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Brachyspira intermedia strain diversity and relationships to the other indole-positive *Brachyspira* species

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The aims of this study were to use multilocus sequence typing (MLST) to i) investigate the population structure, diversity and molecular epidemiology of the weakly haemolytic anaerobic intestinal spirochaete *Brachyspira intermedia*, and ii) determine the relationship of the species to the other two indole-positive but strongly haemolytic *Brachyspira* species - *B. hyodysenteriae* and "*B. suanatina*". Seventy-seven *B. intermedia* isolates from pigs and chickens were analysed, with the nucleotide sequences of seven conserved genomic loci examined for each. *B. intermedia* was genetically diverse, with the 77 isolates being divided into 71 sequence types (STs) and 64 amino acid types (AATs). Many distinct groups of *B. intermedia* isolates were identified, with some isolates being separated from others by large genetic distances. Although both pig and chicken isolates were found in most groups, suggesting that cross-species transmission of such isolates may occur, some isolates from pigs were located in small groups that did not include chicken isolates, and vica versa. Eight clonal complexes (Cc) of STs were identified by e-Burst analysis. The Ccs contained between 2 and 5 STs, and between 2 and 9 isolates. Five Ccs contained multiple isolates from the same farms, collected at the same time, indicating the existence of ongoing minor genetic change amongst isolates at the farm level. On the other hand, isolates with quite different STs also were found amongst multiple isolates collected from some farms. By comparison with the much more restricted diversity observed for 111 isolates of *B. hyodysenteriae*, and four isolates of "*B. suanatina*", it is difficult to justify including all weakly haemolytic indole-positive *Brachyspira* isolates in the single species *B. intermedia*.

Keywords: Brachyspira intermedia; MLST; pigs; chickens; molecular epidemiology; spirochaete

1. Introduction

Brachyspira intermedia is one of seven officially named species of anaerobic intestinal spirochaetes in the genus *Brachyspira* (Hampson and La, 2006; Stanton, 2006). Members of this species are indole-positive, as are the related pathogenic species *Brachyspira hyodysenteriae* (the agent of swine dysentery) and the proposed species "*Brachyspira suanatina*" (Råsbäck et al., 2007a); however, *B. intermedia* isolates are weakly haemolytic while the other two species are strongly haemolytic. *B. intermedia* isolates can be recovered from the faeces of pigs, and from laying chickens and breeding hens. There is some evidence from the field to suggest that *B. intermedia* isolates may cause colitis and diarrhoea in pigs (Binek and Szynkiewicz, 1984; Fellström and Gunnarsson, 1995; Komaret et al., 2009), although this suggestion has not been supported by the results of several experimental infection studies (Hudson et al., 1976; Neef et al., 1994; Jensen et al., 2000). On the other hand, *B. intermedia* infections in chickens have been associated with diarrhoea and/or reduced egg production in the field (Stephens and Hampson, 1999; Bano et al., 2008; Feberwee et al., 2008), and have caused diarrhoea, reduced egg production and/or reduced growth rates in experimentally infected chickens (Dwars et al., 1992a, 1992b, 1993; Hampson and McLaren, 1999).

Studies analysing *B. intermedia* isolates using multilocus enzyme electrophoresis (MLEE) or pulsed field gel electrophoresis (PFGE) have suggested that the species is diverse (Lee et al., 1993; McLaren et al., 1997; Suriyaarachichi et al., 2000; Stephens et al., 2005). Relationships between the *Brachyspira* species have been studied using16S rDNA sequencing, but it can be difficult to differentiate between them with this technique (Stanton, 2006). MLEE is useful for distinguishing *Brachyspira* species, but the technique is time consuming. On the other hand, multilocus sequence typing

(MLST) is easier to perform, and, even using small numbers of isolates, it seems to be a promising approach to *Brachyspira* speciation (Råsbäck et al., 2007b).

The purpose of the present study was to use MLST with a view to i) studying the population structure, diversity and molecular epidemiology of *B. intermedia*, and ii) investigating the relationship of this species to the other two indole-positive species, *B. hyodysenteriae* and "*B. suanatina*".

2. Materials and methods

2.1. Brachyspira intermedia isolates

A total of 70 isolates of *B. intermedia* were obtained as frozen stock from the culture collection at the Reference Centre for Intestinal Spirochaetes at Murdoch University. These had been identified as *B. intermedia* based on their phenotypic properties (weakly haemolytic, indole-positive, alpha-glucosidase positive, alpha galactosidase negative), and amplification in a specific polymerase chain reaction assay based on the NADH oxidase gene (Phillips et al., 2006), but with a minor modification of the forward primer to 5'-AGAGTTTGAAGACACTTATGAC-3', as this primer improves the performance of the original *B. intermedia* PCR (Phillips ND and La T, Murdoch University, unpublished data). The isolates included 49 from chickens and 21 from pigs. Data for another seven porcine isolates that were examined in a previous MLST study of the whole genus (Råsbäck et al., 2007b) were obtained from PubMLST (http://pubmlst.org/) and were included in the analysis. The isolates originated from Australia (n=62), Sweden (n=5), the UK (n=4), New Zealand (n=2), Finland (n=2), the Netherlands (n=1), and the USA (n=1). Multiple isolates were obtained at the same time from 7 Australian farms and one New Zealand farm, with between 2 and 12 isolates examined. Six of these were farms with laying hens and two were pig farms. The names

of the isolates and their origins are presented in Table 1. Some of the isolates previously had been examined using PFGE (Suriyaarachchi et al., 2000) and/or MLEE (Lee et al., 1993; McLaren et al., 1998; Stephens et al., 2005).

2.2. MLST data for B. hyodysenteriae and "B. suanatina"

MLST data for 111 isolates of *B. hyodysenteriae* (La et al., 2009), and for four isolates of "*B. suanatina*" (Råsbäck et al., 2007b) were obtained from PubMLST, and were included in the analysis.

2.3 Bacterial culture, DNA extraction and MLST

The methods used for anaerobic spirochaete culture, DNA extraction and MLST at seven loci were exactly as previously reported by us for analysis of *B. hyodysenteriae* by MLST (La et al., 2009).

2.4. Data analysis

The aligned sequences for each of the loci were analysed using the non-redundant databases (NRDB) program (http://pubmlst.org/analysis/) to identify isolate sequences that were identical. Each unique nucleotide sequence was assigned a unique allele number. The allelic profile for each isolate was determined and consisted of a line listing the allele number for each locus in turn. Isolates were assigned a sequence type (ST) according to their allelic profiles. Isolates were considered genetically identical and hence of the same ST if they were identical at all 7 loci. The sequences of the *B. intermedia* MLST alleles for each locus were deposited at the PubMLST site (http://pubmlst.org/). Unique amino acid types (AATs) predicted from the nucleotide sequences also were recorded. A consensus MLST dendrogram was constructed from

the data matrix of allelic mismatches using START2 with 1000 bootstrap replicates (Jolley et al., 2001), using the unweighted pair group method with averages (UPGMA) method. Isolates were grouped into clonal complexes (Cc) by the BURST algorithm using the eBURST v3 program (Feil et al., 2004). Within the program, a population snapshot was viewed by setting the group definition as 0/7 shared alleles. The same analysis was used for the AATs. The degree of linkage disequilibrium in the population was estimated by calculating the Index of Association (I_A) for the 77 isolates and the 71 STs, using the START2 program. A Diversity Index (DI) based on Simpson's index of diversity was calculated for the results, as previously described (La et al., 2009). Data from the PubMLST site for *B. hyodysenteriae* and *"B. suanatina"* were compared with the *B. intermedia* data over the same seven loci.

Allele sequences for each *B. intermedia*, *B. hyodysenteriae* and "*B. suanatina*" sequence type (ST) also were concatenated using Geneious Pro version 3.8.5 (http://www.geneious.com/) in the gene order *adh*, *pgm*, *est*, *glp*, *gdh*, *thi* and *alp* used by Råsbäck et al (2007b). Nucleic acid and deduced amino acid sequences were concatenated in the same order. The concatenated sequences were aligned using ClustalW2 (Larkin et al., 2007) and converted to the MEGA format (Tamura et al, 2007). Phylogenetic trees for the aligned nucleic acid and amino acid sequences were constructed using the UPGMA method in MEGA version 4.0. The maximum likelihood model was used for the nucleic acid sequences and the Poisson correction model for the amino acid sequences, both with 1000 bootstrap replicates. An unrooted radiation tree was also constructed to help visualise relationships between isolates from the three species. Sequence type designations used in the concatenated trees were the same as in the consensus trees. On the trees where are three species were shown, the prefixes T, H or S were added to differentiate STs of *B. intermedia*, *B. hyodysenteriae* and "*B.*

suanatina", respectively, and the prefixes aT, aH and aS were used to differentiate the amino acid types.

3. Results

3.1. B. intermedia sequence types, population structure and molecular epidemiology

The 77 *B. intermedia* isolates analysed were divided into 71 STs and 64 AATs (Table 1). The corresponding allele numbers assigned for all the STs are shown in the supplementary table, and these raw sequences are recorded in the PubMLST site. Allelic frequency over the seven loci ranged from 19 (for *adh*) to 43 (for *pgm* and *thi*), with a mean of 34. Based on the number of isolates tested the population had an I_A value of 1.31, whilst based on the number of STs the standardised I_A was 0.76. Significant linkage disequilibrium (P <0.001) was detected in both analyses.

[Table 1 about here]

A dendrogram based on consensus data showing the relative relationships of the 71 STs of *B. intermedia* is presented as Figure 1. The STs are arranged in the same order as they appear in Table 1. There was some clustering of *B. intermedia* isolates, but most of the clusters were small, and many clusters were separated from each other by large genetic distances. *B. intermedia* isolates from pigs and chickens were located in STs throughout the tree, although there was a tendency for some isolates from pigs to be found towards the periphery of the tree, being more distantly related than the majority of the chicken isolates (the names of the STs containing porcine isolates are marked in bold). The number of *B. intermedia* isolates in an ST varied from 1 to 4 (ST23), and the isolates in ST23 were all recovered from chickens on the same farm on the same day. A dendrogram based on the translated amino acid sequences demonstrated a more

stepwise increase in distance between the AATs for *B. intermedia* in the middle of the tree, somewhat resembling the pattern seen with *B. hyodysenteriae* (supplementary Fig.1). In addition, there were other groups of *B. intermedia* AATs that appeared only distantly related to the other AAT groups. The relationships between *B. intermedia* isolates according to their ST and AAT in these two trees did not always appear the same. For example, the two isolates in ST1 and ST2 were at the periphery of the ST tree (Fig.1), but the corresponding AATs, aT47 and aT48, fell more centrally in the AAT dendrogram (supplementary Fig. 1). The number of *B. intermedia* isolates in an AAT varied from 1 to 4 (AAT13).

[Fig 1 about here]

Eight clonal complexes (Cc) of *B. intermedia* STs were identified by e-Burst analysis, and these are highlighted in Table 1 and marked and named in Figure 1. The Ccs contained between 2 and 5 STs, and between 2 and 9 isolates. Cc36 contained one isolate from a UK chicken and an isolate from a Swedish pig, while the other Ccs contained either isolates from Swedish pigs (Cc3), Australian pigs (Cc26), or Australian chickens. Five of the Ccs contained multiple isolates from the same farms that were collected at the same time.

For each of the eight farms where multiple *B. intermedia* isolates from the same sampling time were available, many of the isolates belonged to different STs (Table 1). Farm A had 9 isolates in STs 21, 22, 23, 54 and 56; farm B had 3 isolates in STs 52 and 53; farm C had 2 isolates in STs 39 and 46; farm D had 12 isolates in STs 29, 30, 33, 40, 41, 42, 43, 44, 45, 63 and 64; farm E had 2 isolates in STs 31 and 32; farm F had 3 isolates in STs 26, 27 and 28; farm G had 2 isolates in STs 25 and 50; farm NZA had two isolates in STs 59 and 60.

In a population snapshot obtained by using AATs rather than STs, 21 (33%) of the AATs containing 30 (38%) of the *B. intermedia* isolates were located in one major clonal complex, consisting of two sub-clusters (supplementary Fig. 2). The larger sub-cluster had AAT13 as the founder member, and the other sub-cluster had AAT22 at its centre. Twenty-eight of these isolates were from chickens, of which 26 were from Australia, with one isolate each from the UK and Sweden. There was one other complex with 3 AATs, and four complexes with 2 AATs. The other AATs were all separate. The relationship of the AATs of *B. intermedia* to those for *B. hyodysenteriae* and "*B. suanatina*" are also demonstrated in the supplementary figure. Overall, the *B. intermedia* AATs were far more heterogenous and diverse than those of the other two species.

3.2. Diversity

The diversity index (DI) for *B. intermedia* using MLST at 7 loci was 0.984, and this was reduced to 0.983 when *adh* and *alp* were removed from the analysis, so that just 5 loci were examined.

3.3. Comparisons between indole-positive species using concatenated trees

A dendrogram of concatenated ST data for the three indole-positive species is shown as Fig. 2, and for concatenated AATs as supplementary Fig. 3. The three species were clearly delineated in both trees, but the *B. intermedia* isolates showed much more diversity and heterogeneity than the other two species. Within the ST tree the *B. intermedia* isolates could be separated into 16 groups at a distance greater than that delineating the whole of *B. hyodysenteriae* (*B. intermedia* groups marked a through p on Fig. 2). Only groups "b" and "m" contained relatively large numbers of STs (12 and

27 respectively). Porcine isolates were found in all groups except for the small groups e-i and o, and they exclusively made up the small clusters e, k, l and p (STs containing porcine isolates of *B. intermedia* are marked in bold on the tree). In the AAT tree, five main branches of *B. intermedia* were apparent that diverged from each other at a distance greater than that separating *B. hyodysenteriae* and "*B. suanatina*". The first two branches were exclusively made up of porcine isolates. Only the two bottom branches contained large numbers of AATs, and the lower branch that contained the majority of the AATs itself was separated into a number of groups, including some that predominantly contained porcine isolates, and some avian isolates.

[Fig. 2 about here]

The relative relationships and clustering of individual *B. intermedia* STs and AATs that were observed in the concatenated trees were not always equivalent to those seen in the consensus trees. For example, considering the 37 STs from T12 through T44 in the consensus tree (Fig. 1), in the concatenated tree these made up all of the STs in groups j and m, and there were also one or two of the STs in each of groups a, b, g, and n (Fig. 2).

The concatenated ST data for the three species are also presented as an unrooted radiation tree (Fig. 3), which more clearly emphasises the distances and differences between them, and the tendency for there to be clustering of some of the pig isolates of *B. intermedia*. In this tree, the *B. hyodysenteriae* isolates formed a tight species group, with only UK isolate KF9 in ST H10 being more distantly related to the majority of isolates. The four "*B. suanatina*" isolates were similarly closely grouped, and were distinct from *B. hyodysenteriae*. In comparison, there was a considerable distance from these to the isolates identified as *B. intermedia*, which themselves showed great variation. The tree also helps to illustrate the tendency for some of the porcine isolates

to cluster within the groups (STs containing porcine isolates are marked with a filled circle).

[Fig. 3 about here]

4. Discussion

This study had two main aims, the first of which was to use MLST to analyse the population structure, diversity and molecular epidemiology of *B. intermedia*. Based on the I_A values that were calculated from the data, the *B. intermedia* population appeared to be clonal. In part, this finding may be biased by the non-random selection of isolates, as many of the chicken isolates were derived from a small number of Australian farms. Furthermore, it is evident that the isolates identified as *B. intermedia* were extremely diverse, with many forming small groups of isolates that would give an overall impression of clonality. Hence, to help confirm that the species has a clonal population structure, it would be useful to analyse additional *B. intermedia* isolates from other sources.

Consensus trees based on the allelic profiles at seven loci were constructed to examine diversity within *B. intermedia*, and to investigate aspects of its molecular epidemiology. Examination of the ST tree (Fig. 1) demonstrates the great diversity of the species. Used in this way, the technique was highly discriminatory, and this discriminatory power was not significantly diminished when only five loci were examined. The method proved useful for strain typing and studying the molecular epidemiology at the farm level. As can be seen in Table 1, used with reference to Figure 1, in some cases multiple isolates from a given farm were shown to belong to quite different STs (eg on farms A and D). Such diversity of isolates on a farm potentially could complicate any attempts to control infection. In other cases, some isolates from the same farm were different, but closely related within the same clonal complexes (eg

on farm B). As has been pointed out for *B. hyodysenteriae* (La et al., 2009), the existence of such closely related isolates provides evidence for the likely rapid "microevolution" of strains on the farm. These minor changes presumably are mainly caused by mutations, although there also is potential for genetic exchange between *Brachyspira* isolates and species via the *Brachyspira* prophage-like gene transfer agents (Motro et al., 2009).

To achieve the second aim of the study, to evaluate relationships between the three indole-positive species, used trees based on concatenated sequences, as recommended by Gadagkar et al (2005). On these trees (Figs 2 and 3), a large number of individual groups of *B. intermedia* isolates were identified (groups a-p). Based on the large genetic distances between these groups, and in comparison with the tight clustering of the *B. hyodysenteriae* isolates, it could be argued that some of these actually represented distinct species that have not previously been identified. As previously stated, in order to define these various groups better, and to sample the full diversity that exists amongst isolates with this phenotype, it would be useful to examine additional *B. intermedia* isolates. DNA-DNA reassociation assays between representatives of the different groups also could be undertaken in the future to help determine whether or not the groups do represent different species.

The potential existence of multiple species of weakly haemolytic indole-positive *Brachyspira* has important implications. For example, by analogy to the other *Brachyspira* species, it seems quite probable that isolates belonging to different "species" or clusters of these spirochaetes may differ in their biological properties. As an instance of this, whilst pig and chicken isolates were found throughout many of the groups that were identified, implying the potential for cross-species transmission, some groupings were exclusively or predominantly made up of isolates from either pigs or

chickens. Such differences amongst *B. intermedia* isolates related to their species of origin were identified in an earlier study, where a PCR that was based on the NADH oxidase gene (*nox*) amplified DNA from 10 of 10 porcine *B. intermedia* isolates, but only from four of 10 chicken isolates (Atyeo et al., 1999). Indeed, these results then encouraged the development of a *nox*-based PCR that identified all weakly haemolytic indole-positive isolates as *B. intermedia* (Phillips et al., 2006). These differences may also explain why it has been difficult to reproduce disease in pigs using *B. intermedia* strains; for example, strains from different groups may have different pathogenic potential, and perhaps only representatives of non-pathogenic groups were used in the small number of experimental infection studies that have been reported. On the other hand, the published studies of experimental infections of chickens with *B. intermedia* that have resulted in disease have only involved strains 1380 (Dwars et al., 1992a, 1992b, 1993) and HB60 (Hampson and McLaren, 1999), and both these strains are located in ST cluster "m" in the concatenated ST trees (Figs. 2 and 3). Had isolates from other clusters been used, it is possible that disease may not have occurred. Indeed, this suggestion may explain why "*B. intermedia"* has been identified in laying hen flocks that do and those that do not show clinical signs (Myers et al., 2009). Although host factors also may clearly influence whether or not disease occurs in an individual animal, it will be important to try to assess the pathogenic potential of isolates from the different groups. This could be done by collecting additional field data that relate specific groups of *B. intermedia* with disease or absence of disease, and/or by using specific strains from the various groups to experimentally infect pigs and chickens under standard conditions, such as those that are used to induce experimental swine dysentery. It also will be important to try to develop simple diagnostic tests that can be used to differentiate the different groups.

Based on the close 16S rDNA sequence similarities of the *Brachyspira* species, it seems that speciation in the *Brachyspira* lineage has occurred comparatively recently and rapidly. Consistent with this, the radiation tree (Fig. 3) gives the impression of a burst of evolution of numerous distinct variants and groups of related isolates. Pathogenic *B. hyodysenteriae* represents one such specialised group, and "*B. suanatina*" another. In the case of *B. hyodysenteriae*, it has been deduced that the fully sequenced strain WA1 has acquired genes from other enteric species, and presumably this transfer has increased the fitness of the species for the environment of the porcine colon (Bellgard et al., 2009). It remains to be determined whether acquisition of similar genes by other *Brachyspira* species has occurred, and whether this gene flow is necessary to help stabilise closely related clonal groupings into coherent species.

In conclusion, this study has provided novel insights into the diversity and the broader genetic relationships amongst the indole-positive *Brachyspira* groups. The weakly haemolytic isolates currently identified as *B. intermedia* are extremely diverse, and clearly form several groups, some of which are at least as genetically distinct from each other as are *B. hyodysenteriae* and "*B. suanatina*". Future studies are required to determine how well these groups are adapted to their hosts, their genetic stability, pathogenic potential, and whether some of them would be better regarded as being representatives of distinct species.

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Figure captions

Fig. 1. Dendrogram based on consensus sequences constructed from combined individual distance matrices of nucleotide sequences from the seven genes *adh*, *alp*, *est*, *gdh*, *glpK*, *pgm* and *thi*. The dendrogram shows 77 *B. intermedia* isolates divided into 71 sequence types (STs), arranged in the same order as they appear in Table 1. The names of the STs containing isolates from pigs are outlined in bold, while the other STs contain isolates from chickens. The branches of the STs that form clonal complexes are marked in bold, and the clonal complex is named. The length of the scale bar represents one nucleotide substitution in 10 base pairs of the sequenced gene fragment.

Fig 2. Dendrogram based on concatenated nucleotide sequences at seven loci showing the relationships of 71 sequence types (STs) of *B. intermedia*, as well as 67 STs of *B. hyodysenteriae* and four STs of "*B. suanatina*". The *B. intermedia* STs have been divided into 16 groups (marked a though p) at a distance approximately equivalent to that separating *B. hyodysenteriae* and "*B. suanatina*". The names of the *B. intermedia* STs containing isolates from pigs are outlined in bold, while the non-bolded STs are made up of isolates from chickens. The divisions on the scale bar represent one nucleotide substitution in 100 base pairs of the sequenced gene fragment.

Fig. 3. Unrooted radiation tree based on concatenated nucleotide sequences showing the relationships of the 71 STs of *B. intermedia*, and the 67 STs of *B. hyodysenteriae* and the 4 STs of "*B. suanatina*". The groups of *B. intermedia* STs marked a through p correspond to the groups marked in Fig. 2. The location of STs containing porcine isolates of *B. intermedia* are marked with filled circles. The length of the scale bar represents one nucleotide substitution in 100 base pairs of the sequenced gene fragment.

Table 1. *Brachyspira intermedia* isolate names, origin, sequence type (ST) and amino acid type (AAT) in multilocus sequence typing. The isolates are listed numerically according to their STs, corresponding to the order that they appear in Figure 1.

Isolate[a]	Origin[b]	ST	AAT
AN519/97*	Finland, pig, 1997	1	47
AN517/97*	Finland, pig, 1997	2	48
AN885/94*	Sweden, pig, 1994	3	49
AN621/97*	Sweden, pig, 1997	4	49
Q94/0354/0/3	Australia, chicken, 1994	5	57
Q94/0348/2/4	Australia, chicken, 1994	6	33
OR2	Australia, pig, 1990s	7	55
OF11	Australia, pig, 1990s	8	56
Q98/0037/20	Australia, chicken, 1998	9	60
UNL/4	USA, pig, 1980s	10	41
PWS/A^T*	UK, pig, 1970s	11	43
FF3	Australia, chicken, 1990s	12	8
AN520/93	Sweden, pig, 1993	13	42
HB60	Australia, chicken, 1994	14	29
A3	Australia, chicken, 1994	15	38
E2	Australia, chicken, 1994	16	16
A7	Australia, chicken, 1994	17	16
Q98/0446/2	Australia, chicken, 1998	18	9
MU08/01	Australia, chicken, 2008	19	14
GLD3	Australia, chicken, 2008	20	14
Q08/B85	Australia, chicken, 2008, farm A	21	11
Q08/B93	Australia, chicken, 2008, farm A	21	11
Q08/B82	Australia, chicken, 2008, farm A	22	12
Q08/B124	Australia, chicken, 2008, farm A	23	13
Q08/B139	Australia, chicken, 2008, farm A	23	13
Q08/B46	Australia, chicken, 2008, farm A	23	13
Q08/B98	Australia, chicken, 2008, farm A	23	13
Q97/000/6/22	Australia, chicken, 1997	24	28
B52/iii	Australia, chicken, 1994, farm G	25	15
Whites/3	Australia, pig, 1990s, farm F	26	39
Whites/2	Australia, pig, 1990s, farm F	27	40
Whites/4	Australia, pig, 1990s, farm F	28	37
WACM04/24	Australia, chicken, 2004, farm D	29	22
WACM04/22	Australia, chicken, 2004, farm D	30	22
Q97/2224/3/2	Australia, chicken, 1997, farm E	31	22
Q97/2224/3/1	Australia, chicken, 1997, farm E	32	24
WACM04/21	Australia, chicken, 2004, farm D	33	21
1380	the Netherlands, chicken, 1980s	34	23
Q98/0029/26	Australia, chicken, 1998	35	17
B230	UK, chicken, 1980s	36	32

AN983/90*	Sweden, pig, 1990	37	30
90/21643/0	Australia, pig, 1990	38	31
Q97/2110/4/5	Australia, chicken, 1997, farm C	39	10
WACM04/6	Australia, chicken, 2004, farm D	40	20
WACM04/2	Australia, chicken, 2004, farm D	41	34
WACM04/4	Australia, chicken, 2004, farm D	41	34
WACM04/18	Australia, chicken, 2004, farm D	42	11
WACM04/12	Australia, chicken, 2004, farm D	43	18
WACM04/8	Australia, chicken, 2004, farm D	44	19
WACM04/10	Australia, chicken, 2004, farm D	45	46
Q97/2110/4/3	Australia, chicken, 1997, farm C	46	25
Histo/6	Australia, chicken, 1994	47	26
2A/10	Australia, chicken, 1994	48	27
P1	Australia, pig, 1990s	49	36
B22/ii	Australia, chicken, 1994, farm G	50	3
3B/1	Australia, chicken, 1994	51	61
22/6	Australia, chicken, 1994, farm B	52	5
22/8	Australia, chicken, 1994, farm B	52	5
22/5	Australia, chicken, 1994, farm B	53	5
Q08/B136	Australia, chicken, 2008, farm A	54	6
MUP69	Australia, pig, 2008	55	7
Q08/B130	Australia, chicken, 2008, farm A	56	4
N96/2968/3A	Australia, pig, 1996	57	1
B52/1	Australia, chicken, 1994, farm G	58	2
NZ96/0485	New Zealand, pig, 1996, farm NZA	59	53
NZ96/0479	New Zealand, pig, 1996, farm NZA	60	54
WestB	Australia, pig, 1980s	61	51
2818/5	Australia, pig, 1990	62	52
WACM04/26	Australia, chicken, 2004, farm D	63	58
WACM04/25	Australia, chicken, 2004, farm D	64	59
Wand403	Australia, pig, 1997	65	64
P280/1	UK, pig, 1980s	66	63
889	Australia, pig, 1980s	67	62
Linc	UK, pig, 1980s	68	44
AN1707/96*	Sweden, pig, 1996	69	50
V992/2F	Australia, pig, 1990s	70	35
V245/3	Australia, pig, 1990s	71	45

[a] Adjacent isolates in the same clonal complex are highlighted with the same background shade, and these correspond to the bolded branches in Figure 1. Unshaded isolates are not included in a clonal complex. The seven isolates previously investigated by Råsbäck et al (2007b) are marked with an asterisk.

[b] The year or approximate year of isolation, and the farm of origin, if known, are shown.