provide sufficient analytical power for between group comparisons. Sources within the non-human NEPSS dataset with less than 50 samples within a category were aggregated resulting in four groups for comparison with the human and chicken-meat study isolates: bovine (n = 256), poultry (n = 200), companion animal (n = 44) and other (n = 102).

2.5. MLVA profile analysis

MLVA analysis and visualization of the resulting single and double locus variant minimum spanning trees (MST) was conducted using the goeBURST algorithm in "Phyloviz 2.0" (Feil et al., 2004; Nascimento et al., 2017; van Belkum et al., 2007).

2.6. Cluster analysis

The *S.* Typhimurium typing matrices, PT, MLVA or PT/MLVA, were normalized using a species profile transformation for each typing method to remove the effect of extremely high or low abundance typing profiles while maintaining the original composition of the source matrices (Legendre and Gallagher, 2001). The adequacy of normalization was tested using the Shapiro test and examination of the qqplot (Borcard et al., 2011). Euclidean distances were calculated on these transformed matrices. The difference in *S.* Typhimurium population

diversity was evaluated by measuring the ecological distance (dissimilarity) between sources, where the ecological distance between sources sharing many *S.* Typhimurium variants was small and the distance between sources sharing only a few variants was large. This is a measure of the correlation between two locations, or sources in this case. Hierarchical clustering was conducted using complete linkage on the transformed matrix. The significance of the clustering correlation was conducted using multiscale bootstrapping (n = 1000) to estimate the approximately unbiased (AU) P-value and 95% confidence intervals. If the AU P-value was greater than 95% then clustering was supported by the data (Suzuki and Shimodaira, 2015).

2.7. Principal component analysis

The ecological distance between sites was further evaluated using principal component analysis (PCA). The number of significant components was assessed using the broken stick test (Legendre and Legendre, 2012). Principal components were significant where the cumulative eigenvalue was greater than the equivalent broken stick value, sufficient to include no less than 75% of the total variance and visualized using a screeplot (Borcard et al., 2011). Typing profiles significantly contributing to the ordination were identified using the circle of equilibrium and their square cosine values (Kassambara, 2017; Legendre and Legendre, 2012).

3. Results

3.1. Chicken meat study

A total of 421 *S.* Typhimurium chicken-meat isolates were used for this comparison. Briefly, phenotyping (PT) and genotyping (MLVA) of the *S.* Typhimurium isolates identified 8 phage types with 41 MLVA profiles or 62 PT/MLVA combinations. Nearly 80% of these isolates were either DT135 (19.6%) or PT135a (59.9%) (Table 2). MLVA profiles were not specific to individual phage types with more than 83% of the MLVA profiles detected in more than one phage type.

3.2. Human isolates (NEPSS dataset)

Between January 2013 and September 2014, 3950 human isolates of *S* Typhimurium were fully characterized in the NEPSS dataset. Sixty-two phage types were reported and the top five most frequently identified phage types, DT9, DT135, PT135a, DT170 and DT44 comprised 85% of the isolates (Table 2). Phage types DT12, DT3, DT141 and DT193 were the next most frequently reported (7.8%) phage types, with the remaining 53 phage types (Other) comprising less than 1% of the isolates each. A total of 918 MLVA profiles and 1076 PT/MLVA combinations were described in this human dataset (Table 3).

Table 3
Number of unique *Salmonella* Typhimurium phage types and MLVA type detected within the human and non-human NEPSS dataset and the chicken meat study (2013–14).

Source Population	No. unique *Salmonella* Typhimurium phenotypes identified			
	Phage Type	MLVA Types	PT/MLVA combinations	Total Isolates
Human[a]	62	918	1076	3947
Bovine[b]	23	156	163	256
Poultry[b]	19	113	123	200
Companion[b]	13	35	35	44
Other[b]	17	50	51	102
Chicken Meat[c]	8	41	62	421
Total	66	1066	1286	4970

[§]No isolates of this phage type detected.
[a] Human NEPSS.
[b] Non-human NEPSS.
[c] Chicken meat study.

3.3. Non-human isolates (NEPSS dataset)

For the coincident sampling period of the chicken meat-study, 558 non-human isolates of *S.* Typhimurium from 25 different sources were submitted to the NEPSS database; Bovine and poultry sources comprised 82% of all isolates (Table 2). A total of 34 phage types with 313 MLVA profiles and 371 PT/MLVA combinations were described (Table 3). As with the human dataset, the top five most frequently identified phage types accounted for a large proportion of the cases (72%). The top four phage types were the same as the human isolates (DT9, DT135, PT135a, DT170), but the fifth most frequently detected phage type was DT141. The remainder of the top ten most frequently reported phage types differed by source and were not reported with the same frequency as those detected in humans: DT197, RDNC, DT29, DT8, DT4.

3.4. Phage typing and MLVA profiling

A comparison of the most frequently reported phage types in the NEPSS dataset identified a large amount of MLVA diversity within each phage type (H' = 5.89) (Supplementary Table 1). Phage types with the most MLVA diversity were DT9 and PT135a, while DT44 had the least diversity. The most frequently detected MLVA profiles in 2013 were also the most frequently detected profiles in 2014, but not all the MLVA profiles detected in 2013 were present in 2014. The proportion of profiles present in both years varied by phage type and ranged from 9 to 29%. Hierarchical cluster analysis identified a single cluster containing DT193 and DT170 PT/MLVA combinations (AU > 0.991, 95%CI: [0.985, 0.997]) but did not strongly support clustering of the other PT/MLVA combinations within the dataset (Fig. 1).

Table 2
Comparison of *Salmonella* Typhimurium phage types (%) detected in the human and non-human NEPSS dataset and the chicken meat study (2013–14).

Source Population	No. Salmonella Typhimurium isolates (%) detected in each source						
	DT9	DT170	DT135	PT135a	DT44	Other	Total
Human[a]	992 (25)	643 (19)	745 (19)	787 (20)	201 (5)	579 (15)	3947
Bovine[b]	58 (23)	39 (15)	9 (4)	34 (13)	12 (5)	104 (41)	256
Poultry[b]	54 (27)	48 (24)	41 (21)	22 (11)	14 (7)	21 (11)	200
Companion[b]	11 (26)	ND[d]	10 (23)	10 (23)	2 (5)	11 (26)	44
Other[b]	18 (18)	4 (4)	18 (18)	23 (23)	3 (3)	36 (35)	102
Chicken Meat[c]	9 (2)	ND[d]	82 (20)	251 (60)	ND[d]	79 (18)	421

[a] Human NEPSS.
[b] Non-human NEPSS.
[c] Chicken meat study.
[d] No isolates of this phage type detected.

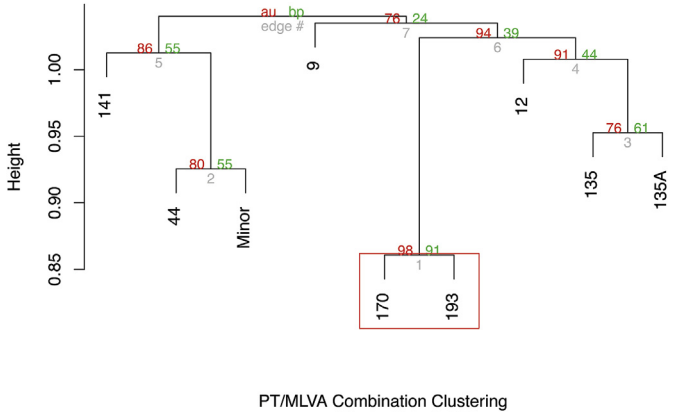

Fig. 1. Hierarchical cluster (correlation) dendrogram of PT/MLVA combinations using multiscale bootstrapping support for clusters. Clusters with approximately unbiased P-values (AU) greater than 95% are strongly supported. A single cluster containing DT170 and DT193 is statistically supported by the dataset (Highlighted in red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

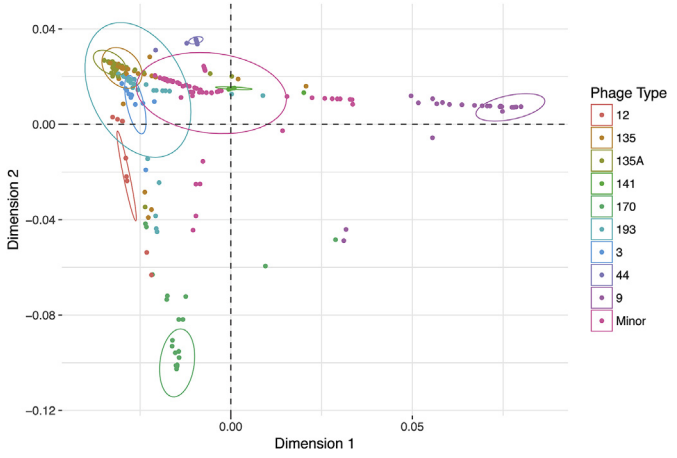

Fig. 2. Principal Component Analysis of individual samples by PT and MLVA profile. Individual locations represent the position of the MLVA profile and phage type in a two-dimensional space with 95% confidence ellipses around individual MLVA patterns with the same phage type. The variation explained within each dimension is low, this is highlighted by the large confidence ellipses around many of MLVA profiles and overlapping of phage type ellipses, indicating that MLVA profile is not unique to specific phage types.

There was significant overlap between phage types and MLVA profiles, with multiple MLVA profiles contained within each of the phage type confidence ellipses (Fig. 2). Only subsets of the PT/MLVA combinations (DT9, PT135a and DT170) were well defined within the PCA. Ten significant principal components explained only 24.7% of the variance between phage type and MLVA profiles (Supplementary Table 2), indicating that MLVA profile is poorly correlated with phage type.

3.5. Comparison of S. Typhimurium sources

All MLVA patterns were linked into a single MST; 984 MLVA profiles were linked by a single locus, 72 by two loci and 9 by three loci. Phage types within the MST were largely clustered but DT135 and PT135a were identifiable within multiple clusters within the MST (Fig. 3). Non-human NEPSS isolates were scattered within the human isolates (Fig. 4), particularly the bovine origin isolates which appear across all branches of the MST. In contrast, the chicken-meat study isolates were tightly clustered within the tree. Hierarchical clustering

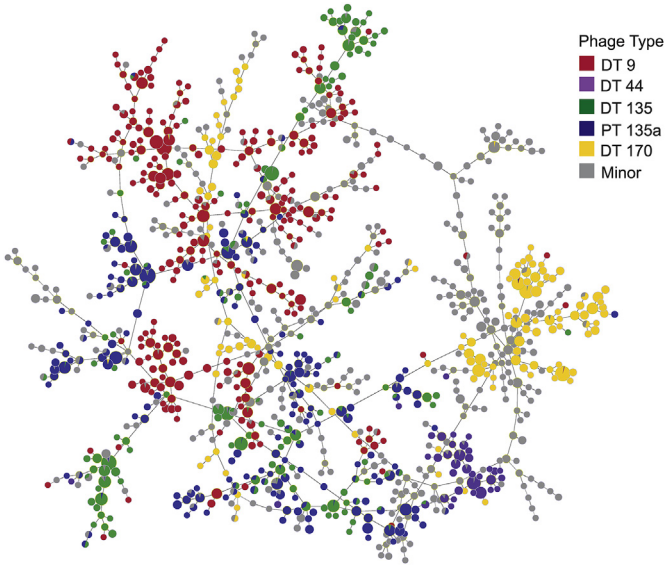

Fig. 3. MLVA profile minimum spanning tree (MST) with all NEPSS human and non-human plus chicken-meat study isolates. All data combined into a single MST with 984 MLVA profiles joined by a single locus within the MLVA profile. MLVA profiles are coloured by the *Salmonella* Typhimurium phage type of the isolate.

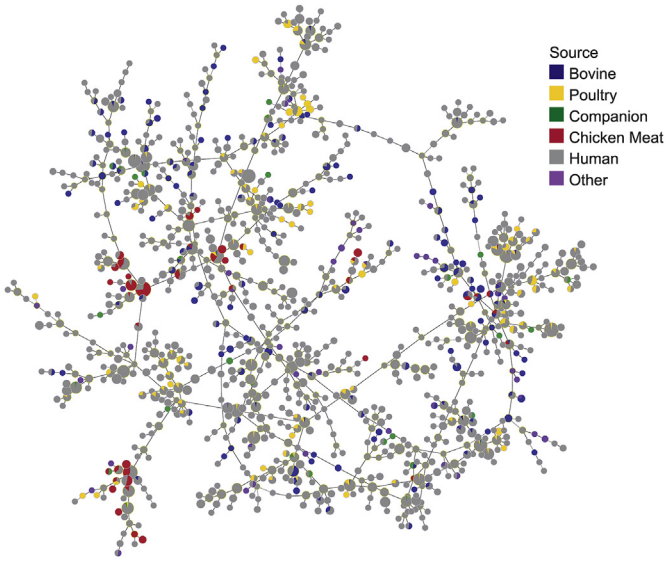

Fig. 4. MLVA profile minimum spanning tree (MST) with all NEPSS human and non-human data plus the chicken-meat study isolates. All data is combined into a single MST with 984 MLVA profiles joined by a single locus within the MLVA profile. MLVA profiles are colored by source of isolate.

and PCA demonstrated that there was a difference between the typing methods (PT, MLVA or PT/MLVA combinations) in the clustering of *S.* Typhimurium isolates by source. Clustering of companion animal and other source isolates (Supplementary Fig. 1A) were strongly supported by phage typing (AU > 0.980, 95% CI: [0.970, 0.990]), the clustering of companion animal and chicken-meat study isolates (Supplementary Fig. 1B and Supplementary Fig. 1C) were strongly supported by MLVA typing (AU > 0.972, 95% CI: [0.962, 0.982]) and PT/MLVA combinations (AU > 0.988, 95% CI: [0.980, 0.996]). PT/MLVA combination clustering (Supplementary Fig. 1C) also supported clustering of human and poultry isolates (AU > 0.862, 95% CI: [0.854, 0.870]), but clustering of bovine and other sourced isolates was poorly supported (AU > 0.816, 95% CI: [0.806, 0.826]).

3.6. Principal component analysis by source

PCA determined that the chicken-meat study and companion animal isolates clustered separately from isolates from all other sources. All the variance in the *Salmonella* Typhimurium typing (PT/MLVA combination) by source was explained in 5 principal components, with 2 components explaining 82% of the variation (Supplementary Table 3). The chicken-meat study isolates and the companion animal cases are clustered separately (different quadrants) from the bovine, human, poultry and other sources of *Salmonella* Typhimurium (Supplementary Fig. 2). The chicken-meat study isolates contained more variation than any other source, indicating that the chicken-meat study isolates were significantly different from the remaining sources.

3.7. Comparison of chicken-meat isolates with NEPSS dataset

Four of the five most frequently detected *S.* Typhimurium phage types identified in the chicken-meat study were also detected in the human NEPSS dataset (PT9, DT135, PT135a and DT193) (Table 2). The number of isolates (511 (12.9%)) in the NEPSS dataset exactly matching those found in the chicken-meat study were identified in seven other sources, including humans, but only 24 of the 62 PT/MLVA combinations identified matched those in the NEPSS dataset exactly. The four largest clusters containing chicken-meat study isolates identified in the MST were assessed further (Table 4). These four clusters included 465 human cases, from 4 to 6 sources including the chicken-meat study. Between 14 and 23 PT/MLVA combinations were identified within each of the clusters, indicating that limiting cluster identification or putative source to a single matching PT or PT/MLVA combination is flawed. Only 74/465 (15.9%) of these human cases could be temporally (occurring within a month of detection in either the chicken-meat or the human population) associated with cases from the chicken-meat study.

4. Discussion

This study highlights several critical limitations with the epidemiological investigation of potential sources of *S.* Typhimurium using the available passive surveillance data, particularly for the non-attributable cases which comprise up to 93% of human salmonellosis in Australia (Green and Fitzsimmons, 2013; OzFoodNet, 2015). Firstly, the variation in phage types present in the human and non-human datasets is small. Four phage types (DT9, DT135 and PT135a, DT108/170) were the most frequently detected in both years in all datasets, including the chicken-meat study (except DT108/170). These dominant phage types are routinely identified in Australia from many sources as indicated in the results presented here, and in other surveillance reports and studies (Heuzenroeder et al., 2013; Powling, 2012, 2013). Ninety percent of the phage types (56/62) comprised only 10% of the isolates reported within the NEPSS dataset. The predominance of a few phage types means that additional typing methodologies are essential for further differentiation of isolates, to enable clustering of isolates to a common source.

Hierarchical clustering and principal component analysis demonstrated the non-specificity of the phage type and MLVA profiles. Only 24% of the variation between isolates is explained by the use of MLVA profiles for describing the *S.* Typhimurium isolates reported to the NEPSS database, indicating that MLVA type is not specific to phage type, which is consistent with the biology of the tandem-repeats region. MLVA profiling increases the apparent diversity of the phage types, but as the MLVA profiles are not unique to phage type, it does not improve attribution of the cases to a single source. These results are consistent with the findings of our previous study (Crabb et al., 2018a, 2018b) and demonstrate that the use of MLVA profiling and phage typing on their own or in combination are insufficient, in the absence of strong additional epidemiological evidence such as temporal or spatial clustering, for source attribution.

This analysis is limited by the quantity of the source information obtained from the non-human NEPSS data. There were nearly as many cases from a single source, the chicken meat study (n = 421), as from the NEPSS non-human dataset (n = 558), indicating the relative paucity of non-human data available for comparison for the coincident period. Despite 25 isolate sources being present within the non-human NEPSS dataset, most isolates were from bovine (46%) and poultry (36%) sources. Grouping of source isolates ($n \cong 50$) was required to ensure that clustering or PCA analysis was conducted with sufficient power to determine any associations between sources. This consequently caused loss in resolution within the dataset as most groups contained fewer isolates than required for robust analysis and consequently attribution is biased to those isolate sources comprising the majority of the dataset. The results of source attribution using similarly sparse datasets should therefore be interpreted with caution in light of these results. The data contained within the non-human database is comprised of opportunistically collected isolates that may have been obtained during trace-back outbreak investigations, or from animal disease cases submitted for *Salmonella* typing (Department of Health and Ageing, 2007; Forsyth et al., 2006). There is no nationally coordinated structured surveillance for *Salmonella* spp. in food animals in Australia (Department of Health and Ageing). Thus, the non-human NEPSS dataset is not a representative sample of all possible sources for source attribution of human cases. Therefore, the attribution of human cases to only those sources contained within the non-human dataset should be made with extreme caution in the absence of supporting epidemiological data.

The comparison of the chicken meat isolates with those identified within the NEPSS database confirmed that isolates detected in the chicken-meat study may be entering the human population and contributing to human infection. However, the identification of multiple potential sources of the same or very similar *S.* Typhimurium isolates (PT/MLVA combination) at the same time indicates that incorrect source attribution is probable when utilizing retrospectively collected

Table 4
Cluster identification and temporal comparison of chicken meat study isolates with matching MLVA profiles and phage types to human cases.

Cluster ID	Year	Human Cases	Human and Non-Human NEPSS Data				Chicken Meat	
			No. Sources	MLVA	Phage Types	PT/MLVA Combinations	Attributable cases/total cases (within clusters)	No. variants present/total variants
1	2013	80/2109	6	8	6	19	0/54	0/5
2	2013	76/2109	5	28	8	33	0/76	1/1
3	2013	45/2109	4	12	6	19	25/39	1/11
	2014	75/1841	4	14	7	21	49/63	2/8
4	2013	65/2109	5	8	6	14	0/25	0/8
	2014	124/1841	6	17	8	24	0/109	0/10
All	2013–2014	511/3950	7	1066	66	1286	111/171	11/51

*2014 is only partial to the end of September.
†MLVA presented using Australian reporting method as reported to NEPSS.

passive surveillance data. The low temporal association between a well sampled potential source and human cases emphasizes the limitations of passive surveillance and the current tools available for typing.

The rapid introduction of tools such as whole genome sequencing is necessary to differentiate their true relationships. The disparity between the NEPSS poultry and the chicken-meat study with the human isolates (demonstrated by different clustering) and confirmation bias (human isolates clustered with poultry isolates) indicated within the MST, indicates that substantial source information is missing. All comparisons were made using both the chicken meat study isolates from the field study, plus all the non-human results available in the NEPSS surveillance system for the contemporaneous period. The NEPSS chicken isolates, comprise more than one third of the isolates available for comparison. The addition of the study isolates does not improve the resolution of the human cases indicating that unknown sources remain. The disparity between the NEPSS poultry and the chicken-meat study with the human isolates (demonstrated by different clustering) and confirmation bias (human isolates clustered with poultry isolates) indicated within the MST, indicates that substantial source information is missing. These results support some previous findings (Fearnley et al., 2011; South Australia Health, 2014) and confirm that much remains unknown about sources of *S.* Typhimurium in Australia. This would likely explain why human cases mostly remain unattributed to a source.

5. Conclusion

A single large source of isolates (chicken-meat study) available for comparison demonstrated with high confidence that these isolates were not a significant source of human infection and that multiple sources of *Salmonella* Typhimurium with the same typing profiles exist at the same time. This study highlights the limitations of the current epidemiological tools available for the identification of salmonellosis cases non-attributable to a source and the necessity of the rapid introduction of alternate typing methods, such as whole genome sequencing, for the identification of all isolates and attribution of cases to a potential source.

Acknowledgments

The authors would like to acknowledge the support of the producers and farm managers without whom this study could not have been conducted. We also acknowledge the support of the typing laboratory (Microbiological Diagnostic Unit, Victoria) for their assistance in serotyping and MLVA profiling of the chicken meat study isolates and Joan Powling for her assistance in providing the human and non-human surveillance data for this study. H. Crabb was funded by a Postgraduate Training Award and this project was supported by funding from the Cybec Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.06.001.

References

Achtman, M., Wain, J., Weill, F.-X., Nair, S., Zhou, Z., Sangal, V., Krauland, M.G., Hale, J.L., Harbottle, H., Uesbeck, A., Dougan, G., Harrison, L.H., Brisse, S., the *S. enterica* MLST study group, 2012. Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. PLoS Pathog. 8, e1002776.

Animal Health Australia, 2017. Animal Health in Australia 2016. (Canberra, Australia).

Baggesen, D.L., Sorensen, G., Neilsen, E.M., Wegener, H.C., 2010. Phage typing of *Salmonella* Typhimurium - is it still a useful tool for surveillance and outbreak investigation? Euro Surveill. 15, 19471.

Biosecurity Australia, 2008. Generic Import Risk Analysis Report for Chicken Meat: Final Report. Bioscurity Australia, Canberra, Australia.

Borcard, D., Gillet, F., Legendre, P., 2011. Numerical ecology with R. In: Gentleman, R., Hornik, K., Parmigiani, G.G. (Eds.), Use R!. Springer, New York, pp. 315.

Boxrud, D., Pedersen-Gulrud, K., Wotton, J., Medus, C., Lyszkowicz, E., Besser, J., Bartkus, J.M., 2007. Comparison of multiple-locus variable-number tandem repeat analysis, pulsed-field gel electrophoresis and phage typing for subtype analysis of *Salmonella enterica* serotype Enteritidis. J. Clin. Microbiol. 45, 536–543.

Communicable Diseases, 2019. *Salmonella* Enteritidis Outbreak Linked to Contaminated Eggs. NSW Government Ministry of Health.

Crabb, H.K., Allen, J.L., Devlin, J.M., Firestone, S.M., Stevenson, M.A., Gilkerson, J.R., 2018a. The use of social network analysis to examine the transmission of *Salmonella* spp. within a vertically integrated broiler enterprise. Food Microbiol. 71, 73–81.

Crabb, H.K., Allen, J.L., Devlin, J.M., Firestone, S.M., Wilks, C.R., Gilkerson, J.R., 2018b. *Salmonella* spp. transmission in a vertically integrated poultry operation: clustering and diversity analysis using phenotyping (serotyping, phage typing) and genotyping (MLVA). PLoS One 13, e0201031.

Department of Health and Ageing, National notifiable diseases surveillance system, in: Australia, C.D. (Ed.), http://www.health.gov.au/internet/main/Publishing.nsf/Content/cda-surveil-nndss-nndssintro.htm.

Department of Health and Ageing, 2007. Surveillance systems reported in CDI, 2007. Comm. Dis. Intell. 31, 159–164.

European Food Safety Authority, 2012. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. EFSA Journal 10, 2597.

European Food Safety Authority, 2014. The European Union summary report on trends and sources of zoonotic agents and food-borne outbreaks in 2012. EFSA Journal 12, 3547.

European Food Safety Authority, 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA Journal 13, 3991.

Fearnley, E., Raupach, J., Lagala, F., Cameron, S., 2011. *Salmonella* in chicken meat, eggs and humans; Adelaide, South Australia, 2008. Int. J. Food Microbiol. 146, 219–227.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., Spratt, B.G., 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. 186, 1518–1530.

Food Standards Australia New Zealand, 2012. Primary Production and Processing Standard for Poultry Meat. Food Standards Australia New Zealand.

FoodNet, 2015. Foodborne Diseases Active Surveillance Network (FoodNet): Tables and Figures 2014. http://www.cdc.gov/foodnet/trends/2014/number-of-infections-by-year-1996-2014.html#table2b.

Forsyth, J., Lightfoot, D., Powling, J., Tan, A., 2006. *Salmonella* surveillance in Australia - created from adversity. J. AOAC Int. 89, 560–565.

Green, M., Fitzsimmons, G., 2013. The OzFoodNet story: 2000 to present day. Microbiology Australia 34, 59–62.

Heuzenroeder, M.W., Ross, I.L., Hocking, H., Davos, D., Young, C.C., Morgan, G., 2013. An Integrated Typing Service for the Surveillance of *Salmonella* in Chickens. Rural Industries Research and Development Corporation, ACT, Australia.

Kassambara, A., 2017. Practical Guide to Principal Component Methods in R Statistical Tools for High-Throughput Analysis. http://www.stdha.com.

Kassambara, A., Mundt, F., 2017. Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. http://www.sthda.com/english/rpkgs/factoextra.

Kindt, R., Coe, R., 2005. Tree Diversity Analysis. A Manual and Software for Common Statistical Methods for Ecological and Biodiversity Studies. World Agroforestry Centre (ICRAF), Nairobi.

Kirk, M.D., Pires, S.M., Black, R.E., Caipo, M., Crump, J.A., Devleesschauwer, B., Dopfer, D., Fazil, A., Fischer-Walker, C.L., Hald, T., Hall, A.J., Keddy, K.H., Lake, R.J., Lanata, C.F., Torgerson, P.R., Havelaar, A.H., Angulo, F.J., 2015. World health organization estimates of the global and regional disease burden of 22 foodborne bacterial, Protozoal, and viral diseases, 2010: a data synthesis. PLoS Med. 12, e1001921.

Le, S., Josse, J., Husson, F., 2008. FactoMineR: an R package for multivariate analysis. J. Stat. Softw. 25, 1–18.

Leekitcharoenphon, P., Nielsen, E.M., Kaas, R.S., Lund, O., Aerestrup, F.M., 2014. Evaluation of whole genome sequencing for outbreak detection of *Salmonella enterica*. PLoS One 9, e87991.

Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129, 271–280.

Legendre, P., Legendre, L., 2012. Numerical Ecology, third ed. Elsevier, Oxford, UK.

Magurran, A.E., 1998. Ecological Diversity and its Measurement. Springer United Kingdom.

Nascimento, M., Sousa, A., Ramirez, M., Francisco, A.P., Carriço, J.A., Vaz, C., 2017. PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. Bioinformatics 33, 128–129.

Oksansen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Szoecs, E., Wagner, H., 2017. Vegan: community ecology package. https://Cran.R-project.org.

OzFoodNet, OzFoodNet - Enhancing Surveillance for Foodborne Disease in Australia. www.ozfoodnet.gov.au.

OzFoodNet, 2010. Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet network, 2009. Comm. Dis. Intell. 34, 396–426.

OzFoodNet, 2015. Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet network, 2011. Comm. Dis. Intell. 39, E236–E264.

Powling, J., 2012. In: Hogg, G. (Ed.), National Enteric Pathogen Surveillance Scheme Non-human Annual Report 2012. The University of Melbourne, Victoria, Australia, pp. 34.

Powling, J., 2013. In: Vulcanis, M. (Ed.), National Enteric Pathogens Surveillance Scheme Non-human Annual Report 2013. The University of Melbourne, Victoria, Australia, pp. 34.

R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for statistical computing, Vienna, Austria.
Rabsch, W., 2007. *Salmonella* Typhimurium phage typing for pathogens. In: Schatten, H., Eisenstark, A. (Eds.), Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 177–212.
Ross, I.L., Davos, D.E., Mwanri, L., Raupach, J., Heuzenroeder, M.W., 2011. MLVA and phage typing as complementary tools in the epidemiological investigation of *Salmonella enterica* serovar Typhimurium clusters. Curr. Microbiol. 62, 1034–1038.
Sabat, A.J., Budimir, A., Nashev, D., Sá-Leào, R., Van Dijl, J.M., Laurent, F., Grundmann, H., Friedrich, A.W., on behalf of the ESCMID Study group of Epidemiological Markers, 2013. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveill. 18, 20380.
South Australia Health, 2014. Food Act Report: 2014. Government of South Australia, Adelaide, South Australia.
Suzuki, R., Shimodaira, H., 2015. Pvclust: Hierarchical Clustering with P-Values via Multiscale Bootstrap, 2.vols. 0–0 (Cran-R-project).
The Institute of Environmental Science and Research Ltd, 2010. Notifiable and Other Diseases in New Zealand. (Annual Report, Porirua, New Zealand).
The Institute of Environmental Science and Research Ltd, 2015. Notifiable Diseases in New Zealand. Annual Report 2014, Porirua, New Zealand.
van Belkum, A., Tassios, P.T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N.K., Fussing, V., Green, J., Feil, E., Gerner-Smidt, P., Brisse, S., Struelens, M., 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin. Microbiol. Infect. 13, 1–46.
Wingstrand, A., Sørensen, A.I.V., Helwigh, B., Müller, L., 2015. Annual Report on Zoonoses in Denmark 2014. DTU Food, Sørborg, Denmark.
Wuyts, V., Mattheus, W., De Laminne de Bex, G., Wildemauwe, C., Roosens, N.H.C., Marchal, K., De Keersmaecker, S.C.J., Bertrand, S., 2013. MLVA as a tool for public health surveillance of human *Salmonella* Typhimurium: prospective study in Belgium and evaluation of MLVA loci stability. PLoS One 8, e84055.