Exploring the molecular mechanisms of antimicrobial resistance in *Brachyspira hyodysenteriae* using whole genome sequencing

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

1. In chapter 3 twenty isolates were purified by Jon Rodgers at APHA Bury St Edmunds (js01, js21, js34, js35, js39, js41, js47, js51, js63, js64, js65, js68, js70, js78, js79, js83, js86, js89)

- The sequencing department at the APHA performed all sequencing conducted at the APHA, this involved one MiSeq metagenomic sequencing run and all NextSeq sequencing
- A Perl script written by Nicholas Dugget was used to automate the identification of SNPs by Snippy
- A python script designed by Richard Brown was used in chapter 3, 4 and 5 to automate processes
- Novel MLST sequence types were assigned by Tom La

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Abstract

Brachyspira hyodysenteriae is the causative agent of swine dysentery, a disease characterised by bloody diarrhoea. It is endemic to the UK, and if untreated it can cause severe economic cost to farmers. Currently, it is treated with antibiotics including the pleuromutilin antibiotics tiamulin and valnemulin. *B. hyodysenteriae* has become more resistant to the antibiotics used to treat infections , and increasing levels of pleuromutilin resistance has been observed in some countries.

In this study, 84 clinical isolates, from 2004 to 2015, were sequenced on an Illumina MiSeq. From this, the population structure of *B. hyodysenteriae* in the UK was constructed. In addition, the phenotypic resistance of 47 sequenced isolates was obtained using a commercial broth dilution assay. The use of sequenced isolates enabled detection of a recently identified pleuromutilin resistance gene and enabled prediction of the resistance phenotype of all sequenced isolates. The use of whole-genome sequencing has increased our knowledge of *B. hyodysenteriae* in the UK, highlighting potential regional differences and has created a reference database of all *B. hyodysenteriae* isolates from 2004 to 2015. This will improve surveillance and increase the power of outbreak analysis.

Direct sequencing from clinical samples could further strengthen outbreak analysis and surveillance. This would improve the speed of identification and could provide useful information. To explore swine dysentery, positive samples were sequenced on an Illumina MiSeq and *B. hyodysenteriae* reads were extracted. From this, it was possible to identify the closest sequenced strains. This study highlights the potential uses of whole genome sequencing to analyse *B. hyodysenteriae*.

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List of Abbreviations

AIAO:	All-in-all-out production
ATP:	Adenosine triphosphate
APHA:	Animal and Plant Health Agency
AMR:	Antimicrobial resistance
BHI:	Brain heart infusion
DDGS:	Distillers dried grains with solubles
DNA:	Deoxyribonucleic acid
ELISA:	Enzyme-linked immunosorbent assays
ETEC:	Enterotoxigenic *Escherichia coli*
EU:	European Union
HKY:	Hasegana, Kishino and Yano
LDA:	Linear discrimination analysis
LOS:	Lipooligosaccharides
MIC	Minimum inhibitory concentration
MIU:	Microbiology and infection unit
MCMC:	Metropolis-Hastings Markov chain Monte Carlo
MLEE:	Multilocus enzyme electrophoresis
MLVA:	Multiple-locus variable-number tandem-repeat analysis
MSLT:	Multilocus sequence typing
na:	Not applicable
NCBI	National Center for Biotechnology Information
NSP:	Non-starch polysaccharide
NUTS:	Nomenclature of territorial units for Statistics
PCA:	Principle component analysis
PCR:	Polymerase chain reaction
PRRS:	Porcine reproductive and respiratory syndrome
RNA:	Ribonucleic acid
rRNA:	Ribosomal RNA
RUMA:	Responsible use of medicines in agriculture
SNP:	Single nucleotide polymorphism
ST:	Sequence type

SVARM:	Swedish veterinary antibiotic resistance monitoring
TCP	Toxic precursor pilus
UK:	United Kingdom
UN:	United Nations
USA:	United States of America
USSR:	Soviet Union
WGS:	Whole-genome sequencing

Chapter 1. Introduction

1.1. The domestic pig and pig farming

1.1.1. Historical overview

Pigs were independently domesticated in Anatolia and the Mekong valley in the sixth millennium BCE and pig farming reached Western Europe by the fourth millennium BCE [1-3]. Traditionally pigs have been raised in a sty in small holdings [4]. This changed during the 19th century with an increased intensity of pig farming [4-8]. In the UK, outdoor farming was followed by the development of indoor pig farming [4]. Indoor farming increased the efficiency of pig fattening and reduced costs, but there were increased rates of diarrhoea [4]. The drive towards increased efficiency was in part driven by international competition: for example, by 1960 approximately 300,000 tonnes of bacon and ham were being imported from Denmark [4]. Intensification of pig production has continued leading an increase in average herd size in the UK from 70 pigs in 1966 to 600 in 2000 [9]. This is part of a global trend especially evident over the last twenty years [5-8].

1.1.2. World production and trade

The United Nations Food and Agriculture Organization estimates that 1.5 billion pigs were slaughtered in 2016 [10]. The largest pig producer is China, followed by the EU and the USA [11]. In the EU alone, around 260 million pigs were slaughtered in 2016 [12]. In addition to the large volumes produced each year, there is a trend towards fewer and larger producers, with the largest five producers responsible for 30% of pigs slaughtered in the UK in 2015 [13]. These trends are seen in other pig-producing regions— by 2014, over 80% of herds in the USA contained over 5000 pigs, while in China the proportion of pig production taking place in backyard farms fell from 74% in 2000 to 27% in 2015 [7, 8].

There are several types of pig production. In farrow-to-finish herds, pigs are produced from breeding to slaughter. In weaner herds, pigs are bred and kept until weaning (at 8 weeks or at 20 kg) and then sold to finisher sites, where pigs are grown

to 70 kg before being slaughtered [14-16]. These production systems are used worldwide and form the majority farms in the EU, the USA and compose an important section of Chinese production [5, 17, 18]. However, there are a number of regional differences; for example, many breeding herds in China are run by the state. In the EU, the degree of intense production varies—with Italy and Romania having far more smaller holdings than countries such as Spain or France [19]. In addition, some countries have specialised in a particular aspect of production—for example, in 2016 Denmark exported 13 million piglets to be fattened elsewhere [5, 17, 20].

In the UK, most pigs are farmed in England, chiefly in Yorkshire and the east of England. The size of companies varies between regions:

- Large companies (defined as having more than 50 farms) predominate in the east of England [21].
- Yorkshire has many more small companies (with fewer than five farms) and medium-sized companies (with between five and 50 farms) [21].
- Other regions, in particular, the South West, have a large number of farms per kilometre but these are mostly small farms [22].

Analysis of the UK pig network indicates that most nodes in the network, for example, pig farms, markets and slaughterhouses, are not directly connected to each other, but have a small number of highly connected nodes; this is consistent with analysis of the Danish, Belgian, French, Spanish and Swedish pig industry [19, 23-26]. These highly connected nodes present key areas of vulnerability through which a pathogen could spread rapidly.

1.1.3. Role in UK agricultural economy

Agriculture contributes £8.2 billion to the gross domestic product of the UK and accounts for 1.35% of the workforce, employing 466,000 people [27]. The UK pig industry encompasses 4.8 million pigs covering over 10,000 hectares of land, and the value of pig meat produced in 2016 was £1 billion [27].

1.1.4. Current challenges in pig farming

Currently, there is a severe price pressure on the pig industry in the UK, and the pork market is volatile, with long periods where meat prices are very low [28, 29]. Between 2012 and 2017 the profit margin varied between a loss per pig of £12 and a profit of £23 [29]. For the whole of 2012, 2015 and the first two quarters of 2016 the cost of pig production was higher than the value of the slaughtered pig [29]. In 2012, this was due to the high price of feed, while in 2015 the value of pig meat fell due to oversupply exacerbated by a Russian ban on EU pig meat [30].

1.1.5. The pig gut microbiota

The gut microbiome is defined as the community of microorganisms that inhabit the gut [31]. In the pig gut, the composition of the microbiome varies in response to a wide range of factors, including age, breed and antibiotic use [32, 33]. Initially, the microbiome is dominated by *Bacteroides* genus, but after weaning this declines and is replaced by *Prevotella* [34, 35]. Over time there is a decrease in *Bacteroidetes* and an increase in *Firmicutes*, in particular, *Anaerobacter*, *Streptococcus* and *Lactobacillus* [35]. In addition, there are genera present at lower levels that contribute to the health of the host. These include butyrate-producing genera such as *Roseburia* and *Megasphaera* [36]. Butyrate is an important energy source for enterocytes and is associated with a healthy gut, while *Megasphaera* has an extensive metabolic potential that includes the production of short chain fatty acids, amino acids and vitamins [37].

1.1.6. Infectious diseases of pigs

Each quarter, diseases that threaten the UK pig industry are summarised in surveillance reports from the Department of Environment, Food and Rural Affairs. These include

- emerging threats, such as African swine fever, a viral infection with a mortality rate of 90% [38]. An outbreak of African swine fever originating in Georgia has spread as far as the Czech Republic [38].
- endemic diseases such as porcine reproductive and respiratory syndrome (PRRS), which is caused by a viral pathogen and has recently devastated herds in China [39]. The endemic population in the UK has a different

genotype from that in China, but still remains a serious problem for the pig industry [40].

- septicaemia caused by *Klebsiella* species, in particular, *Klebsiella pneumoniae* subspecies *pneumoniae*, and swine dysentery [40].

1.2. Swine dysentery

1.2.1. Epidemiology and clinical features

Swine dysentery was first documented in 1921 [41]. The causative agent was identified in 1971 as *Treponema hyodysenteriae*, now classified as *Brachyspira hyodysenteriae* [42-44]. Recently, two novel *Brachyspira* species have been implicated in swine dysentery: *B. suanatina* and *B. hampsonii*; however, the majority of incidents are caused by *B. hyodysenteriae* [45, 46].

B. hyodysenteriae has a global distribution, while the novel species are more local: *B. suanatina* has been isolated in Scandinavia and Germany, while *B. hampsonii* was initially isolated in the USA and Canada, but now appears to now be present in Belgium and Germany as well [45, 47-50].

Although swine dysentery is found globally, the incidence is often unknown. In the USA, it appears to be increasing, with the number of cases in Iowa rising from three in 2005 to 466 in the first nine months of 2010 [51, 52]. Swine dysentery is also a problem in South America, with 35.3% of diarrhoeal cases in Brazil attributable to swine dysentery [53]. Swine dysentery is also present on large pig holdings in Australia and has been detected in commercial pig herds in China [53, 54]. Swine dysentery is also found across Europe, although it is not known if the rate is increasing. In Poland and in Spain, swine dysentery was responsible for 34.8% and 38.3% of diarrhoea cases respectively [53]. In the UK, swine dysentery remains a persistent problem, although there has been a small reduction in reported cases since 2015 [40].

The most significant risk factor for swine dysentery is a previous history of the infection on the premises [55]. Introduction of new finisher pigs, or pigs from sale yards, also contributes to the risk of swine dysentery [55]. Continuous pig

production may be a factor, as the infection cycle is unbroken [56]. Pigs typically become infected after ingestion of infected faeces [53], so inadequate decontamination (cleaning or disinfection) could trigger an outbreak.

The typical incubation period for swine dysentery is 10 to 14 days [53]. Infected pigs have yellow to soft grey faeces and, as diarrhoea develops, faeces become watery and contain mucus and blood. On post-mortem, large lesions are evident in the colon [53]. Subsequent outbreaks may occur over three to four-week periods due to the removal of therapeutic agents. Morbidity of 90% has been reported and, in experimental models, mortality can reach 80%, although in clinical cases a mortality rate of 30% is more typical [53, 57].

1.2.2. Microbiology

Brachyspira are anaerobic Gram-negative bacteria belonging to the phylum Spirochaetes [58], a diverse taxon, most members of which have long spiral-shaped cells. The phylum is also home to *Borriela burgdorferi*, the causative agent of Lyme disease [59]. The genus *Brachyspira* is composed of both pathogenic and commensal species:

- *B. innocens* and *B. murdochii* are considered commensals of pigs, although *B. murdocchi* has infrequently been implicated in disease episodes in pigs [60-63].
- *B. intermedia* may also be commensal in pigs and causes diarrhoea in chickens [62, 64].
- *B. pilosicoli* causes porcine intestinal spirochetosis, a milder diarrhoea than swine dysentery, in pigs [65].

Species of *Brachyspira* that produce swine dysentery can be distinguished by their high haemolytic activity when grown upon blood agar [45, 49, 53]. *B. hyodysenteriae* is also motile and has a loosely coiled appearance when viewed under a microscope [53].

Although *B. hyodysenteriae* chiefly colonises the colon of pigs, it has been found in other animals. Mallard ducks (*Anas platyrhynchos*) are asymptomatic carriers and have been hypothesised to be a vector species [66, 67]. However, *B. hyodysenteriae*

isolates from mallard ducks were not able to successfully colonise pigs in a challenge experiment [66]. In addition, the porcine *B. hyodysenteriae* reference strain B204R was unable to colonise mallard ducks [67]. *B. hyodysenteriae* can potentially colonise other animals typically found on a farm, such as

- rats and mice—the sequence type ST8 has been recovered from a farm mouse [68].
- chickens—although colonisation is rare, this could represent another animal reservoir [69].

However, *B. hyodysenteriae* has not been found in other farmed poultry or ruminant species.

B. hyodysenteriae can survive in soil for 10 days at 10 °C, so the pathogen could perhaps persist in untreated soil before re-infecting a herd. *B. hyodysenteriae* can also survive in pig faeces for 112 days at 10 °C [70]. As production of pigs, especially intensive farming, results in large volumes of faeces being flushed into large open-storage facilities [71], the resulting lagoons could act as a reservoir for *B. hyodysenteriae*. Pig farmers also produce manure to fertilise their crops, which could re-infect pigs [72]. This may explain why isolates of the same sequence type occur on the same farm 19 months apart [73].

1.2.3. Pathogenesis

B. hyodysenteriae produces axial flagella, which appear to be important to virulence, as mutants unable to produce flagella have an attenuated ability to cause infection [74]. The flagella reside within the periplasmic space and are attached to the two poles of the cell, which gives Spirochaetes their characteristic spiral shape [75, 76]. Like all bacterial flagella, these periplasmic flagella are composed of a filament, a hook and a basal-motor complex [77, 78]. However, the motor appears to be larger than in the canonical *Salmonella* system and lacks an L ring [79]. The filament itself is composed of a sheaf, *FlaA*, and at least three core proteins *FlaB1*, *FlaB2* and *FlaB3* [80]. The flagellar sheaf is a dynamic structure, enabling motility in materials of varying viscosity [80]. Flagellar rotation produces helical waves that drive *B. hyodysenteriae* forward in a corkscrew-like motion through linear polymers such as

mucin [81]. Chemotaxis enables migrations to the preferred environment, as well as to potential sources of energy [74, 82, 83].

B. hyodysenteriae preferentially colonises the mucus above the caecal and colonic epithelium [76]. However, it is also able to adhere to the epithelium and invade goblet cells [76, 84]. To obtain nutrients, *B. hyodysenteriae* produces proteolysins and haemolysins that cause damage to the epithelium, resulting in the lesions seen in swine dysentery [85, 86].

The composition of mucus in the colon is altered during *B. hyodysenteriae* infection. The mucus in the colon is largely composed of mucins, in particular, mucin 2, which forms a gel-like matrix near the epithelium [87]. Mucins may represent an important metabolic source of sulfur, and it has been observed that there is a reduction of sulfated mucins during swine dysentery [88]. However, *B. hyodysenteriae* does not possess a sulfatase gene and may rely on other bacteria to degrade the mucin [88]. This could explain why other bacteria are important for colonisation by *B. hyodysenteriae* [89].

After *B. hyodysenteriae* colonisation of the colon, there is an increase in mucin 2 and *de novo* production of mucin 5AC [87, 88]. Mucin 5AC may aid in the clearance of pathogens. However, the increase in mucin 2 provides more binding sites for *B. hyodysenteriae,* resulting in increased binding of the bacterium to mucin [87, 90, 91]. As well as increased production of mucin 2, there is a reduction in mucin 4, a mucin important in renewing the epithelium [88]. Degradation of mucin 4 could exacerbate the damage caused by haemolysins, enabling migration of luminal bacteria into the epithelium and resulting in a severe inflammatory response. The lipooligosaccharide (LOS) of *B. hyodysenteriae* and other bacteria will also contribute to the inflammation [92].

It is likely that *B. hyodysenteriae* is more diverse than previously thought, with some strains pathogenic, while other weakly haemolytic strains are asymptomatic. This could be an evolving phenotype, or it may be that the techniques used to detect *B. hyodysenteriae* are becoming more sophisticated, and therefore *B. hyodysenteriae* is being detected more often. Crucially, there have been recent reports of *B.*

hyodysenteriae in healthy pigs that do not exhibit any signs of swine dysentery [93-96]. Bacterial isolates from these cases often carry single nucleotide polymorphisms (SNPs) in haemolysin genes, chiefly *tylA*, haemolysin III and *hylA*. It has been suggested that the associated amino-acid substitutions have inactivated the haemolysins, although this has not been proven [95]. SNPs have also been detected in the promoter region of some weakly haemolytic isolates [95].

The emergence of weakly haemolytic *B. hyodysenteriae* presents a problem for farmers, as it is not easy to distinguish between pathogenic and commensal forms of *B. hyodysenteriae*. If action is delayed until one can establish the virulence of the strain, an outbreak could have already occurred. Also, there could be implications for a farmer's reputation if it is established that a farmer sold pigs colonised with *B. hyodysenteriae* to customers. In a recent case, some farmers decided to stop selling live pigs when *B. hyodysenteriae* was found in their herd [95].

1.2.4. Immune response

Infection by *B. hyodysenteriae* results in the activation of T helper cell types 1 and 2 [97]. Increased levels of IL-17A mRNA have been detected in the colonic epithelium [98, 99]. This cytokine results in the production of mucin 5AC in airways and is likely to do so in the colon [100]. There is an increase in IL-1B, TNF-α and IL-6. Secretion of cytokines such as IL-1B results in the recruitment of neutrophils to the site of infection [101] leading to phagocytosis of *B. hyodysenteriae* and production of neutrophil elastase [99]. Neutrophil elastase can result in degradation of bacterial virulence factors, direct killing of pathogens and modulation of the inflammatory response [102]. Colonisation with *B. hyodysenteriae* triggers an IgG response to the commensal microbiota, perhaps due to enhanced permeability of the colonic epithelium [97].

1.2.5. Diagnosis

Definitive diagnosis of swine dysentery requires culturing of *B. hyodysenteriae* from clinical samples through serial passage on blood agar under anaerobic conditions [53]. Adding antibiotics to the agar enables selection, typically 400 μg/ml of

spectinomycin, 25 μg/ml of vancomycin and 25 μg/ml of colistin [53, 103]. Pure cultures display a zone of strong haemolysis with a film of growth [53]. Biochemical tests are typically used in diagnosis to speciate isolates of *Brachyspira*. Culture and biochemical tests (Table 1.2.5.1) has enabled accurate identification of *Brachyspira* species and detection of novel *Brachyspira* species [45, 66]. However, purification of *Brachyspira* species is time-consuming and biochemical tests may miss unusual isolates, for example, indole-negative *B. hyodysenteriae* isolates [104].

Enzyme-linked immunosorbent assays (ELISAs) have been proposed, but none are used routinely [53, 94], as some react only with a subset of serotypes, while others may not even be specific to a *Brachyspira* species [105, 106].

Table 1.2.5.1: Biochemical tests used to speciate *Brachyspira.*

Abbreviations:

S: Strong, W: Weak, Ind: Indole production, Hip: Hippurate production, ∝-gal: ∝-galactosidase activity, ∝-gluc: ∝-glucosidase activity, β-gluc: β-glucosidase activity, nk: not known. Based on a table by Fellstrom *et al*, 1995 [107].

Species	Haemolysis	Ind	Hip	∝-gal	∝-gluc	β -gluc	Ref
B. hyodysenteriae	S	+	-	-	+/-	+	[107, 108]
B. intermedia	W	+	-	-	+	+	[107, 108]
B. murdochii	W	-	-	-	-	+	[107, 108]
B. innocens	W	-	-	+	+/-	+	[107, 108]
B. pilosicoli	W	-	+	+/-	-	-	[107, 108]
B. hampsonii	S	-	-	-	-	+/-	[45]
B. suanatina	S	+	-	-	nk	nk	[66]

Table 1.2.5.2: Suggested MIC breakpoints for *B. hyodysenteriae.*

Note there are two common MICs for tiamulin * denotes the breakpoint for VetMic Brachy plates and the other MIC is for agar dilution.

Antibiotic	Wild type MIC (µg/ml)	Clinical resistance MIC (µg/ml)
Tiamulin	<0.25* [109] <1 [110]	>2* [109] >4 [110]
Valnemulin	>0.125 [109]	na
Doxycycline	<0.5 [109]	>4 [111]
Tylvalosin	<1 [109]	>16 [110]
Lincomycin	< 1 [109]	>16 [110]
Tylosin	< 16 [109]	>16 [110]

A real-time PCR can distinguish between *B. hyodysenteriae*, *Lawsonia intracellularis* and *B. pilosicoli*, while a duplex PCR can distinguish between *B. hyodysenteriae* and *B. pilosicoli* [112, 113]. Commonly, *B. hyodysenteriae* is identified by sequence analysis of the NADH gene, while other species are identified by sequencing of the 16S gene [112, 113]. Although PCR offers a rapid method of identification, performance with faecal samples can be problematic, and there is no gold standard diagnostic PCR for identification of *B. hyodysenteriae* [112, 113].

For *B. hyodysenteriae*, minimum inhibitory concentration (MIC) testing is done by agar dilution or via a commercial broth dilution test, the VetMIC Brachy (SVE, Sweden) [114, 115], which works for six antibiotics: tiamulin, valnemulin, doxycycline, tylosin, lincomycin and tylvalosin [114]. There are no universally acknowledged clinical resistance breakpoints for *B. hyodysenteriae,* but a number have been suggested (Table 1.2.5.2). In addition, wild-type cut off values have also been suggested for *B. hyodysenteriae*; this enables differentiation between sensitive isolates and isolates that are developing tolerance towards antibiotics (Table 1.2.5.2) [109]. Isolates below the wild-type breakpoint are classed as susceptible, isolates above wild-type and below the clinical resistance breakpoint are classed as intermediate, and all isolates with MICs above the clinical resistance breakpoint are classed as clinical resistant [109, 110].

MICs provide important epidemiological information and may influence the treatment regime. However, MIC testing is slow, and MICs can vary quite considerably; in a ring trial for the VetMIC Brachy plates, the manufacturer's recommended quality control strain (B78) varied between three concentrations [116]. For agar dilution methods in-house methods make comparison between labs difficult, and if MICs are one dilution higher with agar dilution, this could affect the treatment given [115].

Although multiple-locus variable-number-tandem-repeat analysis (MLVA) and multilocus enzyme electrophoresis (MLEE) have been used to type isolates of *B. hyodysenteriae* [117, 118], MLST on seven housekeeping genes is the most widely used epidemiological typing technique[119], which has shed light on the distribution of *B. hyodysenteriae* strains [120]. MLST has been used to investigate the population

structure of *B. hyodysenteriae* in Australia, Belgium, Germany, Italy, Spain and the USA [52, 60, 73, 93-95, 121-125].

Most *B. hyodysenteriae* STs show a local distribution, restricted to a single country. However, there are a few STs that have been found in multiple countries, notably ST 8 and 52, which are present in several European countries. No STs have a global distribution. Although an ST 52 isolate has been identified in Japan, *B. hyodysenteriae* is only infrequently transferred between continents [73]. Weakly haemolytic *B. hyodysenteriae* isolates belong to different STs to strongly haemolytic isolates [93, 94].

1.2.6. Infection control

Carrier pigs represent the most likely source of infection and persistence of *B. hyodysenteriae* on a farm. *B. hyodysenteriae* is typically transported into finisher farms by asymptomatic weaner pigs [118]. However, in farrow-to-finisher farms, sows who have survived the disease act as reservoirs [126]. Visitors or trucks from *B. hyodysenteriae*-positive farms can introduce it into a new herd [120, 127], as *B. hyodysenteriae* can be cultured from trucks even after decontamination [128]. It is also possible that *B. hyodysenteriae* could be introduced to farms by unidentified animal vectors.

A number of safeguards to prevent *B. hyodysenteriae* infections have been suggested. In all-in-all-out production (AIAO) systems, batches of pigs are grown in a succession of pens, with pigs of the same age being kept together [15, 129]. In between batches of pigs, occupied pens are left empty for a period of time before cleaning and disinfection [129]. Additionally, in multi-site farms, pigs at different stages of the production cycle are kept on separate sites [5]. This helps to break the infection cycle.

After swine dysentery has been verified, the herd will be treated with antibiotics, reduced in size, and in some cases, completely depopulated [53]. The site will be thoroughly decontaminated and outside areas used for pig production not be used for

several months. After successful decontamination, new pigs will be sourced from swine dysentery-free stock [53].

Unfortunately, efforts to develop a vaccine to prevent swine dysentery in pigs have not been successful [60, 130].

1.2.7. Treatment

In the EU, tylvalosin and the pleuromutilin antibiotics, tiamulin and valnemulin, are used for treatment of swine dysentery as well as for prophylaxis and metaphylaxis [131-133]. Most commonly tiamulin and valnemulin are used, and the treatment lengths are shown in table 1.2.7.

Table 1.2.7: Dosage and treatment length of tiamulin and valnemulin.
Modified from a table by Zimmermann *et al,* 2012 [53]. Valnemulin is not administered inter-muscularly or in drinking water in the EU [134].

	Inter-muscular	Drinking water	In-feed
Tiamulin	10 mg/kg body weight 1 to 3 days	8 mg/kg body weight 5 to 7 days	100 ppm for 7 to 10 days followed by 30 to 40 ppm for 2 to 4 weeks
Valnemulin	na	na	10 mg/kg body weight 1 to 4 weeks

1.3. The Challenge of antimicrobial resistance

Overuse of antibiotics has led to the development of antimicrobial resistance, which has meant that important antibiotics are becoming less effective. To compound the problem, there are few new antibiotics available to replace them, given that no significant new classes of antibiotics have hit the market since the late 1980s, [135]. Antimicrobial resistance (AMR) has been reviewed extensively, and mechanisms of AMR are summarised in table 1.3. [136, 137]. Briefly, resistance can be

- *intrinsic*, resulting from existing genes in the chromosome; for example, *Pseudomonas aeruginosa* contains an efflux pump, MexAB-OprM, that makes its outer membrane only weakly permeable to β-lactams [138].
- *acquired*, through SNPs in genes associated with antimicrobial resistance or via AMR genes obtained by horizontal gene transfer.

AMR genes can be found on plasmids; for example, the pNDM-CIT plasmid carries the metallo β-lactamase gene NDM-1 [139]. In addition, this plasmid carries a gene encoding a multi-drug efflux pump [139]. AMR genes can be transferred between cells by transposons and temperate bacteriophages: for example, in *Streptococcus agalactiae*, the lincomycin resistance gene *lnuC* is transferred between strains by the transposon MTnSag1 [140], while bacteriophages have been implicated in the transfer of bla-CTXM genes to sensitive strains of *Escherichia coli* [141].

Table 1.3: Mechanisms of antibiotic resistance

LPS refers to Lipopolysaccharide.

Mechanisms	Example	Reference
Alteration of target pathway	Substitution of the vancomycin target acetyl-D-alanyl-D-alanine with acetyl-D-alanyl-D-alanine by Van genes	[142]
Degradation of antibiotic	NDM-1 is a plasmid-born β-lactamase that degrades cephalosporins	[143]
Efflux pumps pump antibiotics out of the cell	The RND/MDR efflux pump on the pNDM-CIT plasmid enables efflux of multiple antibiotics from the cell.	[139]
Alteration of the targets site	SNP in the 23S rRNA at position 2058 (*Escherichia coli* numbering) results in resistance to lincomycin and tylosin in *B. hyodysenteriae*.	[144]
Reduced Uptake	Modification of Lipid A of LPS results in an alteration of the charge across the cell membrane reducing the ability of polymyxcin to bind.	[145]

1.3.1. Antimicrobial use and resistance in human medicine

The WHO has released a list of critically important bacteria that should be priority targets for the development of new drugs, while the Public Health Agency Canada and the Centers for Disease Control and Prevention have published lists of AMR pathogens that are a severe threat to human health [146-148]. It is likely that the number of multi-drug resistant pathogens important to human health will increase. Indeed, it has been estimated that by 2050, the number of deaths attributable to AMR infections will be higher than those attributable to cancer [149]. As the supply of new antibiotics is limited, it is essential that currently available antibiotics are well stewarded and used only when needed. This focus on stewardship has also become a key aspect of government policy towards antibiotic resistance [150, 151].

1.3.2. Antimicrobial use and resistance in veterinary and agricultural practice

Antibiotics have been used in agriculture for most of the last century. Over this time, they have been used to treat bacterial infections, in prophylaxis or in metaphylaxis. Antibiotics have been used in subclinical concentrations as growth enhancers since the 1940's [152]. Antibiotic usage in agriculture is changing rapidly due to new legislation; such as the 2006 EU ban on antibiotics as growth enhancers and new restrictions on antibiotic use in agriculture in the UK [153]. There are also changes in industrial practices, such as the establishment of the Targets Task Force to aid in reducing antibiotic use in agriculture by the Responsible Use of Medicines in Agriculture (RUMA) [154, 155].

The scale of antibiotics usage in animal health globally is unknown, but it has been estimated that the volume of antibiotics used is greater in animal medicine than human medicine [156]. In the USA, it has been estimated that 70% more antibiotics are sold for animal use than for human medicine [156]. In the UK in 2013, 532 tonnes of antibiotics were prescribed for human use, and 419 tonnes were sold for animal use [157]. RUMA have suggested that the pig industry is associated with the highest usage of antibiotics, accounting for 192 tonnes of antibiotics in 2015 [155].

1.3.3. Antimicrobial resistance in *B. hyodysenteriae*

Resistance to pleuromutilins is most likely to occur by chromosomal SNPs followed by clonal expansion of the resistant strain and dissemination by carrier pigs and other vectors. Pleuromutilin resistance is thought to develop in a stepwise manner, via a series of SNPs in AMR genes [158]. SNPs potentially involved in resistance to pleuromutilins have so far only been found in domain V of the 23S ribosomal subunit and the L3 50S ribosomal subunit protein [159, 160]. Domain V of the 23S ribosomal subunit is the target site of pleuromutilins; a conformational change here could prevent binding of the antibiotic. The L3 50S ribosomal subunit protein restricts the conformational freedom of domain V of the 23S ribosomal subunit [161]. Mutations in the L3 protein result in resistance to tiamulin, but SNPs in the 23S rRNA are needed for reduced susceptibility to valnemulin [161, 162].

Pringle *et al* selected for resistance in sensitive isolates in 2004 by repeated subculture in sublethal concentration of tiamulin; other studies have identified SNPs in clinical isolates of *B. hyodysenteriae* from Germany, Spain, Italy, Belgium and Taiwan (table 1.3.3) [73, 159, 160, 163-166]. Some candidate SNPs may be phenotypically neutral or associated with resistance to other antibiotics, e.g. lincomycin, and therefore might have no impact on pleuromutilin sensitivity [144]. However, some SNPs appear clearly to be responsible for pleuromutilin resistance. For example, a SNP at position 2032 in the 23S rRNA (*E. coli* numbering) has been consistently found only in resistant isolates [159, 164, 167]. There are instances of phenotypic resistance not associated with known AMR SNPs in the 23S rRNA and the L3 protein; it is possible this could be due to SNPs in as yet unidentified genes in *B. hyodysenteriae* [159].

Horizontal gene transfer is an important mechanism of disseminating antimicrobial resistance within bacteria [168]. However, there are few known mechanisms of horizontal gene transfer in *B. hyodysenteriae* and no evidence that the *B. hyodysenteriae* plasmid is conjugative. The best-studied example in this species is the gene transfer agent VSH1 [169, 170]. Gene transfer agents are non-lytic phages that have lost the ability to self-replicate but remain able to package and transport

DNA for their host bacteria [171]. VHS1 has been shown experimentally to transfer coumermycin A(1) resistance, but this has not been implicated in the development of AMR in clinical isolates [172].

Recently the transposon MTnSag1 has been identified in an Italian *B. hyodysenteriae* isolate that contains the lincomycin resistance gene *lnu(C)* [165]. This transposon has previously been identified in a *B. pilosicoli* isolate in Australia and was first identified in the *S. agalactiae* isolate UCN36 [140, 165]. This is an IS-1-like transposon that lacks transfer genes and requires the presence of a co-resident conjugative element for mobilisation. In *S. agalactiae* this function is performed by Tn916 transposon [173]. This transposase is not present in *B. hyodysenteriae*, and currently, there is no known mechanism of mobilisation in *B. hyodysenteriae*.

Table 1.3.3: Potential pleuromutilin-resistance SNPs

SNPs in the 23S rRNA are *E. coli* (Genbank accession J01695) numbering and SNPs in the L3 protein are in *B. pilosicoli* numbering (Genbank accession AF114845). Many SNPs have been identified in pleuromutilin-resistant and pleuromutilin-sensitive isolates.

Gene	Present in Resistant Isolate	Present in Sensitive Isolate
A2031T (23S rRNA)	✓[160], ✓[166]	
G2032A (23S rRNA)	✓[159] , ✓[160], ✓[164], ✓[166]	
C2055A (23S rRNA)	✓[159]	
G2057A (23S rRNA)	✓[160], ✓[166]	
A2058T (23S rRNA)	✓[160] , ✓[164], ✓[166]	✓[160] , ✓[164], ✓[166]
A2058G (23S rRNA)	✓[164]	
A2059G (23S rRNA)	✓[160]	
G2087T (23S rRNA)	✓[160]	✓[160]
G2116A (23s rRNA)	✓[164]	
C2146T (23S rRNA)	✓[160] , ✓[165]	✓[160]
G2201C (23S rRNA)		✓[160], ✓[164]
G2201T (23S rRNA)	✓[164]	
C2362T (23S rRNA)	✓[160]	✓[160]
G2365C (23S rRNA)	✓[160]	✓[160]
G2447T (23S rRNA)	✓[159]	
C2449A (23S rRNA)	✓[159]	
T2402C (23S rRNA)	✓[164]	
T2504G (23S rRNA)	✓[159]	
T2528C (23S rRNA)	✓[164]	✓[164]
G2535A (23S rRNA)	✓[160] , ✓[164]	✓[160] , ✓[163] , ✓[164]
A2572T (23S rRNA)	✓[159]	
C2611T (23S rRNA)	✓[160]	
N148S (L3 protein)	✓[159] , ✓[160] , ✓[163], ✓[73], ✓[166]	✓[160]
S149I (L3 protein)	✓[159]	
S149T (L3 protein)	✓[159], ✓[160], ✓[166]	

1.4. New opportunities: whole-genome sequencing

1.4.1. Sequencing technologies

The British scientist Fred Sanger invented the first widely used sequencing approach, which exploits chain termination with a known labelled nucleotide base followed by gel electrophoresis [174]. Subsequent refinements such as capillary gel electrophoresis, shotgun sequencing and use of computers greatly increased the utility of sequencing allowing much higher throughput [175, 176]. This led to the first whole genome sequencing of a bacterium, *Haemophilus influenzae* in 1995, and the first human genome sequence in 2003 [177, 178].

Early genome projects were time-consuming and expensive; for example, it cost $878,450 to sequence the *H. influenzae* genome [177], far beyond what the average lab can afford. In 2004, the National Institutes of Health in the USA launched a $70 million funding program to develop technologies to sequence human genome for $1000, and other funding bodies followed suit [179]. The resulting second-generation sequencing machines use a variety of techniques. For example, Illumina has developed a sequence by synthesis method whereby each of the four nucleotides has a different fluorophore attached at the three prime end. After each nucleotide is added fluorescence is detected, and the fluorophore is removed. This enables identification of the incorporated base. To increase the speed of sequencing the genome is cut into multiple small sections, with a maximum size of 700 base pairs, and sequenced in parallel [180, 181]. The result of these advancements is the rapid sequencing of a whole genome. Many of the more recent sequencing machines, including the Illumina MiSeq, are small enough to fit on a laboratory bench, making them easy to incorporate into existing laboratories. Recently, there have been new developments in the WGS field with the development of two quick long read sequencers: the PacBio and the Nanopore [181, 182]. Long read sequencers can sequence much longer read lengths than short read sequencers. For example, the PacBio can produce sequences of up to 10,000 bases long compared to 700 for the MiSeq increasing the simplicity of re-assembling the genome after sequencing and downstream analysis [181].These are the first third-generation sequencing machines produced, and it is likely that more advanced sequencing machines will be produced

in the future. However, the majority of sequencing projects are currently still done using second generation sequencing platforms such as the MiSeq due to cost implications.

With the development of second-generation sequencing machines, the cost of sequencing a genome has decreased substantially. The National Human Genome Research Institute has estimated that the cost of sequencing the human genome currently stands at $1,121 [183]. It is much cheaper to sequence bacteria due to a smaller genome. Furthermore, the speed of sequencing has increased dramatically, and it is now possible to sequence the genome of multiple strains of *H. influenzae* in a few days as opposed to the three months it took in 1996 [177].

1.4.2. Bacterial genome biology, evolution and epidemiology

The reduced cost and speed of second-generation sequencing machines make them ideal for the analysis of bacterial species diversity. Typically, this is done by comparing SNPs in the core genome, which represents the genes shared by all isolates of interest [184, 185]. From this comparison, the relationship between strains can be established. In addition, by sequencing the whole genome novel virulence factors may be identified.

WGS analysis has been used in the analysis of both bacterial and viral pathogens. For example, it identified a 30 amino acid deletion in the non-structural protein 2 in a PPRS viral strain responsible for an epidemic in China in 2006 [39]. This protein inhibits the innate immune system by blocking activation of NF-κB; a mutation here may have increased the virulence of the epidemic strain [186]. WGS has also been used to identify the spread of colistin resistance in pigs and humans, to explore the relationship between *Mycobacterium bovis* in five cattle farms and local badgers and to distinguish between isolates of livestock-associated methicillin-resistant *Staphylococcus aureus* that had identical STs [168, 187-189].

A small number of projects have specifically sequenced the genome of *B. hyodysenteriae* [85, 165, 190]. An Australian strain, WA1, was completely sequenced using a combination of 454 sequencing and Sanger sequencing [85]. This

was the first strain sequenced and increased the understanding of *B. hyodysenteriae*. For example, *B. hyodysenteriae* has a higher number of genes involved in amino acid transport compared to other sequenced *Brachyspira* species [85]. This may reflect adaption to the protein-rich environment of the pig gut. In addition, a 36 kb plasmid was also found, containing genes involved in LOS biosynthesis [85]. The importance of the plasmid to *B. hyodysenteriae* is unknown. Initially, it was suggested that this plasmid could play a role in virulence, but this plasmid has subsequently been identified in non-virulent strains [95, 191]. No other transposon-like elements were identified, nor were any pathogenicity islands identified [85]. Subsequent sequencing of an Italian clinical isolate revealed a lincomycin resistance gene *lnu(C)* on a transposon [165]. However, the mechanism of transmission is currently unknown, and it is likely that horizontal gene transfer only occurs infrequently in *B. hyodysenteriae.*

Draft genome sequences of multiple strains of *B. hyodysenteriae* isolates have also been analysed. Analysis of genomes from across the world identified potentially useful vaccine targets and differences between haemolytic and weakly haemolytic *B. hyodysenteriae* isolates [93, 95, 192].

Another study used core genome SNP analysis to investigate the relationship between different species of *Brachyspira*. This was done to investigate the relationship between *B. suanatina* and other *Brachyspira* species [46]. *B. suanatina* was compared to closely related species including *B. hyodysenteriae,* and it was determined that it was distinct from these species and is most likely a distinct species. The genome sizes were also compared, and it was found that *B. hyodysenteriae* had the smallest genome [46]. This could potentially mean that *B. hyodysenteriae* is adapting to the pig gut through reductive evolution through the loss of non-essential genes [193].

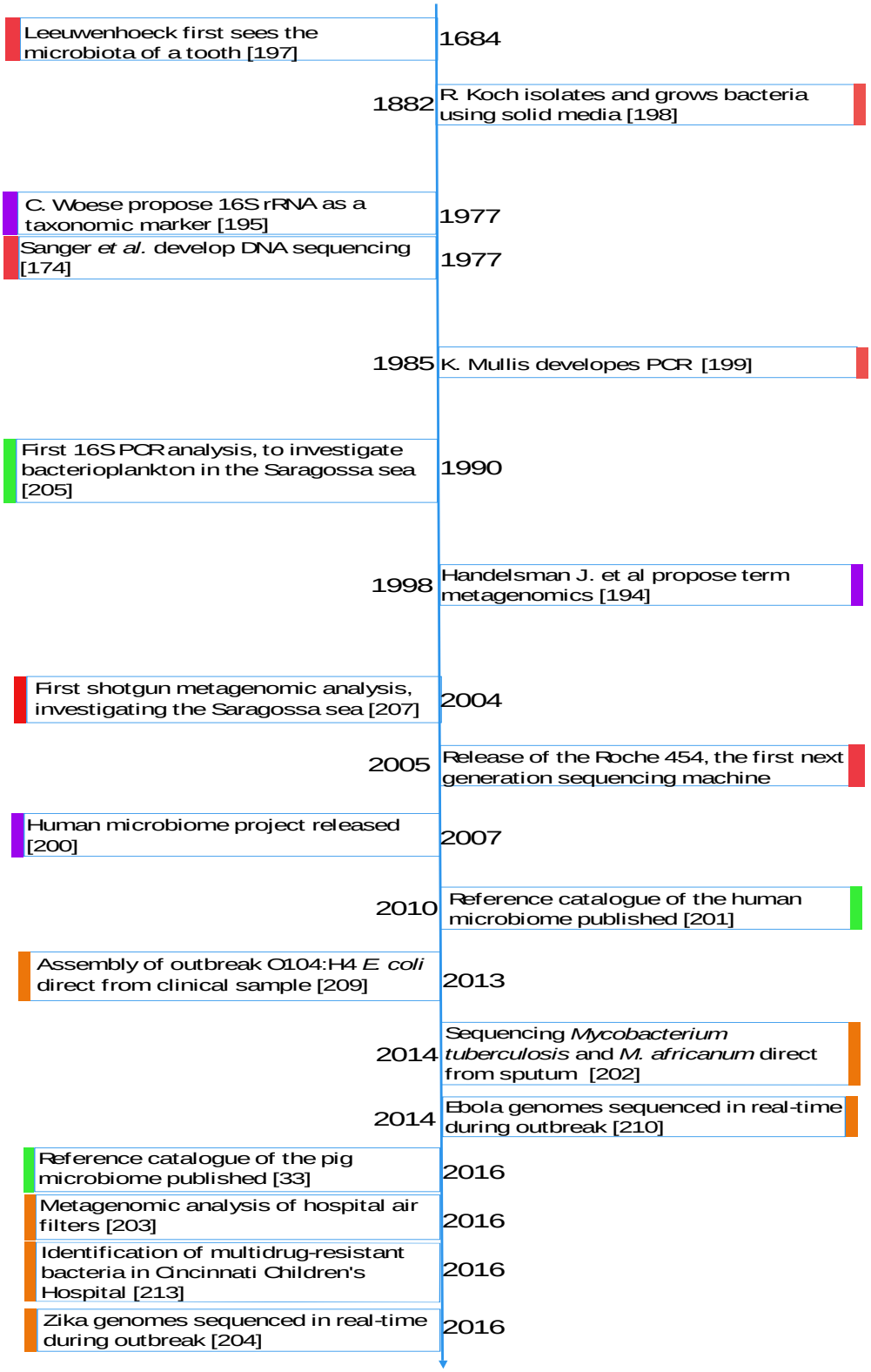
1.4.3. Metagenomics

As sequencing has become steadily cheaper over time, it has become economically feasible to sequence DNA directly from a sample. The analysis of genetic material directly from samples is referred to as metagenomic analysis, a term first used in 1998 [194]. Initially, this was conducted by analysing the 16S ribosomal gene. The 16S rRNA subunit provided a molecular barcode allowing rapid identification of the species in a metagenomic sample and was first proposed as a typing mechanism by Carl Woese [195].

By sequencing total DNA additional genomic information is available, for example, AMR gene presence, as well as species presence. This approach is often referred to as shotgun metagenomics to distinguish it from the 16S analysis, and it is a rapidly developing field driven by improvements in technology. A historical perspective as well as more recent developments are summarised below (figure 1.4.3).

Figure 1.4.3: Timeline of Metagenomics.

Edited from Escobar-Zepeda et al 2015 [196]. Squares signify themes in the timeline. Red: technological development, purple: theoretical development, Green: key publications (in relation to metagenomics in pigs) and orange relates to metagenomic studies that involved clinical samples.



1.4.3.1. Applications in microbial ecology

16S analysis allowed rapid quantification of total species composition in an environment of interest. In 1990 and 1991 Giovannonni and Schmidt respectively demonstrated the potential of metagenomics to thoroughly interrogate the microbial ecology of the Saragossa Sea and the Pacific Ocean [205, 206]. Shotgun metagenomic analysis has also been used to investigate microbial ecology with the first published analysis occurring in 2004 investigating the Saragossa Sea where a number of new species were identified. Additionally, it was also possible to identify rhodopsin-like genes, which are important in photosynthesis [207].

1.4.3.2. Applications in pathogen discovery and detection

16S analysis was initially used to identify pathogens. In the first instance, this was done to identify *Campylobacter* species on chicken products [208]. More recently shotgun metagenomics has become more widely used to investigate pathogens. For example, it was used to identify and re-assemble the genome of the *E. coli* O104:H4 strain responsible for an outbreak of food poisoning in Germany in 2011 [209]. Shotgun metagenomics has also been used to track outbreaks. Recently it was used to sequence multiple Ebola and Zika genomes during the 2014 Ebola epidemic and helped identify cases of co-infection of Zika and Chikungunya during the 2015 Zika outbreaks [210, 211]. As well as uses for outbreak analysis, metagenomics has the potential to elucidate changes in the microbiome. Shotgun metagenomics has been used to investigate the colonisation of bacterial species after a faecal transplant and has identified AMR gene carriage in a children's ward at Cincinnati Children's Hospital Medical Center [212, 213].

With regard to *B. hyodysenteriae* infections, there has been little metagenomic analysis into the interaction of the microbiome with *B. hyodysenteriae*. This is despite the importance of the microbiota potentially in *B. hyodysenteriae* infection [89]. Terminal restriction fragment length polymorphism analysis established that during infection there is disruption of the normal microbiota when pigs are challenged with *B. hyodysenteriae* [214]. A 16S rRNA analysis of the lumen and epithelium of pigs challenged with *B. hyodysenteriae* identified species that are enriched compared to control pigs. Linear discriminatory analysis **(**LDA**)** identified a

number of genera associated with swine dysentery including *Brachyspira*, *Campylobacter*, *Mogibacterium*, and *Desulfovibrio*. *Lactobacillus* and *Bifidobacterium* genera were associated with healthy pigs, *Desulfovibrio* species are able to bind and degrade sulfated mucin possibly providing an energy source for *B. hyodysenteriae* [215]. *Bifidobacterium* and *Lactobacilli* are associated with a healthy microbiome and have been suggested as potential probiotics [216]. It is possible that *B. hyodysenteriae* disrupts the microbiota reducing the gut health and leading to an environment favourable for colonisation by other pathogenic bacteria.

1.5. New opportunities: alternatives to antibiotics

1.5.1. Diet

Feed given to pigs can also help reduce *B. hyodysenteriae* infections. A number of different additives have been investigated for their ability to protect against *B. hyodysenteriae* infection in pigs (table 1.5.1)**.** Inulin has been shown to protect against *B. hyodysenteriae* infection in multiple trials. It has been hypothesised that this protective effect is due to changes in the gut microbiota. Inulin has been associated with an increase in the probiotic species *Bifidobacterium* and *Megasphaera elsdenii* in pigs, which may inhibit colonisation by *B. hyodysenteriae* [217].

Table 1.5.1: Summary of additives added to pig diets.

Pigs were fed the additive and then challenged with *B. hyodysenteriae.* Abbreviation: DDGS refers to Distillers Dried Grains with Solubles.

Additive	Effect	Conclusion	Citation
Cooked Rice	No pigs developed swine dysentery	Low levels of non-starch polysaccharide (NSP) may be protective	[218]
Cooked Rice and Guar Gum	All pigs fed this diet developed swine dysentery	Highly fermentable carbohydrates associated with swine dysentery	[219]
Parboiled Rice	90% of pigs still developed swine dysentery	Low levels of NSP are not protective.	[220]
Treated Wheat or Sorghum	No significant difference between diets	Low levels of NSP are not enough to prevent swine dysentery	[221]
Fermented Liquid Feed	Lower rate of swine dysentery when fed liquid feed	Changes were not due to low levels of NSP; it may be due to changes in the microbiota.	[222]
Dried Chicory Root	Pigs fed chicory did not develop swine dysentery	Chicory seeds contain inulin which may be protective.	[223]
Inulin	17/20 pigs fed inulin diet did not develop swine dysentery	Inulin provides protection against swine dysentery	[224]
Inulin	8/15 pigs were protected from swine dysentery when fed 80 g/kg	Inulin needs to be at high concentrations (80 g/kg) to be protective.	[225]
DDGS	Faster onset of swine dysentery	Insoluble dietary fibre should be reduced in pig feed	[226]

1.5.2. Bacteriotherapy

There are a number of probiotic bacteria preparations that could be useful for the prevention of *B. hyodysenteriae* infection. A combination of *L. rhamnosus* and *L. farciminis* were effective against *B. hyodysenteriae in vivo* [227].

1.5.3. Phage therapy

Phage have been found to lyse critically important pathogens and have been shown to be effective in animal trials against multidrug-resistant pathogens such as *P. aeruginosa* [228-230]. There has also been considerable commercial and governmental interest in phage therapy. The first clinical phase one trial of a phage therapy occurred in 2009 to treat chronic venous leg ulcers and the EU have provided € 3,838,422 to investigate the potential of phage therapy in the treatment of burn patients [231, 232].

Due to the strict legal and regulatory processes that need to be completed before a product can be sold for human treatment, the sector where phage therapy is likely to become more used is in agriculture. There are a small number of treatments that are commercially available. This includes AgriPhageTM used to prevent tomato spot and speck (Omnilytics, USA). While Listex P100 has been classified by the US Food and Drug Administration as "generally recognised as safe" and is used to treat animal products before market [233]. Although there is currently no published research for phage as a treatment for *B. hyodysenteriae* infections it has been used to deal with a number of other important pathogens that colonise pigs, for example, removal of pathogenic *Salmonella* during slaughter and prevention of diarrhoea in pigs [234, 235].

1.6. Aims and Objectives of this Study

There is a growing body of literature on pleuromutilin resistance within *B. hyodysenteriae* in the UK. However, little is known about the population structure within the UK or the mechanisms of pleuromutilin resistance in isolates from the UK. Moreover, there has been little WGS based phylogenetic studies conducted on

B. hyodysenteriae, and it is unknown whether resistant strains are becoming dominant in the UK. In this study we hypothesis

The aim of this study was to characterise the population of *B. hyodysenteriae* in the UK. The objectives of this study were to identify the phylogenetic relationship of clinical isolates of *B. hyodysenteriae* in the UK (chapter 3) and identify resistance mechanisms that may have caused pleuromutilin resistance (chapter 4). In addition, shotgun metagenomics was used to directly sequence *B. hyodysenteriae* from faeces and investigate the changes in the microbiota associated with swine dysentery (chapter 5).

- Establish the population structure of *B. hyodysenteriae* within the UK
- Identify genetic determinants associated with pleuromutilin resistance
- Sequence *B. hyodysenteriae* directly from faeces
- Investigate changes in the microbiome in swine dysentery

2. Material and Methods

2.1 Samples

Samples were provided by the Animal and Plant Health Agency (APHA). *Brachyspira hyodysenteriae* isolates were collected from the APHA Bacteriology Culture Collection held Bury St Edmunds regional laboratory and stored at -80 °C until use. Historic faecal samples were sent from the APHA Penrith regional laboratory and stored at -20 °C until use. Faecal isolates received over the course of the study were sent from the APHA Bury St Edmunds regional laboratory and stored at -80 °C until use. Pig caecal samples collected from healthy pigs at abattoirs as part of a separate APHA project (VM0518) were stored at -80 °C. All of the samples were stored at APHA Weybridge.

2.2 Media

Media was prepared according to manufacturer's instructions and sterilised by autoclaving at 121 °C for 15 minutes at 15 psi (Table 2.3).

Table 2.2 Media used in this study

Media	Atmosphere	Antibiotics	Notes
Fastidious Anaerobic Agar + 5% Sheep Blood	Anaerobic		
Brachyspira Enrichment Media	Aerobic		Used to store *B. hyodysenteriae* at -80 ℃
Brain Heart Infusion Broth + 10% Horse Serum	Anaerobic		
Brachyspira Selective Media	Anaerobic	Spectinomycin (400 µg/ml), vancomycin (25 µg/ml) and colistin (25 µg/ml)	Used at Bury Regional Lab to isolate *B. hyodysenteriae*. Ref: [236]

2.2.1 Bacterial Media

Fastidious Anaerobic Agar plates, supplemented with 5% horse blood (FABA) made at the APHA media department, were used to culture *B. hyodysenteriae* isolates routinely. Brain Heart Infusion (BHI) supplemented with 10% horse serum (v/v) (EO Labs) was used for liquid cultures of *B. hyodysenteriae*.

2.2.2 Bacterial Growth Conditions

B. hyodysenteriae was grown in an anaerobic cabinet (Don Whitley, Shipley, UK) on FABA plates for 3 to 5 days at 37 °C. The gas mix was 10% hydrogen, 10% carbon dioxide and 80% nitrogen. For broth cultures, *B. hyodysenteriae* was grown in pre-reduced BHI with 10% horse serum in an anaerobic cabinet at 37 °C overnight on a mini orbital-shaking platform (Stuart, Staffordshire, UK) at 80 rpm.

Isolates were subcultured from the initial culture a minimum of two times before being used. At this point plates were sub-cultured for 4 days and were used to make either a cell pellet, a frozen stock or a broth culture.

The overnight culture was used to perform MIC tests (Chapter 4, section 4.2.3). Contaminated *B. hyodysenteriae* samples were purified by repeat subculturing on FABA plates. If this was not sufficient, isolates were purified by repeat subculturing from additional frozen stocks on *Brachyspira* Selective Media, made in-house (Table 2.3), under anaerobic conditions at 37 °C at the APHA Bury St Edmunds regional laboratory.

For cell pellets, a plate was scraped into a McFarland tube containing 2 ml of Phosphate-buffered saline (PBS) (APHA media department) and resuspended by pipetting. A McFarland reading was obtained using a Den-1 McFarland Densitometer (Grant Bio, Cambridge, UK), and the contents were transferred to a 1.5 ml or 2 ml microcentrifuge tube and centrifuged for 7 minutes at 13**,**500 rpm. The supernatant was removed and the cell pellet stored at -20 °C until the DNA was extracted.

For frozen stocks, a plate was scraped into a McFarland tube containing 2 ml of *Brachyspira* Enrichment Media (APHA Newcastle regional laboratory) and re-suspended by pipetting. A McFarland reading was obtained using a Den-1 McFarland Densitometer and 660 µl was transferred to three cryovials (ThermoFisher) and stored at -80 °C.

2.3 Isolation of DNA

2.3.1 Extraction of DNA by Blood and Tissue Kit

The DNase Blood and Tissue Kit (Qiagen) was used for isolation of DNA from *B. hyodysenteriae*. Cell pellets were re-suspended in 180 µl ATL buffer and 20 µl of proteinase K was added. This was mixed by vortexing and incubated at 56 °C under shaking conditions (80 rpm) in a shaking heating block (Eppendorf, Stevenage, UK)

for 1 hour until the tissue was completely lysed. Samples were then vortexed for 15 seconds and 200 µl buffer AL was added and vortexed to mix. To the mixture, 200 µl of ethanol (100%) was added and the sample vortexed. This was added to a DNeasy Mini spin column and centrifuged at 6000 x g for 1 minute. The collection tube and flow-through were discarded, and the spin column was placed in a new collection tube. To the spin column, 500 µl of buffer AW1 was added and centrifuged at 6000 x g for 1 minute. The collection tube and flow through were discarded, and the spin column was added to a new collection tube, and 500 µl of buffer AW2 added and the column centrifuged for 3 minutes at 20,000 x g. The collection tube and flow through were again discarded and the spin column was added to a 1.5 ml microcentrifuge tube. Finally, 100 µl of buffer AE was added directly to the DNeasy membrane and incubated at room temperature for 1 minute before centrifugation at 6000 x g for 1 minute. To maximise DNA yield this step was repeated by adding another 100 µl of buffer AE to the DNeasy membrane and centrifuging at 600 x g. The DNA was stored at – 20 °C until needed.

2.3.2 DNA extraction using the MacMAX CORE Nucleic Acid Purification Kit and Kingfisher DUO Prime

Buffers were supplied as part of the MagMAX CORE nucleic acid purification kit (ThermoFisher). To rows B and C of a 96-deep-well plate, 500 µl of wash buffers 1 and 2 were added respectively. To wells of a separate elution strip 90 µl of elution buffer was added. A working solution of 350 µl of lysis buffer and 350 µl of binding buffer per sample was prepared. Beads were vortexed for 10 seconds before 20 µl of beads were then added to 10 µl of proteinase K per sample and pipetted into row A. Cell pellets were prepared as described previously (Materials and methods, section 2.2) and then re-suspended in 270 µl PBS and added to row A. The 96-deep-well plate was then incubated for 2 minutes at room temperature before 700 µl of the lysis-binding buffer mix was added to row A. A tip comb was then placed in row H before the 96-deep-well plat was placed in the Kingfisher Duo Prime and the following program was used: MagMax CORE Duo heated script MagMAX CORE DUO (ThermoFisher) .

2.3.3 DNA Quantification by Qubit 2.0

A working solution of 1:200 solution of high sensitivity dye (Life technologies, USA) and high sensitivity buffer was made. To individual Qubit tubes, 10 µl of each high sensitivity standards 1 and 2 or 2 µl of sample DNA were added. 190 µl of the working solution was added to each standard and 198 µl of working solution was added to the sample DNA. The tubes were then mixed by vortexing for 3 seconds and incubated in the dark for 2 minutes at room temperature. They were then quantified on the fluorometer and the concentrations recorded.

2.3.4 DNA quantification with Quantifluor

A DNA dilution series of Quantifluor DNA standard (Promega) was made by adding 1188 µl of 10 nM Tris, 1 mM Ethylenediaminetetraacetic acid (TE buffer; Sigma) to a 1.7 ml microcentrifuge tube and 600 µl to seven other tubes. To the initial tube, 12 µl of DNA standard was added, mixed by vortexing and 600 µl was added to the next tube. This was repeated for the next five tubes, and the last tube was left as a negative control. 1 µl of DNA to be quantified was added to wells of a black half-area clear flat bottom 96-well optical plate (Sigma, Gillingham, UK) with one sample per well; 49 µl of TE buffer was then added. One column was left for the DNA standard and 50 µl of the dilution series was added to each well. Picogreen (Thermofisher) was diluted 1 in 200 with TE buffer and 50 µl was added to each well. Fluorescence was detected by a Polarstar Galaxy Plate reader (BMG labtech, Aylesbury, UK) with quantifluor test selected and gain adjustment specified. Results were analysed in Excel 2013 (Microsoft). If the standard curve was not straight or the R^2 value was less than 0.95, the test was repeated with the same DNA dilution series.

2.4 DNA Sequencing

2.4.1 Illumina Nextera XT Library Preparation

Except for EB buffer (Qiagen), all buffers were supplied as part of the Nextera XT library preparation kit (Illumina). DNA from 2.3.1 was diluted to 0.2 ng/µl using EB

buffer; from this 5 µl of sample DNA was then added to 10 µl of TD buffer before being centrifuged at 280 x g for 1 minute at room temperature. A microfuge tube of the mixture was then placed in a thermocycler with a heated lid and heated for 5 minutes at 55 °C before being incubated at room temperature for 5 minutes. After 5 minutes 5 µl of NT buffer was mixed by pipetting five times and centrifuged at 280 x g for 1 minute. 15 µl of NPM and 5 µl each of an i7 and an i5 adapter was added and mixed by pipetting before being centrifuged at 280 x g for 1 minute at room temperature. The mixture was then placed in a thermocycler, and the following settings were used:

- 72 °C for 3 minutes
- 95 °C for 30 seconds
- 15 cycles of:
    - 95 °C for 10 seconds
    - 55 °C for 30 seconds
    - 72 °C for 30 seconds
- 72 °C for 5 minutes
- Hold at 10 °C

Samples were then cleaned using the AMPure bead method (Materials and methods, section 2.4.2), and Qubit 2.0 was used to determine the concentration of DNA (Materials and methods, section 2.3.4). Samples sequenced at the APHA followed this protocol with the following modifications: samples were diluted in TE buffer; 13 µl instead of 15 µl of NPM was used; samples underwent 12 cycles of amplification and DNA was quantified using Quantifluor (Materials and methods, section 2.4.4). DNA was stored at – 20 °C.

2.4.2 Cleaning of Nextera XT libraries with AMPure Beads

To 50 µl of Nextera XT library DNA (Materials and methods, section 2.5.1) 30 µl of AMPure XP beads were added and incubated at room temperature for 10 minutes before being put on a magnetic particle concentrator (MPC). Beads formed a pellet at the side of the tube, and the supernatant was discarded. The beads were washed

twice with 200 µl of 80% ethanol. Ethanol was then removed using a pipette, and the samples were left with lids open to dry for 5-10 minutes. Samples were then removed from the magnetic stand and 20 µl of resuspension buffer (Illumina) was added before being vortexed briefly to mix. This was followed by a 2-minute incubation before samples were put on the MPC and 18 µl was removed and placed into a new microcentrifuge tube. The tube was stored at -20 °C until needed.

2.4.3 Preparation of PhiX control

PhiX library (Illumina) was diluted to 4 nM by mixing 2 µl of 10 nM PhiX library with 10 mM Tris-CL (Sigma), pH 8.5 with 0.1% Tween 20 (Sigma). This solution was diluted further by mixing 5 µl of 4 nM PhiX library and 5 µl of 0.2 N NaOH before being vortexed briefly and centrifuged at 280 x g for 1 minute. This was then incubated at room temperature for 5 minutes and 990 µl of pre-chilled HT1 (Illumina) was added. This was further diluted to 12.5 pM PhiX by diluting 375 µl of 20 pM PhiX library in 225 µl of pre-chilled HT1. Samples sequenced at the APHA followed this protocol with the following modifications: PhiX library was diluted to 12.5 pM by mixing 10 µl of the PhiX library with 1590 µl of pre-chilled HT1.

2.4.4 Sequencing isolates on a MiSeq

Samples were diluted to 4 nM using EB buffer and 5 µl of each sample was pooled. From the pooled samples 5 µl was then denatured by adding it to 5 µl of fresh 0.2 N NaOH solution before being vortexed and centrifuged at 280 x g for 1 minute at room temperature. After a 5-minute, incubation at room temperature 10 µl was added to 990 µl of pre-chilled HT1 buffer. Pre-chilled HT1 buffer was then used to dilute the pool to 12 pM by mixing 360 µl of the pool with 234 µl of pre-chilled buffer. To this, 6 µl of PhiX control were added (Materials and methods, section 2.5.4), and the diluted pool was then sequenced on an Illumina MiSeq using either the Illumina Miseq V2 2x250 bp or MiSeq V3 2 x 300 bp paired-end protocols.

Samples sequenced at the APHA followed this protocol with the following modifications: samples were diluted to 2 nM in TE buffer instead of being diluted to

4 nM; and 7.5 µl of the library pool was mixed with 2.5 µl of 0.2 N NaOH and incubated for 5 minutes before 940 µl of pre-chilled HT1 and 50 µl of PhiX library (Materials and methods, section 2.5.4) was added.

2.4.5 Sequencing isolates on a NextSeq

Samples were diluted to 2 nM using TE buffer and 5 µl of each sample was pooled. From the pooled samples 6 µl was then denatured by adding it to 0.5 µl of fresh 2 N NaOH solution. To this 994 µl of pre-chilled HT1 buffer was added before being vortexed and centrifuged at 280 x g for 1 minute at room temperature. An aliquot of 150 µl was made, and 1150 µl of pre-chilled HT1 and 2 µl of 12.5 pM PhiX library (Materials and methods, section 2.4.3) was added. The diluted pool was then sequenced on an Illumina NextSeq using the Illumina NextSeq 2x150 bp paired-end protocol.

2.5 Bioinformatic analysis

2.5.1 Assembly and annotation

Adapters and low-quality reads were removed using default Illumina software. Isolates were assembled using the Nullabor pipeline (version 1.2) [237, 238] with SPAdes, (version 3.10.1) specified as the assembly software [238]. In the Nullabor pipeline, reads were trimmed with Timmomatic (version 0.36) [239] before contaminants were identified with Kraken (version 0.10.5-beta) [240]. Any contaminated samples were regrown from frozen stock, purified and re-sequenced. If the amount of contaminant was less than 1%, Samtools was used to remove all reads that did not map to the WA1 reference using the command *samtools view -f4 –bS.* Bam files were then converted into fastq with bam2fastq [241] and then assembled by Nullabor. After samples had been assembled, they were aligned to the WA1 reference genome using Mauve (version 5.0**)** [242] and annotated with prokka (version 1.11) [243].

2.5.2 Phylogenetic trees

An alignment of SNPs in the core-genome was constructed by Snippy (version 3.1) using default settings [244]. SNPs in recombinant sites were removed with Gubbins (version 2.2.0) [245] using default settings, and maximum likelihood trees were bootstrapped using RAxML (version 8.2.11) [246]. GTRGAMMA was used as the nucleotide substitution model, and 1000 bootstraps were conducted. The number of SNPs between isolates was obtained using snp-dist (version 0.2) [247], and trees were viewed in Interactive tree of life (version 3) [248].

Chapter 3. Population structure of *B. hyodysenteriae* in the UK

3.1 Introduction

Analysis of the population structure of pathogens can reveal valuable information and aid in the development of strategies to limit their impact. The population structure of *B. hyodysenteriae* has been investigated using a variety of techniques, including Multilocus enzyme electrophoresis (MLEE) and Multiple-locus variable number tandem repeat analysis (MLVA) [117, 118, 249, 250]. MLEE characterises isolates based on the electrophoretic mobility of a core set of enzymes, but it is time-consuming, and for recombinant species, hundreds of electrophoresis gels may be required [251]. MLVA is a faster alternative and relies upon the amplification of specific repeating sections of the chromosome, the number of varying repeats differs between strains and therefore can be used for characterisation [118]. Multi-locus sequence typing (MLST) has become the most commonly used technique and has been found to be slightly more discriminatory than MLVA [118].To visualise MLST data minimum spanning trees are most commonly used; these are simple trees that link closely related individuals [252]. It has been used to describe the population structure of *B. hyodysenteriae* in Australia, Belgium, Germany, Italy, Spain and the USA [52, 60, 73, 93-95, 121-125]. The dominant STs vary between countries, and there is little spread of STs between geographically distant countries such as Germany and the USA, probably due to a limited trade in live pigs between these countries (in 2018 only 4,024 live pigs were transported between the USA and the EU) [52, 73, 253]. In Europe, some STs are shared between countries. This may be due to movement of pigs throughout the European pig industry through trade. Analysis of SNPs in the core-genome can enhance MLST-based phylogenetic studies as it provides higher resolution. It has been used to distinguish between isolates of the same ST in pathogens such as *S. aureus*, *P. aeruginosa* and has also been used to track an outbreak of a ST 258 carbapenem-resistant *Klebsiella pneumoniae* at the US National Institute of Health Clinical Center that resulted in 18 infections [189, 254, 255]. It has also been used to investigate how the novel species *B. suanatina* related

to seven other *Brachyspira* species, including *B. hyodysenteriae*; although the population structure of *B. hyodysenteriae* isolates was not studied [46].

This study was done as part of a larger study investigating *B. hyodysenteriae* in the UK (VM0516) [256]. In a separate part of this project, 39 isolates of *B. hyodysenteriae* were analysed by WGS, and antibiotic sensitivity testing performed on these isolates using the VetMic Brachy panel [256]. The remaining ninety-nine *B. hyodysenteriae* isolates held at the APHA were sequenced as part of this study [256]. These are the first WGS studies explicitly investigating the population structure of *B. hyodysenteriae* in the UK.

SNP based core-genome analysis provides information on the phylogeny, but other techniques are needed to investigate transmission over time. Understanding how *B. hyodysenteriae* has transmitted between regions may highlight areas where transmission can be prevented. To investigate transmission Bayesian inference, more specifically Metropolis-Hastings Markov chain Monte Carlo (MCMC), was used [257]. By using Bayesian inference metadata, such as the date of sampling, can be incorporated into analysis, and from this transmission can be inferred. There are a variety of programs that have been designed that infer transmission events from metadata and can be included in the MCMC. The most commonly used program is the BEAST2 environment; BEAST2 is a development of a customisable BEAST program that enables third-party programs to be used [258, 259]. For this study, SCOTTI was used to predict the movement of *B. hyodysenteriae* between different regions of England and Wales [260]. SCOTTI is an implementation of an approximate structured coalescence framework, developed in the BASTA program, to predict transmission events during outbreaks [260, 261]. SCOTTI does not assume that all isolates involved in an outbreak have been found, this makes it useful for investigating a pathogen able to survive in multiple animal vectors [260]. Each host is treated as a different bacterial population, and it is assumed that transmission between different hosts is equally likely [260]. These hosts are based upon metadata provided, and bacterial lineages are only able to migrate to host that are exposed at a similar time (exposure times are also based upon metadata provided) [260]. Nucleotide mutations are modelled using the HKY substitution model, where

nucleotides are present in different amounts, and the rate of transitions and transversions differs [262].

3.1.2. Aim of the study

The population structure *of B. hyodysenteriae* in the UK was investigated in this study. This was done using 99 isolates purified from diagnostic samples submitted to the APHA Reference Laboratory at Bury St. Edmunds, between 2004 and 2015. These isolates were sequenced, then MLST and SNPs in the core-genome were used to investigate the population structure of *B. hyodysenteriae* within the UK. However, as there are few sequenced *B. hyodysenteriae* genomes, core-genome analysis was not the best technique to understand the population structure globally. Instead, MLST analysis, and comparison to published sequences on pubMLST was used to compare more widely outside the UK and provide a global picture of *B. hyodysenteriae* genomics. Such comparisons may identify potential STs moving through Europe or structural differences in the UK *B. hyodysenteriae* population. Core-genome analysis was intended to complement this analysis by identifying clonal populations in the UK, which was combined with transmission prediction to provide information about the persistence of ST and clonal groups in the UK.

3.2. Methods

Ninety-nine isolates were obtained from the APHA culture collection and cultured as specified previously (Table 3.2.1).

Table 3.2.1: Information on the location and year of sequenced isolates in this study.

Holdings have been numbered arbitrarily to show where were originally received from. Where multiple isolates were received from the same holding at different times the same holding number has been given.

Abbreviations: NUTs refers Nomenclature of Territorial Units for Statistics and Yorkshire refers to Yorkshire and the Humber.

Isolate	Year	NUTs Region 1	Holding
js01	2011	South West	1
js02	2009	East of England	2
js03	2013	Yorkshire	3
js04	2013	South West	unknown
js05	2012	South West	4
js06	2009	West Midlands	5
js07	2010	South West	6
js08	2009	East of England	2
js09	2007	South West	7
js10	2008	Wales	8
js11	2010	Yorkshire	9
js12	2011	Yorkshire	10
js13	2005	North East	11
js14	2006	Yorkshire	12
js15	2007	Yorkshire	13
js16	2007	Yorkshire	14
js17	2008	North West	15
js18	2008	Yorkshire	16
js19	2008	Yorkshire	17
js20	2008	Yorkshire	18
js21	2009	Yorkshire	19
js22	2009	North East	20
js23	2009	North East	21
js24	2010	Yorkshire	22
js25	2011	Yorkshire	23
js26	2012	Yorkshire	24
js27	2012	Yorkshire	25
js28	2012	Yorkshire	25
js29	2012	Yorkshire	25

Isolate	Year	NUTs Region 1	Holding
js30	2013	East of England	3
js31	2013	Yorkshire	26
js32	2013	Yorkshire	27
js33	2014	North West	28
js34	2004	North West	29
js35	2006	North West	30
js36	2006	North West	31
js37	2006	North West	32
js38	2007	North West	33
js39	2007	North West	34
js40	2009	North West	35
js41	2009	South East	36
js42	2010	West Midlands	37
js43	2012	South East	38
js44	2012	South East	38
js45	2012	South East	39
js46	2011	East Midlands	40
js47	2008	East of England	41
js48	2008	South West	42
js49	2008	South West	43
js50	2011	East of England	44
js51	2013	Non-UK	45
js52	2011	Unknown	46
js53	2014	East of England	47
js54	2015	Non-UK	48
js55	2006	Wales	49
js56	2007	Wales	50
js57	unknown	unknown	51
js58	unknown	unknown	52
js59	2011	South West	53
js60	2006	South West	54
js61	2004	West Midlands	55
js62	2008	Wales	56
js63	2011	West Midlands	57
js64	2011	West Midlands	58

Isolate	Year	NUTs Region 1	Holding
js65	2011	West Midlands	59
js66	2005	South West	60
js67	2007	South West	61
js68	2009	South West	62
js69	2009	South West	63
js70	2010	South West	64
js71	2010	South West	65
js72	2011	South West	66
js73	2014	South West	67
js74	2015	South West	68
js75	2005	South West	69
js76	2006	South West	70
js77	2008	South West	71
js78	2008	South West	72
js79	2008	South West	71
js80	2008	South West	4
js81	2008	South West	73
js82	2008	South West	74
js83	2009	South West	75
js84	2009	South West	76
js85	2010	South West	77
js86	2011	South West	4
js87	2011	South West	75
js88	2012	South West	78
js89	2012	South West	79
js90	2004	South East	80
js91	2005	South East	81
js92	2006	South West	70
js93	2007	South East	82
js94	2011	South East	83
js95	2010	South East	84
js96	2009	Wales	85
js97	2015	East of England	86
js98	2015	East of England	88
js99	2015	unknown	87

3.2.1. MLST

SRST2 (version 0.1.5) was used to identify the ST from the fastq files [263]. These STs were combined with the 675 MLST profiles published on pubMLST (http://pubmlst.org/bhyodysenteriae/) and profiles from previously published studies [121] [73]. The fasta of novel ST were extracted using Seqfinder [264] by mapping against the *B. hyodysenteriae* PubMLST allele database (http://pubmlst.org/bhyodysenteriae/) and submitted to Tom La for categorisation. All STs were then used to make minimum spanning tree using Bionumerics 6.0 with 1000 bootstraps [265].

3.2.2. Prediction of movement of *B. hyodysenteriae* between regions of the UK

Transmission was predicted for isolates that contained metadata, including a date at which the sample was received at the APHA. Snippy identified core-genome SNPs in isolates with js90 (the oldest isolate in the study) being used as the reference [244]. SNPs in recombinant sites were removed using Gubbins (version 2.2.0) [245]. SCOTTI (version 1.02) was used to construct a phylogenetic tree and predict movement between Nomenclature of Territorial Units for Statistics (NUTS) 1 regions [266]. NUTS 1 regions are the largest statistical regions within a country and enable analysis of movement of *B. hyodysenteriae* without compromising the identity of farmers. For this analysis the NUTS 1 region of isolate origin, an approximate time of infection and the total time of *B. hyodysenteriae* infection in each region were required and provided as csv files. An exact time of infection is unknown, so an approximate time of infection was used. Infection was estimated as two weeks prior to the APHA receiving the initial clinical sample. This was based on an estimated incubation period of 14 days [53, 267]. A maximum of 30 hosts and 100,000 MCMC interations was specified. Trees were visualised in Figtree (version 1.4.2) [268].

3.3. Results

3.3.1 Sequencing of Isolates

Isolates were obtained from the APHA culture collection at Bury St. Edmunds and grown at the APHA Weybridge site. It was not possible to purify eight of the 99 isolates; this was due to overgrowth of contaminants present in the frozen stock. All purified isolates were sequenced on an Illumina MiSeq in four MiSeq runs. There was variation in the sequence quality with eight sequences being rejected due to low quality. Three isolates failed to sequence, three had coverage below 20x and thus failed our quality threshold, one was *B. pilosicoli*, and one appeared to be a mixed culture. The remaining 84 isolates were used for the subsequent analysis (Table 3.3). Between these isolates, the quality of sequences also varied, with coverage ranging between 20x and 226x. The higher coverage for samples js03 to js12 was because there were fewer samples on this MiSeq run. The median number of contigs was 202, but there was a large range in the total number of contigs, with the js20 having the fewest contigs (20) and js02 having the most contigs (1309). Contig number may not correlate with coverage as some isolates with high coverage, for example, js11 (140x coverage), had more contigs than isolates with less coverage, for example, js01 (28x coverage). It is possible that these genomes contain more repeat regions making assembly more difficult. Also, the N50 also varied with the median N50 being 66839 and js02 having the lowest N50 (4058); while js06 had the highest N50 (2533390). With lots of small contigs in some isolates, there are likely to be errors in the assembly of the genome. This may result in Roary estimating an artificially low core-genome. However, Snippy compares the raw fastq file to a reference when creating an alignment of SNPs in the core-genome, therefore issues with assembly should have little impact on the tree produced. In addition, *in silico*, MLST was used to check for consistency with previously published research.

To identify contaminants, Kraken was used; the percentage of sequences identified as *B. hyodysenteriae* varied between 73.09% and 93.43%. The low similarity between strains of the same species is likely to be due to the reference used. The MiniKraken database was used for the analysis, and this database uses the Australian reference strain *B. hyodysenteriae* genome WA1. This is a commonly used reference

genome as it was the first closed *B. hyodysenteriae* genome [85]. However, it is likely that UK isolates have diverged considerably from this isolate over the decades and it is likely in future a complete sequenced UK reference isolate will be needed.

The isolates used for downstream analysis came from 70 individual holdings with 11 holdings containing multiple isolates. For seven holdings isolates originated in the same year and may be multiple isolates taken as part of the same outbreak. There were two holdings (4 and 74) that contained isolates that originated in different years, with the longest distance between two isolates from the same holding being four years (holding 4). It is possible that these represent persistent infections.

Most of these holdings are from England with one Welsh isolate. There was a varying number of isolates from each region for this time period with 31 from the South West, 18 from Yorkshire and the Humber, six isolates from the East of England, seven from the South East, six from the North West, three from the North East, seven from the West Midlands, one from the East Midlands. In addition, there were three isolates (js04, js57 and js58) from the Scottish Agricultural College that may be from Scotland but the origin of these samples is unknown. For the sake of continuity, and because some isolates are not English, isolates will continue to be called UK isolates, but it must be noted that any conclusions drawn will reflect the pig population in England.

Table 3.3.1: Information on all sequenced isolates used in the study.
Note Kraken refers to the percentage identity to the WA1 *B. hyodysenteriae* reference genome by Kraken [240].

Isolate	Total reads (bp)	GC content	Total contigs	Largest contig length (bp)	N50	Genome coverage	Kraken (%)
js01	373752	29.4	111	504553	112075	28x	85.38
js02	305230	30.6	1309	31611	4058	25x	90.42
js03	2318542	28.5	700	1102549	733978	148x	77.79
js04	1559406	26.9	296	1376438	742677	101x	81.31
js05	3581236	27	421	2063853	2063853	226x	79.18
js06	2116200	27.6	264	2533390	2533390	142x	88.9
js07	2599692	29.3	245	1830524	1830524	175x	85.54
js08	1377202	26.8	256	1473884	706770	88x	86.66
js09	2695738	28	93	1095628	742051	168x	81.67
js11	2325680	28.9	334	335020	130706	140x	73.09
js12	3161630	28.2	168	808888	359603	206x	84.69
js13	324316	31	482	49157	11037	28x	91.98
js14	324842	31	531	68101	10145	23x	88.98
js15	308588	30.7	514	44160	9714	21x	89.56
js16	402872	31.2	581	43256	8725	30x	87.46
js17	520484	30	102	250666	99497	42x	90.69
js18	370584	31.1	415	41181	15157	27x	82.63
js19	365860	30.7	266	76419	24565	26x	91.21
js20	538342	29	35	591358	256675	35x	88.85
js21	764754	29.7	118	391757	238745	56x	81.92
js22	361738	31.4	498	47211	10454	25x	90.28
js23	402382	29.8	199	182316	77444	37x	83.72
js24	270670	29.8	253	104319	27137	21x	83.14
js25	542996	30.6	184	115490	43753	29x	88.01
js26	394442	30.8	388	68957	16189	28x	86.25
js27	285206	30.8	584	42209	10774	20x	84.32
js28	319234	31.2	696	35192	8117	23x	86.29
js29	516624	31.2	366	55765	20067	43x	84.24
js30	384962	31.2	573	44729	9744	26x	79.86

Isolate	Total reads (bp)	GC content	Total contigs	Largest contig length (bp)	N50	Genome coverage	Kraken (%)
js31	475520	30	164	131535	37570	29x	80.26
js32	392526	30.6	326	105253	19587	27x	85.26
js33	309364	30.4	222	98700	24723	28x	85.32
js34	985264	29.8	79	431067	263854	67x	86.12
js35	465962	29	79	486736	207585	34x	90.15
js37	441568	30.9	304	63116	17210	31x	81.05
js39	721510	29.9	92	527045	171375	56x	86.62
js41	673458	30.1	111	592505	159299	51x	88.22
js42	554310	30.1	168	146060	48488	41x	85.9
js43	305168	29.9	248	70035	24043	23x	88.02
js44	289314	29.5	144	118478	42215	22x	88.42
js46	341496	28.9	204	481915	188391	32x	88.94
js47	975536	29.2	248	414612	169848	58x	86.06
js48	372608	29	533	186579	61426	35x	85.68
js49	466750	29.9	96	499290	97003	35x	83.62
js50	938502	30	192	346160	112940	64x	89.06
js51	631868	30.3	123	154426	53882	43x	87.36
js52	442304	29.6	81	210780	83875	30x	87.35
js57	323614	30.3	312	96445	22616	24x	85.97
js59	291520	31.2	443	46757	12485	22x	92.27
js60	360778	29.4	91	290249	91413	27x	86.53
js61	340548	29.4	60	524608	95309	27x	91.75
js62	324226	31.4	944	59447	18118	23x	86.78
js63	434854	29.8	107	395554	126577	33x	88.6
js64	573394	29.6	212	383527	167342	42x	92.47
js65	401064	28.9	57	599968	193903	29x	87.55
js66	806810	30.9	363	48919	13292	51x	87.38
js68	906212	29.2	239	636751	359052	58x	80.55
js69	451504	29.6	149	157415	60673	34x	86.08
js70	1705804	29.2	76	698386	609582	118x	83.23
js71	809256	29.8	225	381543	114447	59x	85.75
js72	561014	29.6	124	333885	72252	35x	81.09

Isolate	Total reads (bp)	GC content	Total contigs	Largest contig length (bp)	N50	Genome coverage	Kraken (%)
js73	867790	29.8	74	676289	210585	54x	81.36
js74	396842	29.8	161	126510	41198	30x	85.95
js75	456774	30.2	196	79848	28020	30x	90.09
js77	302770	29.7	157	130356	39261	22x	92.61
js78	763866	29.6	63	612909	364903	56x	91.79
js79	1073338	29.1	67	792347	507727	69x	81.7
js80	747816	29.9	63	696639	434402	51x	90.02
js81	379780	31.8	585	38033	9719	28x	84.09
js82	309372	31.2	648	33474	7975	23x	85.12
js83	493586	29.1	41	622043	439225	37x	92.77
js85	289066	30.5	307	52409	17665	22x	93.00
js86	1356284	29.5	43	1080869	577440	87x	89.37
js87	457888	30.5	114	153392	55710	35x	93.42
js88	380870	30.3	258	71169	24440	28x	84.98
js90	420878	30.7	208	164859	28855	31x	88.01
js91	309742	29.1	76	261471	77214	23x	92.06
js92	581522	29.8	103	421325	117764	44x	86.41
js93	394280	29.8	137	141341	59374	29x	85.38
js94	655276	29.9	119	373705	179869	48x	88.31
js96	407234	30.1	262	127328	57254	31x	86.22
js97	996098	29.8	54	448580	263047	72x	87.34
js99	311002	29.4	119	132722	55701	23x	88.41

3.3.2 *In silico* MLST

In silico, MLST identified nine STs (Table 3.3.2.1). Fifteen isolates could not be typed and may represent novel STs. The mean depth of coverage across all MLST genes for all isolates was above the cut-off of 20-folds, ranging between ~20-fold (js02) and ~227-fold (js12). The depth of coverage for untypable isolates ranged from 25.9 to 156.8 with the median coverage being 48.63. It is likely that the untypable isolates are novel ST with novel alleles of MLST genes. All untypable alleles had a coverage above 20. The coverage of each gene was above 20x for all isolates except for *gdh* in js91 which had a coverage of 16x. These fold coverages are above the cut-off and may imply that these isolates represent new STs, and, given that there are only 675 isolates on the pubMLST database it is unlikely that the total diversity of *B. hyodysenteriae* has been captured in the current database. The fasta sequences of MLST genes for all untypable genes were submitted for review to Tom La, the curator of the *B. hyodysenteriae* PubMLST database (https://pubmlst.org/brachyspira/), and all but js05 were typed. This was because There were two genes in js05 that were too short: *glpK* (missed the first two nucleotides) and *thi* (the gene ended three nucleotides too early). These genes may be shorter in js05, but this could also be an error in the sequencing. To investigate further, it would be necessary to conduct MLST using PCR amplification of the MLST genes and sequencing of these genes [125].

There was considerable allelic variation between the MLST genes. For example, with *adh* allele 2 present in all isolates; while *glpK* contributed the most to the variation between STs. The most common ST was 52 and was present in 23 isolates in which were from seven regions with 18 isolates originating from the South West. These isolates arose from 22 holdings, and were isolated from diagnostic submissions made in 2006 to 2015. The least common STs were 89 (this contained js26, an isolate from Yorkshire and the Humber), and the new STs 242, 243, 244 and 245 (which originated from Yorkshire and the Humber, non-UK, South West and the South East respectively). There were regional differences, with four of the ST 88 isolates being found in Yorkshire, and eight of ST 91 isolates were identified in the South West. These findings could reflect the pig transportation system in the UK as pigs are more likely to be transported a short distance within regions than longer

distances between regions. ST 52 appears to have been able to spread very successfully, and the large number of isolates present in the South West may suggest a clonal expansion caused by a series of biosecurity breakdowns.

Comparison of STs from this study to previously published STs on PubMLST (http://pubmlst.org/bhyodysenteriae/) indicated that a total of five UK STs had previously been found in other European countries, and four STs had previously only been identified in the UK (Table 3.3.2.2). The most common STs from this study, ST 52 and ST 8, were both present in multiple European countries. ST 8 occurred between 2004 and 2015; while ST 52 occurred between 2006 and 2015. Two UK specific STs were also common: ST 88 (eight isolates from Yorkshire and the Humber, the West Midlands and the South West), and ST 91 (10 isolates from the South West and the West Midlands). These STs were present in the latter years of the study. The latest ST 91 isolate (js59) was in 2011, and the latest ST 88 isolate (js27) was in 2013; while ST 8 (js97 and js99), and ST 52 (js74) were both present in 2015. Multiple STs are able to survive in the UK at the same time, with no single ST able to dominate completely. This may be due to the survival of isolates with these STs in regions where they have been isolated in the environment and/or other vectors where they do not have to compete with isolates of different STs.

Of the holdings that had multiple isolates, MLST identified occurrences of potential chronic contamination by *B. hyodysenteriae* at some of these locations. There were two holdings that possibly had persistent infections. Analysis of holding 3, with a potential persistent infection, indicated that the isolates came from different NUTS 1 regions. It is likely that this is a company with multiple sites which had sent multiple samples to the APHA under one submission. As these isolates could have come from farms in different regions, they will be treated as independent infections. On holding four there was a succession of STs from ST8 to untypable, this is likely due to disease outbreaks caused by independent infections. In the other holdings with repeated outbreaks, the ST was the same in subsequent isolates and may indicate persistently contaminated holdings. Also, there were two holdings (71 and 72) where multiple STs were identified within three months of each other; both STs are likely to be co-infecting the pigs.

Table 3.3.2.1: MLST profiles of all isolates.

Isolates that could not be typed were indicated by NF, *indicates mismatches and a question mark denotes uncertainty due to low coverage in parts of the MLST genes. Information on SNPs and low coverage have been included in Appendix 1.

Isolate	ST	*adh*	*alp*	*est*	*gdh*	*glpK*	*pgm*	*thi*	Depth (x coverage)
js01	52	2	13	3	6	8	2	17	35.73
js02	122	2	11	3	5	9	2	28	20.43
js03	240	2	11	3	6	43	26	43	156.8
js04	52	2	13	3	6	8	2	17	77.4
js05	NF*?	2	11	28	1	15*?	2	19*?	127.37
js06	239	2	11	33	10	7	3	3	136.50
js07	8	2	2	3	12	11	1	3	153.65
js08	122	2	11	3	5	9	2	28	63.61
js09	52	2	13	3	6	8	2	17	186.55
js11	87	2	13	3	6	23	2	21	152.88
js12	8	2	2	3	12	11	1	3	227.11
js13	240	2	11	3	6	43	26	43	55.47
js14	122	2	11	3	5	9	2	28	38.66
js15	242	2	11	33	10	43	3	3	34.82
js16	122	2	11	3	5	9	2	28	32.12
js17	239	2	11	33	10	7	3	3	58.55
js18	167	2	11	3	1	10	2	21	46.11
js19	239	2	11	33	10	7	3	3	37.48
js20	239	2	11	33	10	7	3	3	46.62
js21	87	2	13	3	6	23	2	21	73.22
js22	239	2	11	33	10	7	3	3	48.15
js23	87	2	13	3	6	23	2	21	40.66
js24	87	2	13	3	6	23	2	21	22.59
js25	8	2	2	3	12	11	1	3	88.94
js26	89	2	21	3	20	6	1	11	50.76
js27	88	2	21	3	20	6	11	11	27.7
js28	88	2	21	3	20	6	11	11	31.22
js29	88	2	21	3	20	6	11	11	75.88
js30	240	2	11	3	6	43	26	43	42.66
js31	52	2	13	3	6	8	2	17	48.08

Isolate	ST	*adh*	*alp*	*est*	*gdh*	*glpK*	*pgm*	*thi*	Depth (x coverage)
js32	88	2	21	3	20	6	11	11	43.85
js33	240	2	11	3	6	43	26	43	37.02
js34	8*	2	2*	3	12	11	1	3	104.04
js35	122	2	11	3	5	9	2	28	39.33
js37	52	2	13	3	6	8	2	17	48.69
js39	52	2	13	3	6	8	2	17	78.98
js41	8	2	2	3	12	11	1	3	77.2
js42	52	2	13	3	6	8	2	17	65.84
js43	8	2	2	3	12	11	1	3	30.02
js44	8	2	2	3	12	11	1	3	25.69
js47	239	2	11	33	10	7	3	3	79.16
js48	167?	2	11	3	1?	10	2	21	32.21
js49	167	2	11	3	1	10	2	21	50.09
js50	167	2	11	3	1	10	2	21	100.01
js51	243	2	11	3	1	9	2	13	64.64
js52	90	2	11	8	1	8	2	13	36.48
js57	88	2	21	3	20	6	11	11	21.28
js59	91?	2	22	5	4?	24	2	6	34
js60	52	2	13	3	6	8	2	17	21.75
js61	122?	2	11	3	5?	9	2	28	34.52
js62	88	2	21	3	20	6	11	11	34.22
js63	8	2	2	3	12	11	1	3	47.3
js64	91	2	22	5	4	24	2	6	58.86
js65	8	2	2	3	12	11	1	3	39.3
js66	88	2	21	3	20	6	11	11	98.38
js68	52	2	13	3	6	8	2	17	77.85
js69	52	2	13	3	6	8	2	17	42.52
js70	52	2	13	3	6	8	2	17	162.67
js71	52	2	13	3	6	8	2	17	90.13
js72	52	2	13	3	6	8	2	17	49.21
js73	52	2	13	3	6	8	2	17	78.24
js74	52	2	13	3	6	8	2	17	46.52
js75	244	2	7	3	6	24	2	3	49.11
js77	91	2	22	5	4	24	2	6	32.61
js78	91	2	22	5	4	24	2	6	70.21
js79	52	2	13	3	6	8	2	17	94.4
js80	91	2	22	5	4	24	2	6	91.55

Isolate	ST	*adh*	*alp*	*est*	*gdh*	*glpK*	*pgm*	*thi*	Depth (x coverage)
js81	52	2	13	3	6	8	2	17	52.62
js82	52	2	13	3	6	8	2	17	38.77
js83	91	2	22	5	4	24	2	6	44.99
js85	91	2	22	5	4	24	2	6	35.78
js86	91	2	22	5	4	24	2	6	136.51
js87	91	2	22	5	4	24	2	6	48.51
js88	52	2	13	3	6	8	2	17	45.99
js90	8	2	2	3	12	11	1	3	52.16
js91	245	2	31	3	20	4	2	45	25.9
js92	52	2	13	3	6	8	2	17	66.11
js93	52	2	13	3	6	8	2	17	25.5
js94	8	2	2	3	12	11	1	3	73.76
js96	52	2	13	3	6	8	2	17	44.59
js97	8	2	2	3	12	11	1	3	101.73
js99	8	2	2	3	12	11	1	3	25.59

Table 3.3.2.2: Sequence types of UK isolates and comparison to global isolates. ST profiles of isolates from this study compared to global isolates from pubMLST (https://pubmlst.org/brachyspira/) and previously published studies [73, 121]. Untypable refers to isolates that could not be typed.

ST	Isolates from this study	Source of global isolates from the same ST
8	js07, js12, js25, js34, js41, js43, js44, js63, js65, js90, js94, js97, js99	Belgium, UK, Germany, Italy, Serbia, Spain
52	js01, js04, js09, js31, js37, js39, js42, js46, js60, js68, js69, js70, js71, js72, js73, js74, js76, js79, js81, js82, js88, js92, js93, js96	Austria, Belgium, Germany, Italy, Spain
87	js11, js21, js23, js24	Italy, Belgium
88	js27, js28, js29, js32, js57, js62, js66	UK
89	js26	UK
90	js50, js52	UK
91	js59, js64, js77, js78, js80, js83, js85, js86, js87	UK
122	js02, js08, js14, js16, js35, js61	Germany
167	js18, js48, js49	Belgium
239	js06, js17, js19, js20, js22, js47	UK
240	js03, js13, js30, js33	UK
242	js15	New
243	js51	New
244	js75	New
245	js91	New
Untypable	js05	N/A

MLST alleles were used to build a minimum spanning tree using Bionumerics 6 (Figure 3.3.2.) [265]. In general, different STs were identified in different continents, with only three STs present in more than one continent. ST 104 was isolated in the USA and Spain; ST 54 has been identified in the USA and Germany, and ST4 was isolated in Canada and the UK. The lack of shared STs between distant countries may indicate that *B. hyodysenteriae* STs are diverging rapidly, but there are STs that are differentiated by only a few SNPs. This suggests that although there is considerable divergence within the population *B. hyodysenteriae* many STs in

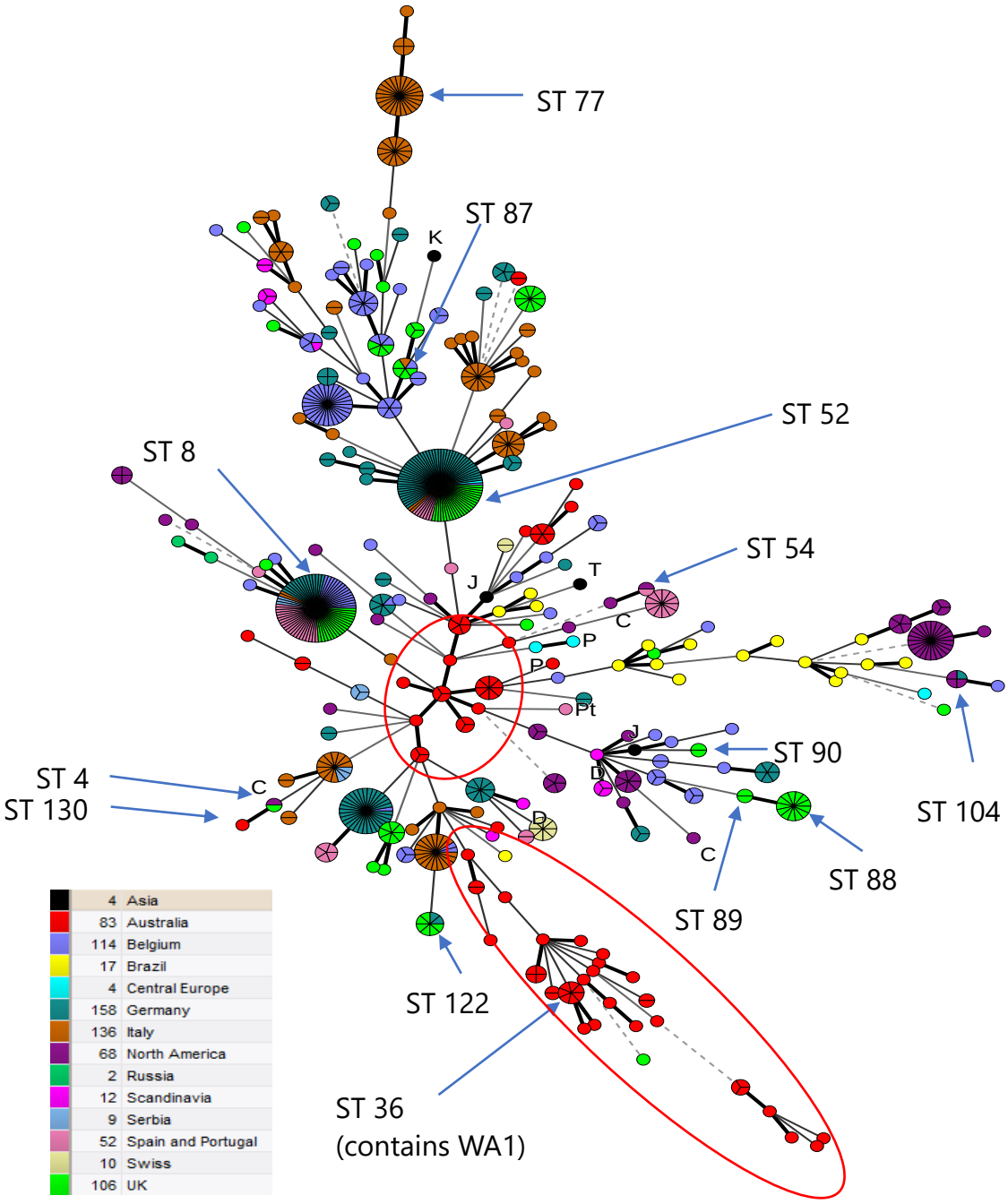
different countries are closely related, and there appears to be a clear structure to the tree. Therefore *B. hyodysenteriae* is not completely recombinant and is likely to be partly clonal.

There are limits to what can be discerned about the global population structure of *B. hyodysenteriae* due to the biased nature of the pubMLST database. Important pig production regions, most specifically China, are absent. However, as much of the tree is composed of Australian, European or American isolates, it is possible to infer details about the population structure of *B. hyodysenteriae* in these regions. Each region appears to have distinct patterns of STs. In Australia, there were two main groups of ST: a group at the centre of the tree and a distinct branch. Some Australian STs were similar to global STs; while another group became divergent and is evolving independently. This was likely due to the isolation of *B. hyodysenteriae* in Australia, although the majority of Brazilian and North American STs are also on a distinct branch. Some STs were dispersed with European STs and dispersed throughout the tree. This potentially indicates greater movement of *B. hyodysenteriae* between these continents. However, the movement of *B. hyodysenteriae* between continents does appear limited.

Within Europe, there were similar STs found in different countries. In total ten STs were shared between different countries in Europe, but only two STs were present in more than two countries: ST 8 and 52. This suggests the movement of *B. hyodysenteriae* across Europe is common; although the relative isolation of Italian STs does indicate that it does not apply to all European countries. These results will have implications for treatment as it is possible that a multi-drug resistant ST could be transported throughout Europe. It should also be noted that the reference strain WA1 (ST 32) does appear divergent from European isolates and therefore for future analysis of *B. hyodysenteriae* it would be beneficial to sequence a new European reference strain.

Figure 3.3.2: Minimum Spanning Tree of all MLST isolate on pubMLST with (http://pubmlst.org/bhyodysenteriae/) and *in silico* MLST isolates from this study.

STs are labelled if present in this study or are mentioned in the results or discussion. C: Canadian isolates (all other North American isolates are from the USA), T: Thailand, J: Japan, K: Korea, Pt: Portugal, D: Denmark (all other Scandinavian isolates are from Sweden), P: Poland (all other Central European isolates are Austrian). The number of isolates from each country, or region, is also given. 1000 bootstraps were conducted. Circles highlight the two Australian clusters.



3.3.3 Core-genome SNP tree

An alignment of SNPs in the core-genome was constructed and filtered using Gubbins to remove recombinant SNPs; this was used to construct a phylogenetic tree (Figure 3.3.3.). There were 116649 SNPs from 1337 genes (53.16% of the genome), and the average number of SNPs per genome was 1090. When compared to MLST there were three discrepancies; ST 88 and ST 89 isolates form the same clade, the Australian isolate, WA100, was very similar to a Canadian isolate, FMV88.9330, and one ST 240 isolate (js13) clustered with ST 239 isolates. The ST for WA100 and FMV88.9330 is not provided on NCBI, but there are isolates on PubMLST with the same name, and these are likely to be the same strains. Comparison of STs indicated that WA100 (ST 130) and FMV88.9330 (ST 4) were separated by nine SNPs in *thi*; while ST 88 and ST 89 were separated by two SNPs in *pgm*. ST 239 and 240 share 6 alleles, and are separated by 6 SNPs in *glpK*. These discrepancies are likely due to greater resolution of core-genome analysis and highlights the potential of WGS to provide additional, more in-depth information on the population structure of *B. hyodysenteriae*.

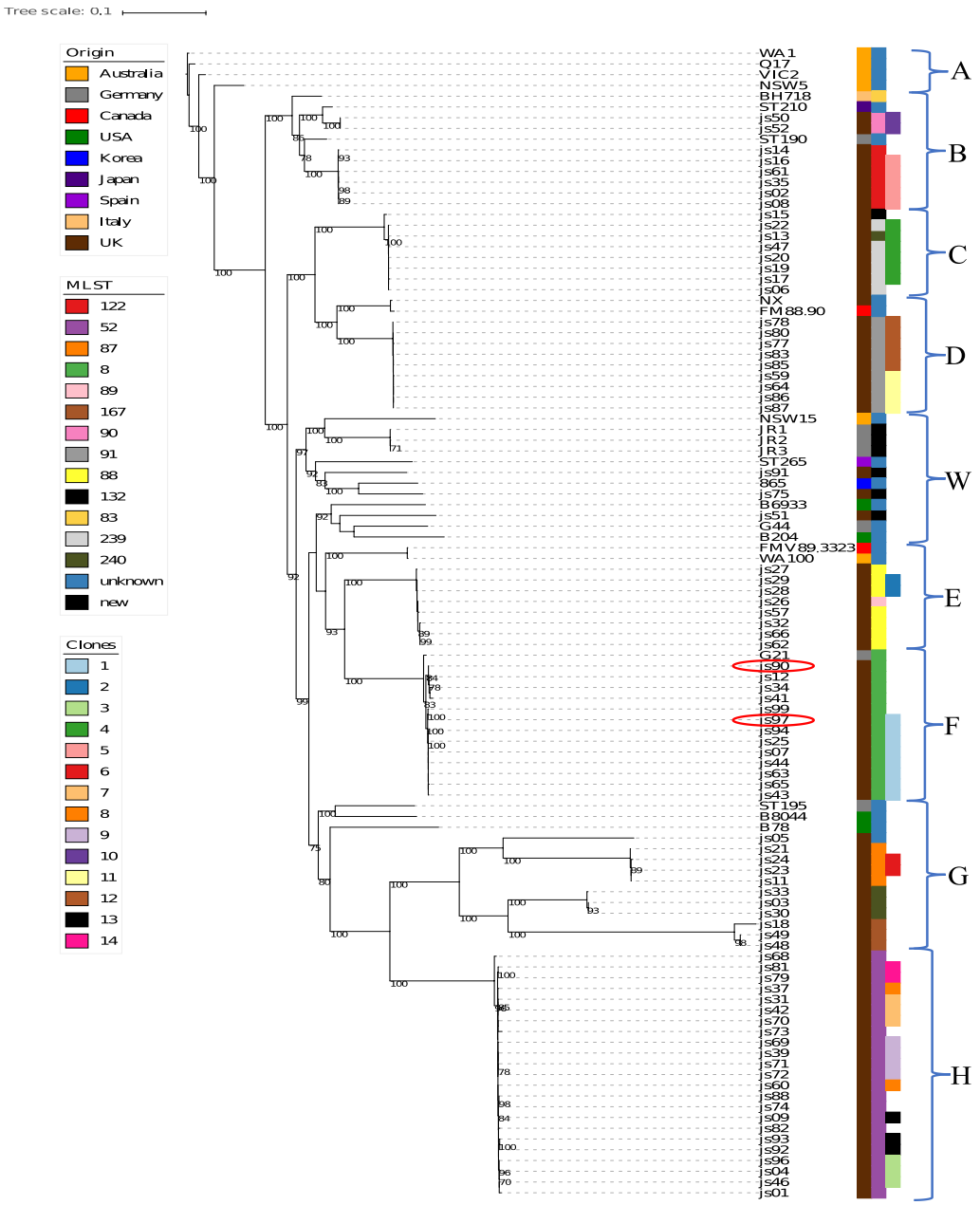
The core-genome tree is dominated by UK isolates as there are only 27 *B. hyodysenteriae* genomes published; despite this, it does appear that the phylogeny was conserved. As in the MLST tree, there was a distinct group of Australian isolates, clade A, and some isolates were present in clades D, and E. Global isolates appeared distinct from UK isolates and did not form a defined cluster. These isolates have long branches indicating greater diversity here, which will become apparent as more global isolates are sequenced. There was only one global isolate, the German isolate G21, that clustered with UK isolates. There does not appear to be a single dominant clonal group in the UK. Instead, there were multiple clades present in the UK; with each clade or subclade aligned with a ST. Each clade contained isolates from multiple regions and holdings. However, smaller clades contained isolates that were recovered closely together in time. The larger clades contained isolates that were isolated over a longer period of time, for example, the ST 8 clade includes js90, from South East England which was isolated in 2004 and js97 from the East of England which was isolated in 2015. The existence of clades that have been

established for years could suggest that there is a reservoir population circulating within the UK, or it may also be due to unintentional transport between holdings.

Within each clade, there were groups if isolates with a small number of SNPs (<20) which could indicate clonal expansion, in total there were 13 groups of isolates with fewer than 20 SNPs between group members. However, as Gubbins removes SNPs that are present in recombinant sites, there may have been an overestimation of the number of clones [245]. Clonal groups were most common in clade H; this corresponds to ST 52. Clonal groups ranged in size from two to eight isolates with the mean clonal group being four isolates. Clonal groups were composed of isolates from different regions, for example, clonal group 1 (js07, js25, js43, js44, js63, js65, js94 and js97) was composed of isolates from the East of England, the South East, the West Midlands and Yorkshire and the Humber. In total 51 isolates comprised 14 clonal groups. It is likely that clonal expansion is an important mechanism for the spread of *B. hyodysenteriae* and that an outbreak in one holding could lead to the rapid spread of swine dysentery to other holdings. Many of these holdings are geographically distant from each other; so, the spread would likely have been facilitated by pig transport trucks or migrating birds.

Figure 3.3.3: Maximum likelihood tree of *B. hyodysenteriae* isolates based on SNP in the core-genome.

Global isolates published on Genbank have been included. STs have been included for isolates of this study. 1000 bootstraps were conducted, and bootstrap values above 70 are shown. Untypable refers to isolates that could not be typed, new refers to STs identified in this study. js90 and js97 have been highlighted in red. Scale bar represents 10 nucleotide substitutions per 100 nucleotides. The global isolates, and js71 and js51, have been shown with a W. Unknown includes global isolates where the MLST was not specified or determined by clustering with UK isolates.



3.3.4 Transmission analysis of *B. hyodysenteriae* in England and Wales

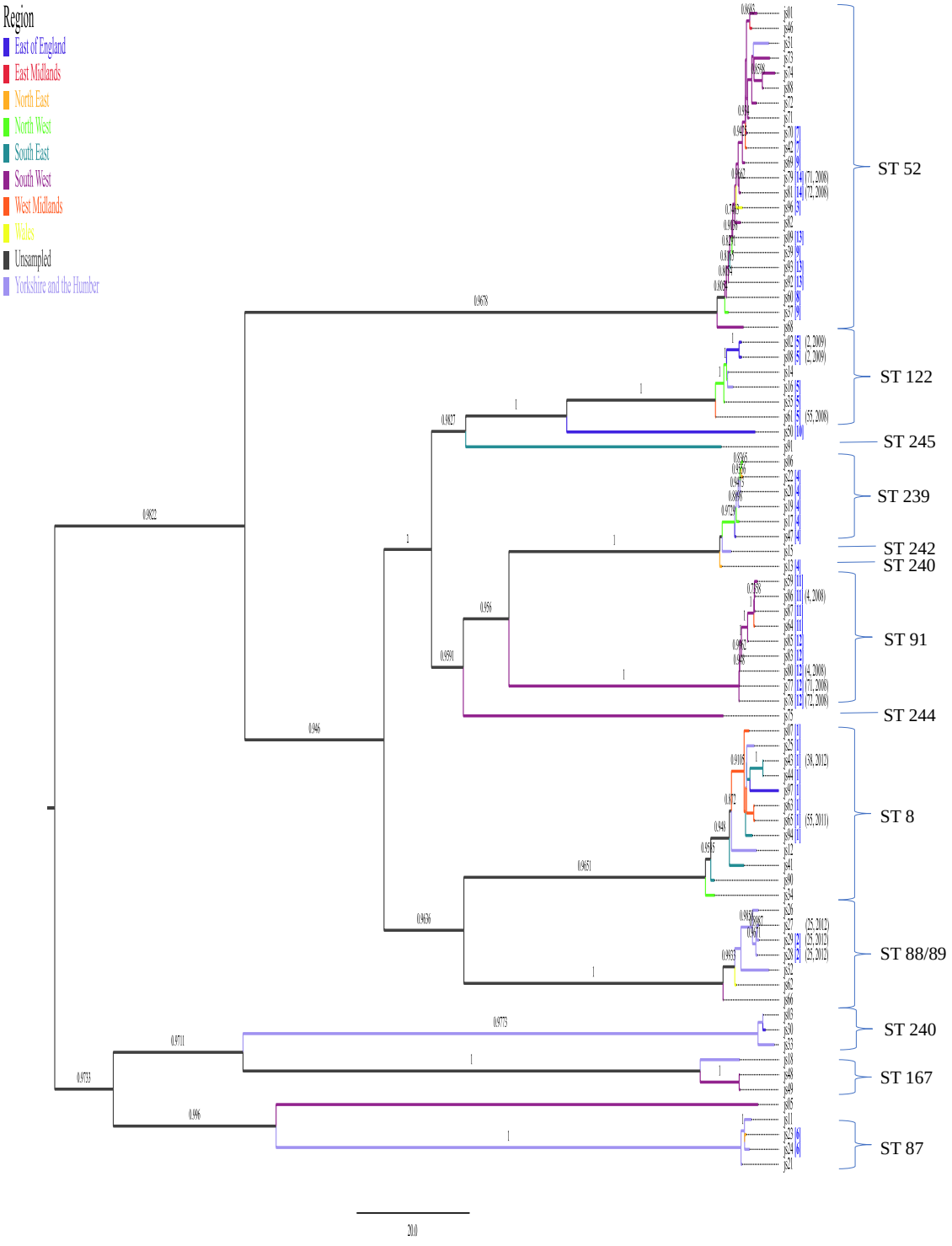
As well as determining the population structure in the UK, transmission events between regions of the UK were predicted. This was done using metadata available for all isolates in combination with the WGS data. In total 79 isolates were used for transmission analysis; the other isolates lacked information on the holding of origin. Like the core-genome tree, SNPs were filtered by Gubbins before being used to construct a phylogenetic tree. However, js90 was used as the reference instead of WA1 as the date of isolation is not known for WA1, and, the aim was to investigate transmission events in the UK alone. The tree contained 62,738 SNPs, and the average number of SNP per isolate was 794 (Figure 3.3.4). Clustering was slightly different to the core-genome tree as transmission between regions over time is predicted. The use of a different reference may also have had an effect. There were two clusters; one cluster composed of ST 167, ST 87, most of ST 240 and js05 isolates, with all other isolates being in the second cluster.

Large amounts of the tree were predicted to be unsampled as the oldest sample used in the study was from 2004; therefore, anything before this date will be classified as unsampled. There does appear to be differences between clades found in different regions. The tree was dominated by the South West, where the majority of isolates come from a small number of clades, principally, STs 52 and 91. These were present for a protracted length of time (nine years for ST 52 isolates and three years for ST 91 isolates). ST 52 was composed of multiple clonal populations; suggesting the expansion of ST 52 being due to multiple outbreaks on holdings, followed by the spread to multiple holdings. In Yorkshire and the Humber, there were seven STs present, but these were detected for a shorter period, for example, ST 88 was only present in Yorkshire and the Humber between 2012 and 2013. Clades appeared to spread within a region more than between regions. This may implicate local transport as an important factor. Also, the clades with few isolates that occurred in Yorkshire and the Humber, and the lack of isolates from the East of England, may suggest that *B. hyodysenteriae* spread was more inhibited in these regions. This could reflect bias in the APHA collection, but it could also suggest that in these regions there were fewer mechanisms available for the spread of *B. hyodysenteriae* between holdings. In part, this could be due to persistently infected holdings acting

as reservoirs. It appears that *B. hyodysenteriae* spreads more readily within some regions than in others, and the spread may be increased by the presence of persistently infected holdings.

Figure 3.3.4 Transmission event predicted by SCOTTI.

Holdings with multiple isolates have been indicated in brackets with the year of isolation). Clones are indicated in square brackets in blue. Note there is only one isolate of clonal groups 7 and 10 as the other isolates did not have sufficient metadata. ST are indicated next to the tree. Scale bar represents 20 years. Abbreviations: Yorkshire refers to Yorkshire and the Humber.



3.4 Discussion

In this study, the aim was to characterise the population structure of *B. hyodysenteriae* in the UK with the aim of identifying clonal groups across pig holdings in England and isolates or STs which may be persistent. It was possible to identify the population structure by MLST and core-genome SNP based analysis, and generally there was an agreement between MLST and SNP based core-genome alignment. A prediction of transmission between regions was also intended and was possible for all isolates where metadata was available. However, it must be noted that submission of isolates to the APHA is voluntary; therefore there will be biases in the data/isolate collection, so caution is needed when drawing conclusions. Also, most of the samples originated were in England, as this is the main pig producing country in the UK; but the lack of samples from other Scotland and Wales means conclusions on the population structure in Scotland and Wales cannot be made [269]. However, there were trends that were of interest and likely reflect important factors affecting the population structure of *B. hyodysenteriae*.

Analysis of *in silico* MLST suggested that *B. hyodysenteriae* is potentially recombinant. Recombination of *B. hyodysenteriae* STs has been seen previously, and analysis of historic European isolates from the 1970s did not identify current common STs such as 8 and 52 [125]. A similar situation has been found in the USA and Australia where there has been divergence of current STs from historic STs. The nature of *B. hyodysenteriae*, whether it is clonal or recombinant, has been debated previously. MLEE analysis indicated *B. hyodysenteriae* was recombinant [117]. However, subsequent analysis by MLST and WGS indicated *B. hyodysenteriae* was clonal [121, 125]. From this work, there is evidence that *B. hyodysenteriae* can be clonal, although it appears to mutate relatively quickly. Therefore, divergence of STs is likely to continue, and likely to lead to a reduction in the current dominance by ST 52.

It is unlikely that any ST will develop a dominant position globally due to limited movement of pigs between continents. This can be seen in the minimum spanning tree (Figure 3.3.1) where many Australian isolates appear distinct, with no ST shared with other countries. This may be due to a ban on pigs being imported into Australia

since the 1980s, and therefore the *B. hyodysenteriae* in Australia has been able to evolve in isolation [123]. A similar situation was seen with most of the Brazilian and North American isolates, which were distinct, with few STs shared with other countries. Again, this most likely reflects the regional isolation and the limited number of pigs transported between America, Oceania and Europe [253].

There were five STs identified in this study that were common in the rest of Europe, chiefly ST 8 and ST 52 [60, 73, 93, 95, 121]. No single ST was completely dominant in the UK; ST 52 was the most common, but ST 8 and 91 were also common. A similar situation has been seen in other phylogenetic studies of *B. hyodysenteriae*, with multiple STs found [52, 60, 73, 93, 121]. The most common STs for the USA (ST 93) and Australia (ST 50, 140 and 150) appeared unique to that continent [52, 123]. However, in some European countries, the dominant ST had a wide distribution. For example, Germany had similar dominant STs to the UK, with ST 52 dominating and ST 8 also being common [73]. ST 8 was also the dominant ST in Spain, and both ST 8 and ST 52 have been found in other European countries including Belgium and Italy [60, 124]. However, not all common STs have been found throughout Europe; in Germany, 25.9% of isolates were ST 122, a German specific ST [73]. In Spain ST 71 was also common, and in Italy, the dominant ST (ST 77) was unique to Italy [73, 121, 122]. In Europe there appeared to be a mix of country-specific STs and STs that have developed a more widespread distribution. As both local and more widespread STs were more common it is unlikely that one ST is significantly fitter than other STs, rather spread of STs may be due to the chance spread of certain STs by a variety of vectors, for example, animal species or biosecurity breakdowns.

The production of pigs is an intensive industry and transport of pigs to slaughter and between farms is a key part of this industry. The transport of pigs between countries could contribute to the circulation of STs. In the EU there is a large amount of movement of pigs between member states. In 2016 9,944,000 pigs were traded for slaughter, 22,451,000 pigs were traded for production and 1,030,000 pigs were traded for breeding [270]. Potentially trucks that have transported *B. hyodysenteriae* positive pigs and have not been adequately cleaned and disinfected could be a source of contamination and a reservoir for *B. hyodysenteriae*. *B. hyodysenteriae* can remain

viable after decontamination, with the risk of *B. hyodysenteriae* survival increasing with increasing numbers of times pigs were transported by a specific truck [128]. Given the large volume of pigs transported across Europe, it is likely that some trucks will be used for multiple trips and could be a vector for *B. hyodysenteriae.*

Also, wild animals could also influence the spread of STs. Migrating birds could spread *B. hyodysenteriae* as they have a wide distribution and migrate across multiple countries [66, 67, 271]. The bird species most often associated with *B. hyodysenteriae* is mallard ducks (*Anas platyrhynchos*) [66, 67]. Reports of ringed mallards recovered by EURING (a European organisation monitoring bird movement by attaching small rings to birds), indicated a high concentration of mallard ducks on both sides of the English Channel between April and August [271]. Mallard ducks migrating across the channel could bring *B. hyodysenteriae* strains with them. Also weakly haemolytic *Brachyspira* species have been identified in a range of other wild birds including Coots (*Rallidae fulica*) [272]. These also have a migratory path that would result in them migrating from the UK to continental Europe and vice-versa [271]. After carriage by birds, *B. hyodysenteriae* could colonise rodents through contact with infected faeces; rodents could act as a reservoir enabling persistence and transmission of *B. hyodysenteriae* in the local area, including pig farms [68, 273]. Our MLST data suggested *B. hyodysenteriae* movement throughout Europe, with some STs dominant in multiple countries. However, the addition of new STs to a region via transport systems or animal vectors is likely to lead to a change in the dominant STs over time. To further analyse the population structure of *B. hyodysenteriae* it is necessary to investigate variation within STs; in this study, this was done using WGS.

The phylogenetic tree produced was dominated by UK isolates due to the small number of isolates currently publically available through Genbank [274]. There are small differences between MLST and core-genome analysis, with some isolates appearing more closely related than would have been suggested by their STs. Which included close similarity between two global isolates WA100 and FMV88.9330; this has been found previously by comparison of the *che* gene [192]. It is possible that some clades may be present globally, but this is unlikely to be common.

Like MLST data most global isolates appeared distinct from the UK isolates. This is consistent with results from MLST studies where the US and Australian isolates appeared different from European isolates [52, 125]. Most of the European isolates do not cluster with UK isolates. This may reflect local dominance by *B. hyodysenteriae* clades that have not spread, for example, ST 77 in Italy [122]. Although the ST of the Genbank isolate, G21 is not stated it is likely it is the ST 8 G21 isolate on pubMLST (http://pubmlst.org/bhyodysenteriae/). This is a ST 8 isolate from Germany, which clustered closely with the UK isolates from this panel. G21 appeared on a different branch to UK ST 8 isolates, suggesting some divergence between ST 8 isolates in the UK and STs from continental Europe. It appeared to have been isolated in Hesse in 2004 [275]. Given that there are several STs that are shared among European countries, it is likely that as more *B. hyodysenteriae* isolates are sequenced there will be greater clustering of UK and European isolates, in particular isolates that belong to the ST 8 and ST 52 groups.

WGS enabled analysis of variation within STs. This led to the identification of 13 clonal groups which aligned with different STs. These were composed of isolates from different regions. This could reflect the spread of *B. hyodysenteriae* from an initial biosecurity failure via pig transport trucks or animal vectors. This spread could occur before a diagnosis of swine dysentery has been made, due to the slow diagnostic time. Potential clonal expansion of *B. hyodysenteriae* has been seen in Sweden where an outbreak of swine dysentery on one farm led to the spread of swine dysentery to four other farms [276]. Three farms had purchased live pigs, and transport trucks were implicated in the spread to the fourth farm [276]. It is likely that there are multiple mechanisms behind the clonal expansion of *B. hyodysenteriae*, prediction of transmission events may help indicate potentially important mechanisms of dissemination of *B. hyodysenteriae*.

Transmission prediction was performed using SCOTTI in the BEAST 2 environment. Although BEAST 2 is a useful tool there are limitations; these relate to the timescale prediction of BEAST2 where it is possible to generate significantly different timescales using the same dataset [260, 277]. Moreover, important inputs such as mutation rates are not known for *B.* hyodysenteriae. Therefore, the timescale generated by SCOTTI must be treated with caution. However, the aim of using

SCOTTI was not to predict a timescale of *B. hyodysenteriae* infections in the UK, but rather the focus was on predicting potential transmission of *B. hyodysenteriae* between regions of the UK. This has not been done previously for *B. hyodysenteriae* and could provide useful information about dissemination of *B. hyodysenteriae* in pig herds in the UK.

The tree of transmission events predicted by SCOTTI suggests that the population structure appears to differ between regions of the UK. The South West appeared to have a reasonably stable population composed of ST 52 and ST 91. It also appeared to be the primary location of ST 52. The situation was different in Yorkshire and the Humber, with many clades present within Yorkshire and the Humber in the timeframe covered by this study. This is one of the main pig producing regions of the UK and the other main pig producing region of the UK is the East of England which also had a diverse range of isolates present [278]. It is likely that a greater number of clades can survive as they are present in more reservoirs. These reservoirs are likely to include wild animals, trucks and may also include persistently infected farms. The variation between regions may also reflect structural differences between the pig industry in different regions of the UK.

Pig production in the UK is concentrated in a few areas (Introduction, section 1.1.2). With larger producers in the East of England, followed by Yorkshire and the Humber; while a large number of small producers are located in the South West (Figure 3.4.1) [21, 22]. The East of England is primarily dominated by large companies, with 72.1% of producers being classed as large companies (companies with >50 farms). This size has an impact on the way pigs are transported. Large companies have integrated systems where 89% of the movements of pigs happens within their company, i.e. pigs are moved from one company site to another company site, compared to 75.6% and 5.3% of pig movement for medium and small companies, respectively [21]. Small companies also use fewer hauliers and abattoirs than other producers, with medium companies using a greater range of hauliers and abattoirs than large companies, and small companies using the greatest range [21]. In a region dominated by large companies, there could be fewer biosecurity vulnerabilities, and it will be easier to coordinate an eradication program, such as the program that occurred in the East of England in 2009 [279]. This may be a

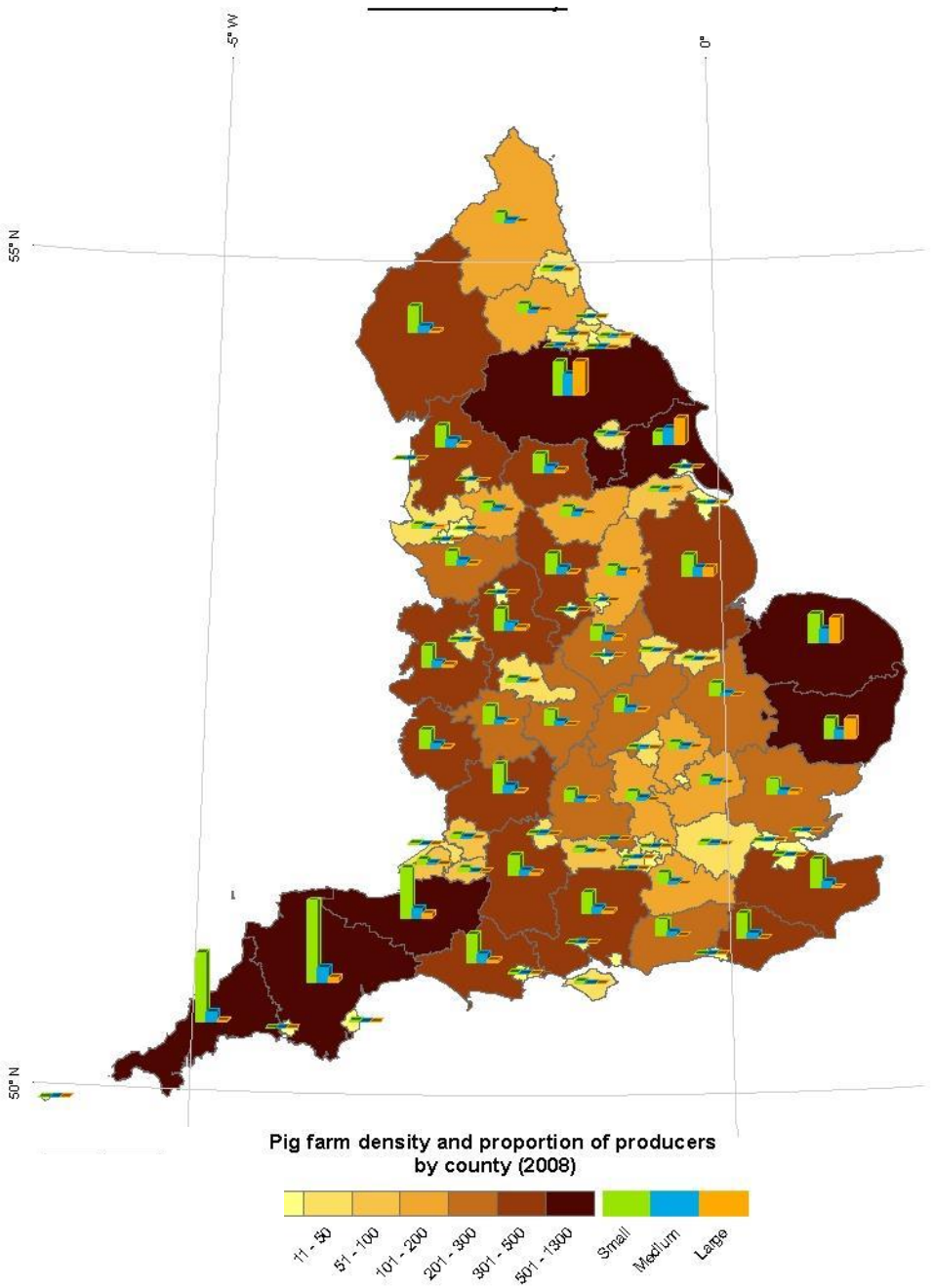
contributing factor to the relatively low number of *B. hyodysenteriae* isolates obtained in the East of England.

A slightly different situation is seen in Yorkshire and the Humber. Of the pig producing companies in Yorkshire and the Humber, 20.7% were large companies, 24.3% were medium companies (companies with 6 to 50 farms), but, crucially 55% of farms were small producers [21]. These small farmers mostly transport pigs outside of the company and rely on a greater number of hauliers [21]. These introduce biosecurity factors outside of the control of the small farmer and may result in biosecurity breakdowns. In addition, there were multiple trade communities present within Yorkshire and the Humber that were connected to holdings in other regions [280]. This could result in the introduction of ST from other regions into Yorkshire.

The South West has the highest number of small companies, and, appears to be somewhat isolated from other regions and appears to form different trade communities [21, 280]. This may be due to the limited distance pigs are typically transported, the average distance pigs are transported in the South West is relatively small at 30 to 31 km, and only 14% of movements were over 100 km [21, 269]. This has continued in recent years with 95% of movements less than 65 km [280]. The spread of ST in the South West could be facilitated by multiple independent hauliers transporting pigs for multiple companies. This could result in the spread of a novel ST, such as ST 52, through the South West.

Figure 3.4.1: A map of the density of pig farms and the proportion of producers of different types in each county in England.

Modified from figure produced by D. Marques and the Scottish agricultural college, 2008 [269]. Large producers: >1000 pigs moved per year, medium: 1000 – 35 and small: <35 pigs moved [269].



This was one of the first studies to investigate the population structure of *B. hyodysenteriae* in the UK. MLST analysis indicated that there were some pan-European STs that have a significant presence in the UK. It was also evident that there were regional differences between regions of the UK. This could be impacted by the biased nature of the sample, but it may also reflect regional differences in pig production in the UK. It is notable that the regions with larger and more integrated systems had less *B. hyodysenteriae*. The pig industry is currently becoming more industrialised with fewer, larger companies; this could result in better control of *B. hyodysenteriae* in the future [13].

Chapter 4. Antibiotic Resistance in *B. hyodysenteriae*

4.1 Introduction

Antibiotic resistance is a major problem for both human and animal health. With the development of multi-drug resistant pathogens, there are fewer antibiotics available to treat important pathogens [281]. Antibiotics are essential for the treatment of veterinary pathogens including SD. In the EU the number of antibiotics that can be used to treat swine dysentery is becoming more restricted, in part due to the development of resistance to antibiotics such as tylosin [282]. Currently tiamulin, valnemulin and tylvalosin are recommended for use [131-133]. However, in the UK if no authorised antibiotics are suitable for a specific case, other antibiotics can be used as part of the cascade system [283]. The pleuromutilin antibiotics have been classified by the World Organisation for Animal Health as critically important for the treatment of swine dysentery and for respiratory infections in pigs and poultry [284].

The level of resistance to pleuromutilins has been investigated in some of the main pig producing countries, including the USA, Germany, Spain, Brazil, and smaller producers such as Japan and five other European countries [73, 109, 122, 167, 285-290]. [115]. Both a wild-type breakpoint and a clinical resistance breakpoint have been suggested for tiamulin for both agar dilution and broth dilution MIC methods (Table 1.2.5.1) [109, 110]. Analysis of pleuromutilin resistance does indicate tiamulin resistant isolates will also have decreased sensitivity to valnemulin [122, 160, 288, 289], and a wild-type breakpoint has been suggested for valnemulin (Table 1.2.5.1) [109]. Isolates with a MIC below the wild-type are classed as sensitive, isolates with a MIC above the wild-type breakpoint and below clinical resistance are classified as intermediate, and isolates with a MIC higher than the clinical resistance breakpoint are classed as resistant. Tiamulin resistance has been observed across the EU with the lowest levels of antibiotic resistance seen in Sweden [276] and the highest levels seen in Germany and Italy where more than 50% of isolates have a

tiamulin intermediate or resistant phenotype [73, 122]. In the UK, tiamulin resistance was first identified in 1998 and has increased over time, but most current isolates are not clinically resistant [291, 292].

SNPs in the 23S rRNA and the 50S rRNA L3 protein (*rplC*) have been implicated in tiamulin resistance; with a SNP at G2032A (*E. coli* numbering) commonly identified in resistant isolates [160, 164]. Other SNPs identified are less clear and have been found in both sensitive and resistant isolates [160, 163-165]. There can be a disagreement between the resistance phenotype (identified by MIC testing) and SNPs identified in the 23S rRNA and *rplC* [160, 163-165]. Also, there have been resistant isolates that do not contain known SNPs in the 23S RNA or *rplC* [159, 167]. Although SNPs in the L2 ribosomal proteins have not previously been associated with tiamulin resistance, a SNP at T50N in the L2 protein has recently been identified that may play a role in tiamulin resistance [163, 293].

Recently two novel antibiotic resistance genes have been identified in *B. hyodysenteriae*. The lincomycin resistance gene *lnu(C)* was identified in on a transposon in an Italian *B. hyodysenteriae* isolate [165]. This is the first potential horizontally transferred gene associated with a stepwise decrease in pleuromutilin sensitivity that has been identified in *B. hyodysenteriae* [165]. In addition, a gene associated with pleuromutilin resistance has recently been identified in *B. hyodysenteriae.* This gene, *tva(A),* is an F-type ATPase [293], which are ABC proteins that do not contain intermembrane domains but contain two nucleotide binding domains, connected by a linker region [294]. They have been implicated in tiamulin resistance in *Staphylococcus aureus, S. haemolyticus,* and *Enterococcus faecalis* [295-297]. F-type ATPases have activity against antibiotic classes that target the ribosome, and it is likely that they bind to the ribosome and can displace the antibiotic from the ribosome [294, 298]. The *tva(A)* gene was identified in *B. hyodysenteriae* isolates with a pleuromutilin intermediate or resistant phenotype [293]. It is possible that the *tva(A)* gene raises tiamulin tolerance slightly, to a similar degree as *vga(A)* does for lincomycin resistance in *S. aureus*, enabling growth of *B. hyodysenteriae* in the presence of pleuromutilins [299]. It is possible that there may be tiamulin resistance genes in *B. hyodysenteriae* strains that have not been identified. WGS could potentially be used in combination with antibiotic

susceptibility testing of clinical isolates to identify other genes in the accessory genome that have caused pleuromutilin resistance.

4.1.1. Study aims

This study builds upon previous work at the APHA where analysis of *B. hyodysenteriae* isolates identified novel SNPs and the *tva(A)* gene, both associated with pleuromutilin resistance [165]. However, in the previous study 34 *B. hyodysenteriae* isolates were analysed. This study aimed to verify these results on a larger set of isolates that had been previously sequenced (Chapter 3) and identify other mechanisms of antibiotic resistance. MIC results of clinical isolates of *B. hyodysenteriae* were also investigated using survival curves; this has not previously been done for UK isolates. In addition, the ability to induce resistance was investigated for tiamulin and valnemulin.

4.2 Methods

4.2.1 Sample Selection for MIC testing

The same isolates that were used previously (Chapter 3) were used in this study. A rational selection of 49 isolates for MIC testing was conducted. Samples were initially chosen from the North East of England and Yorkshire as this is the main pig producing region of the UK. Isolates were then chosen to ensure selection of isolates from across England, and that a range of pleuromutilin resistance genotypes were included.

4.2.2 Identification of SNPs in the 23S rRNA and *rplC* of sequenced *B. hyodysenteriae* isolates

After genomes had been annotated, the 23S rRNA gene and *rplC* of each isolate were isolated using the find function in TextWrangler (Bare Bones Software, USA). These genes were compared to the *E. coli* 23S rRNA (J01685) and the *B. pilosicoli rplC* (AF114845) reference genes, previously used in published literature, to identify SNPs (90, 92, 93). This was done using NUCmer (version 3.1) [300].

4.2.3 Analysis of the accessory genome of sequenced *B. hyodysenteriae* isolates

To identify genes of interest, Roary (version 3.8.2) was used to identify genes present in the accessory genome and absent from closely related strains [301]. These genes were then converted into a fasta file of genes of interest, which were screened against sequenced fasta files using LS-BSR (version last modified 2/9/2016) [302]. LS-BSR preforms reciprocal blast search of all genes in all isolates, and it was used to search the presence of genes in all isolates. Snippy was used to identify SNPs, and domains in genes of interest were analysed using Interpro [303]. To compare two genes nucleotide BLAST was used [304].

4.2.4 MIC testing by broth dilution

VetMIC Brachy plates were used for MIC testing by broth dilution. *B. hyodysenteriae* was grown overnight in pre-reduced 5 ml BHI (10%) in an anaerobic cabinet at 37 °C. This was then diluted to a MacFarland standard of 1.5-2 using pre-reduced BHI (10%) and added to 27 ml pre-reduced BHI (10%) and inverted to mix. To this 0.5 ml of this culture was added to each well of a VetMIC Brachy plate (SVA), and it was incubated at 37 °C under anaerobic conditions with shaking (80 rpm) for four days. Also, the 30 ml culture was used to make a dilution series by performing 1:10 serial dilutions in pre-reduced BHI (10% serum). From this dilution series 100 µl aliquots of the 1:100, the 1:1000 dilutions and the neat culture were plated out on FABA plates. The plates were then incubated for four days. After four days, MICs were obtained, and isolates were categorised into sensitive, intermediate or resistant (Table 4.2.3). Isolates below the wild type breakpoint were classified as sensitive, isolates above the clinical resistance breakpoint were classified as resistant, and all isolates between these two breakpoints were classified as sensitive. Also, colonies on the 1:100 and 1:1000 plates were counted to estimate the bacterial CFU/ml present in the 30 ml culture; while the plate from the 30 ml culture was used to check for any contamination. Plates were classified as contaminated if clear colonies were observed, or if the growth observed was not an off-white colour. If contamination was observed the broth dilution MIC was repeated. In addition, there were three controls that were used each time a series of isolates were tested: an

isolate with a known MIC was tested, there had to be $1x10^6$ CFU/ml cells tested in the VetMIC Brachy plate (the recommended minimum), and growth in the negative control well of each plate (containing only distilled water). The $B78^T$ type strain was tested, as this strain has been used previously; for broth dilution MICs to be accepted the $B78^T$ the MICs for tiamulin, valnemulin, doxycycline, lincomycin and tylosin had to be within the range previously established (tiamulin MIC: 0.063 (μg/ml), valnemulin MIC: 0.031 (μg/ml), tylosin MIC: 2 to 16 (μg/ml), Lincomycin MIC: 1 (μg/ml), and doxycycline 0.125 to 0.5 (μg/ml)) [116]. For a test to be accepted the MICs for $B78^T$ had to be within range, growth had to be observed in the negative control well, and the CFU/ml calculated from the dilution series had to be approximately $1x10^6$ CFU/ml. If these controls failed, the broth dilution MIC was repeated.

Table 4.2.3: Breakpoints used in this study

Antibiotic	Wild type MIC (mg/L)	Clinical resistance breakpoint MIC (mg/L)
Tiamulin	>0.25 [109]	>2 [109]
Valnemulin	>0.125 [109]	N/A
Doxycycline	>0.5 [109]	>4 [111]
Tylvalosin	>1 [109]	>16 [110]
Lincomycin	> 1 [109]	>16 [110]
Tylosin	> 16 [109]	>16 [110, 276]

4.2.5 Making tiamulin and valnemulin disks

Tiamulin and valnemulin disks were made in-house as commercial disks are not produced. Tiamulin fumarate and valnemulin hydrochloride were obtained from Sigma. These were dissolved in sterile water to a concentration of 50 μg/ml. To make lower concentrations the stock solution was further diluted to 10 μg/ml. When the desired stock concentration had been achieved the stock was filter sterilised with a 0.2-micron filter. A working solution was made with a concentration range from 1.6 – 150 μg/μl (table 4.2.5). From the working solution 20 /μl was then added to blank disks (5mm disks: Fisher Scientific), (l0mm disks: Sigma) and left to air dry in a class II MSC for 20 minutes.

Table 4.2.5: Concentrations used to produce tiamulin and valnemulin disks.

Required disk (μg)	Required concentration (μg/μl)	Stock concentration (μg/μl)	Stock to use (μl)	Water (μl)	Disk size (mm)
32	1.6	10	3.2	16.8	5
64	3.2	10	6.4	13.6	5
128	6.4	10	12.8	7.2	5
256	12.8	50	5.12	14.88	5
512	25.6	50	10.24	9.76	5
1000	50	50	20	0	5
2000	100	50	40	0	10
3000	150	50	60	0	10

4.2.6 Mutant selection

Mutant selection in isolates was based on the technique used by *Card. et al.* [293]. Three isolates were grown from frozen stock as previously described (Materials and methods, section 2.3.2) and subcultured at least twice before being used. Isolates were selected based upon MIC and the absence of SNPs associated with pleuromutilin resistance. At this point 200 μl of pre-reduced BHI (10%) was aliquoted into a sterile Eppendorf. A loopful of bacteria was scraped from the plate, added to the BHI (10%) and mixed by pipetting. Of this 100 μl was plated onto two new FABA plates. To one plate an antibiotic disk with an initial disk concentration of 32 μg of valnemulin per disk was added. For isolates grown in tiamulin, MIC strips (Liofilchem) were initially used. The plate containing antibiotics will be referred to as the antibiotic supplemented plate. The other plate to which an antibiotic disc was not added will be referred to as the growth plate. Isolates were incubated for three to four days at 38 °C in anaerobic conditions. After incubation, the growth plate was inspected for visible growth and contamination. For the antibiotic supplemented plate the zone of bacterial growth inhibition was measured from the edge of the disk to the edge of the zone of inhibition of growth. Bacteria were scraped from the edge of the zone of inhibition and suspended into 200 μl BHI (10%), and 100 μl plated onto antibiotic supplemented and antibiotic-free plates. If

the zone of inhibition was <10mm then bacteria recovered from the plates were replated and grown in the presence of discs containing double the concentration of antibiotic. After every fifth subculture of bacteria from the antibiotic-free plate a cell pellet and a frozen stock was prepared (Materials and methods, section 2.3.3). After the 20th subculture, isolates on the growth plate were sequenced at the APHA using an Illumina NextSeq. Sequences were assembled as described previously (Materials and methods, section 2.6.1) with the following exceptions: sequences were not aligned to the WA1 reference genome, also sequences were compared with the isogenic parent using Snippy (version 3.1) [244]. Subculturing to induce resistance was performed over three months.

4.2.7 Statistical analysis

MIC results were used to construct Kaplan-Meier survival curves, for each antibiotic tested. For isolates with a MIC greater or less than the maximum or minimum dilution value of the VetMIC Brachy plate, the MIC was converted to the largest or smallest MIC respectively. MICs were then multiplied by 32, except for valnemulin MICs which was multiplied by 33, and converted into positive log2 values as previously done by *Hidalgo et al.* [167, 305]. Isolates were multiplied by 32, or 33 to ensure positive log2 values were obtained. This was done using SPSS version 22.0 (IBM), and a log-rank test was used for statistical analysis. Isolates were split into two groups: a 2004 to 2009 and 2010 to 2015 group. Significance was determined as a P value of less than 0.05. Specificity and sensitivity were calculated as a two by two table [306].

4.3 Results

Antibiotic susceptibility testing was performed for 47 isolates using the broth dilution method previously described. All isolates that were tested had also been sequenced (Chapter 3). The isolates included in our panel were from 2004 to 2015 and selected from all regions of England.

4.3.1 Antibiotic resistance in *B. hyodysenteriae*

Sensitivities to six antibiotics were tested by broth dilution on the panel of 47 isolates (Table 4.3.1.1). The MIC results were used to construct survival curves (Figure 4.3.1.1). The isolates were split into two group for the analysis: a 2004 to 2009 group with 24 isolates; and a 2010 to 2015 group with 23 isolates. This split was chosen as 2010 which was the end of a British pig executive swine dysentery eradication project which started in 2009 in the East of England; therefore this was a date when industrial attitudes to swine dysentery appeared to have changed [279].

To visualise, and statistically analyse, the susceptibility data survival curves were constructed. The survival curves obtained using MIC data from doxycycline and tylvalosin testing showed there was little difference between the susceptibility of isolates in the two groups. For tylosin and lincomycin, there was a reduction in sensitivity in the 2009 to 2015 group from the 2004 to 2009 group, but this was not statistically significant difference. However, there were significant differences for tiamulin (P value = 0.024) and valnemulin (P value = 0.002), between isolates from the two groups (Figure 4.3.1.1). For tylosin the difference was due to an increase in clinical resistance; this indicates that tylosin resistance is common, with more than 50% of isolates clinically resistant. The percentage of clinical tylosin resistance suggest that this antibiotic is no longer a useful method of treating *B. hyodysenteriae* infections in the UK. For tiamulin and valnemulin, the difference between the two groups was due to an increase in the number of intermediate isolates in the 2010 to 2015 group, which was linked with a decline in sensitive isolates. These results indicate a long-term trend towards decreased susceptibility of UK isolates to pleuromutilins, a trend which likely started around 2010.

Table 4.3.1.1: Tiamulin and valnemulin, doxycycline, tylvalosin, lincomycin and tylosin MICs obtained by broth dilution and SNPs associated with resistance.

Isolates have been ordered based on their tiamulin MIC. Values higher than the wild type breakpoint have been shown in bold.

Isolate	doxycyc-line (mg/L)	tylvalosi-n (mg/L)	Lincomy-cin (mg/L)	tylosin (mg/L)	tiamulin (mg/L)	valnemuli-n (mg/L)	23S rRNA SNPs associated with macrolide and lincomycin resistance		23S rRNA SNPs associated with tiamulin			*rplC*	*tva(A)*	16S rRNA
Range	0.25 to 4	0.24 to 8	0.5 to 64	2 to 128	0.063 to 8	0.031 to 4								
MIC 50	0.5	0.5	0.5	4	0.25	0.5								
MIC 90	2	32	16	128	1	1								
js08	**2**	**16**	**32**	**>128**	≤0.063	≤0.031	A2058T				G2535A			G1058C
js13	0.5	**8**	**16**	**>128**	≤0.063	≤0.032	A2058T							G1058T
js14	**4**	**8**	**32**	8	≤0.063	≤0.031	A2058T				G2535A			
js15	**4**	**16**	**64**	8	≤0.063	≤0.031	A2058T							
js16	0.5	**8**	≤0.5	4	≤0.063	≤0.031	A2058T				G2535A			G1058C
js17	0.5	0.5	0.5	4	≤0.063	0.031								G1058C
js29	0.25	**16**	**32**	8	≤0.063	≤0.031	A2058T					N148S		
js60	≤0.125	**2**	**8**	**>128**	≤0.063	≤0.031	A2058T							G1058C
js61	**2**	**16**	**32**	**>128**	≤0.063	≤0.031	A2058T				G2535A			G1058C

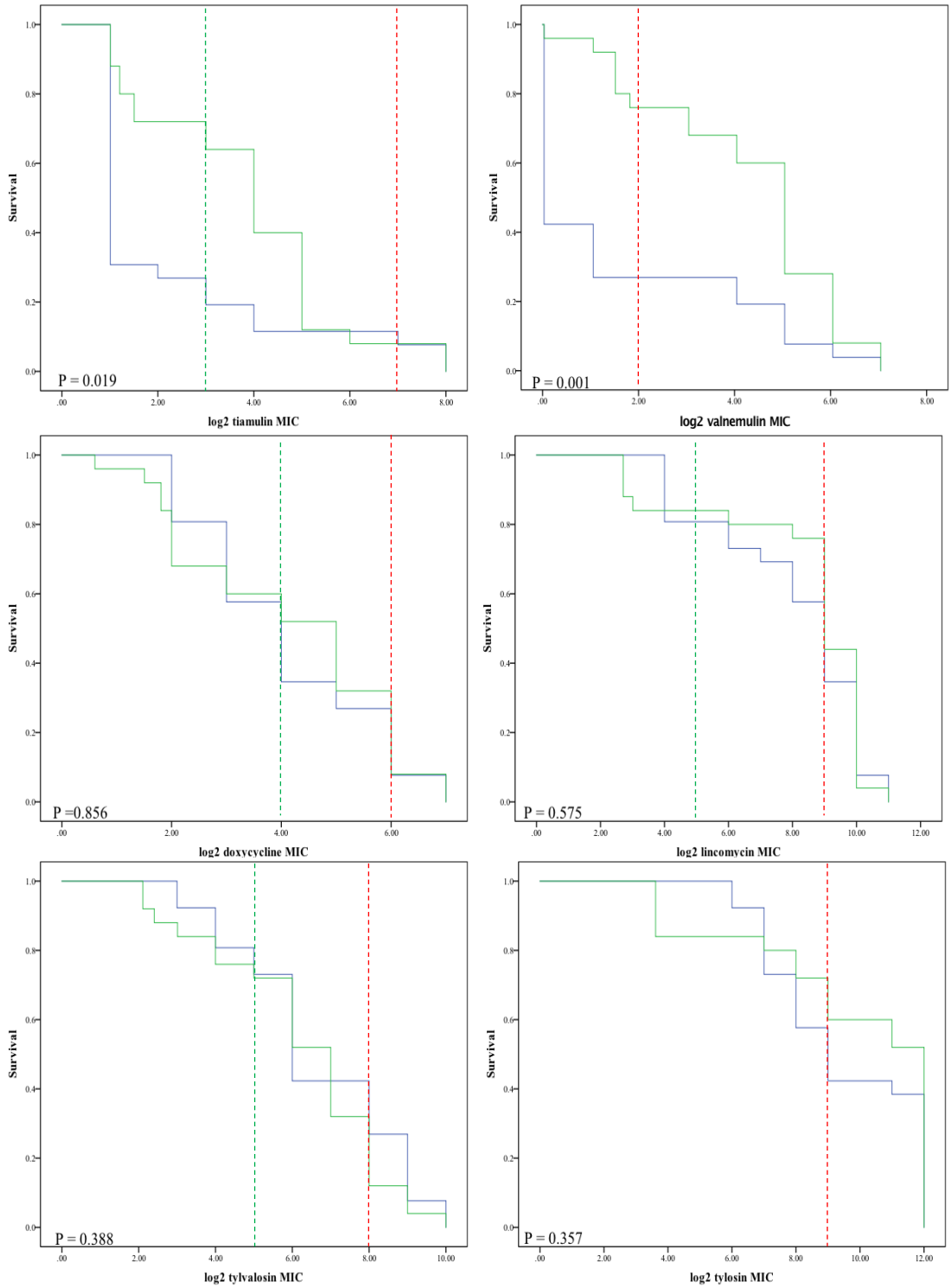
Isolate	doxycyc-line (mg/L)	tylvalosi-n (mg/L)	lincomy-cin (mg/L)	tylosin (mg/L)	tiamulin (mg/L)	valnemuli-n (mg/L)	23S rRNA SNPs associated with macrolide and lincomycin resistance		23S rRNA SNPs associated with tiamulin			*rplC*	*tva(A)*	16S rRNA
js66	**1**	**2**	**16**	8	≤0.063	≤0.031	A2058T							
js77	0.25	0.25	0.5	2	≤0.063	≤0.031								G1058C
js90	≤0.125	≤0.25	≤0.5	≤2	≤0.063	≤0.031								G1058C
js92	≤0.25	1	**4**	**>128**	≤0.063	≤0.031	A2058T							G1058C
js02	**1**	**8**	**16**	8	≤0.063	0.063	A2058T				G2535A			
js05	≤0.125	**16**	**32**	8	≤0.063	0.063	A2058T							
js06	≤0.125	**32**	**16**	16	≤0.063	0.063	A2058T							
js19	0.25	**16**	**32**	16	0.063	0.063	A2058T							
js20	0.5	**16**	**32**	16	0.063	0.063	A2058T							
js22	0.5	**32**	**64**	16	0.063	0.031	A2058T							G1058C
js27	**1**	1	**16**	**64**	0.063	**0.25**	A2058T				G2535A		*tva(A)*	G1058C
js37	≤0.125	**2**	**16**	**>128**	0.063	0.031	A2058T							G1058C
js11	**2**	**2**	**32**	**>128**	0.125	≤0.031	A2058T				G2535A	N148S		G1058C
js07	0.5	**2**	**8**	**64**	0.25	**0.5**	A2058T			C2146T	G2535A		*tva(A)*	G1058C
js18	**2**	0.5	0.5	4	0.25	**0.5**							*tva(A)*	G1058C
js26	**1**	**2**	**16**	**64**	0.25	**0.5**	A2058T						*tva(A)*	G1058C

Isolate	doxycyc-line (mg/L)	tylvalosi-n (mg/L)	lincomyci-n (mg/L)	tylosin (mg/L)	tiamulin (mg/L)	valnemuli-n (mg/L)	23S rRNA SNPs associated with macrolide and lincomycin resistance		23S rRNA SNPs associated with tiamulin			*rplC*	*tva(A)*	16S rRNA
js28	**1**	**2**	**32**	16	0.25	**0.25**	A2058T						*tva(A)*	G1058T
js09	≤0.125	**2**	**16**	**>128**	**0.5**	**2**	A2058T						*tva(A)*	
js12	**2**	**4**	**32**	**128**	**0.5**	**2**	A2058T				G2535A		*tva(A)*	G1058C
js43	**2**	**8**	**32**	**>128**	**0.5**	**1**	A2058T			C2146T	G2535A		*tva(A)*	
js46	0.5	0.5	**2**	4	**0.5**	**0.5**							*tva(A)*	G1058C
js69	0.5	**2**	**8**	**128**	**0.5**	**1**	A2058T						*tva(A)*	G1058C
js71	≤0.125	**4**	**16**	**>128**	**0.5**	**1**	A2058T						*tva(A)*	
js97	**1**	**4**	**32**	**128**	**0.5**	**1**	A2058T				G2535A		*tva(A)*	
js99	**2**	**4**	**16**	**128**	**0.5**	**1**	A2058T				G2535A		*tva(A)*	
js03	**2**	**4**	**32**	**>128**	**1**	**1**	A2058T		G2116A	C2146T	G2535A		*tva(A)*	
js25	0.5	0.5	**8**	16	**1**	**2**					G2535A		*tva(A)*	G1058C
js30	**4**	**2**	**32**	**128**	**1**	**1**	A2058T		G2116A	C2146T		N148S	*tva(A)*	G1058C
js44	**4**	**8**	**32**	**>128**	**1**	**2**	A2058T			C2146T	G2535A		*tva(A)*	
js72	≤0.125	**2**	**16**	**>128**	**1**	**1**	A2058T						*tva(A)*	
js73	**2**	**8**	**16**	**>128**	**1**	**1**	A2058T						*tva(A)*	
js74	≤0.125	**32**	**16**	**>128**	**1**	**2**		A2059C	G2116A	C2146T			*tva(A)*	G1058C

Isolate	doxycyc-line (mg/L)	tylvalosi-n (mg/L)	lincomyci-n (mg/L)	tylosin (mg/L)	tiamulin (mg/L)	valnemuli-n (mg/L)	23S rRNA SNPs associated with macrolide and lincomycin resistance		23S rRNA SNPs associated with tiamulin			*rplC*	*tva(A)*	16S rRNA
js31	0.25	**8**	**32**	**>128**	**2**	**2**	A2058T						*tva(A)*	
js49	0.25	1	**2**	4	**4**	**1**					G2535A		*tva(A)*	
js23	**2**	**2**	**32**	**128**	**8**	**4**	A2058T				G2535A	N148S	*tva(A)*	
js24	**1**	**2**	**16**	16	**8**	**4**					G2535A		*tva(A)*	
js33	**2**	**8**	**64**	**>128**	**8**	**4**	A2058T		G2116A		G2535A		*tva(A)*	G1058C
js48	0.25	0.5	**2**	4	**8**	**1**					G2535A		*tva(A)*	G1058C

Figure 4.3.1.1: Survival curves of MICs obtained by broth dilution.

The 2004 to 2009 group is the blue line, and 2010 to 2015 is the green line. The Red dashed line is the clinical resistance breakpoints used in this study, and the green dashed line is the wild-type breakpoint. Note tylosin only has a red dashed line as the wild type breakpoint, and clinical resistance breakpoints are the same [109, 276].



To identify an antibiotic resistance genotype for all isolates that were MIC tested the sequence for each isolate was compared to previously published SNPs that have been associated with antibiotic resistance for each antibiotic tested by broth dilution. SNPs previously associated with antibiotic resistance were identified in the 23s rRNA and *rplC* to give a predicted resistance genotype (Table 4.3.1.1). This was compared to the resistance phenotype obtained from the MIC. The phenotype was taken to be the gold standard and was compared to the genotype. A true positive was taken to be where both phenotype and genotype matched; where the results didn't match they were classified as false positive or negatives; this was then used to calculate the specificity, sensitivity, positive predicted value, and negative predictive value [165].

The sensitivity and specificity varied between antibiotics (Table 4.3.1.1). For three antibiotics (tylvalosin, tylosin and lincomycin) resistance was caused by a single SNP in the 23S rRNA: A2058T [144]. This SNP was present in 37 isolates for which MIC data was available and was the most common SNP detected. One isolate (js74) had an alternative SNP at A2059G that is also likely to confer resistance to macrolides and lincomycin. These SNPs were mostly present in isolates with MICs above the wild type breakpoint, but, with the exception of tylosin, were also present in sensitive isolates (Table 4.3.1.1). These SNPs were used to predict reduced susceptibility (isolates that are predicted not to have a sensitive phenotype) based on the presence of the A2058T or A2059G SNPs (predicted genotype). The sensitivity and specificity of the correlation between phenotype and genotype was high for tylvalosin (sensitivity: 97.37%, specificity: 88.89%), and lincomycin (sensitivity: 88.1%, specificity: 80%). There was also high sensitivity for tylosin (100%). However, the specificity for tylosin was considerably lower (42.86%), and there was a low negative predicted value for lincomycin (44.44%). For tylosin the low specificity was due to 11 false negatives; while the low negative predicted value was due to the high number of false negatives (five false positives compared to four true negatives). In both cases, this is likely due to isolates with MICs near to the wild type breakpoint. For tylosin, there were two dilutions below the wild type breakpoint; while for lincomycin three out of five false negatives were within two dilutions above the wild type breakpoint. This variation is within the variation previously observed for $B78^T$ [165].

Resistance to doxycycline in *B. hyodysenteriae* has been associated with a G1058C SNP in the 16S rRNA [111]. This SNP was present in 23 isolates, and there was also an alternative SNP (G1058T) identified in two isolates. A comparison of this doxycycline genotypic resistance to the phenotypic resistance showed high correlation with sensitivity of 95.45% and specificity of 84%.

For tiamulin and valnemulin, no single SNP was common to all isolates with reduced susceptibility to pleuromutilins, and SNPs were found in both susceptible isolates and isolates with reduced susceptibility. A comparison of predicted genotypic resistance and phenotypic resistance showed low correlation with the sensitivity and specificity for tiamulin being 66.67% and 65.38% respectively, and for valnemulin, the sensitivity was 61.54%, and specificity 66.67%. The poor sensitivity and specificity may be due to the inclusion of SNPs that do not contribute to pleuromutilin resistance as the role of many SNPs has not yet been clearly established. It may also indicate that other mechanisms may be involved in pleuromutilin resistance in *B. hyodysenteriae*.

Table 4.3.1.2: Specificity and sensitivity of prediction of sensitivity to tiamulin, valnemulin, doxycycline, tylvalosin, lincomycin and tylosin based on genotype.

Previously published SNPs were used to predict genotype. Values above 80% have been shown in bold.

	Tiamulin SNPs	Valnemulin SNPs	Doxycycline SNPs	Tylvalosin SNPs	Lincomycin SNPs	Tylosin SNPs
True Positive	14	16	21	37	37	27
False Positive	9	7	4	1	1	11
True negative	17	14	21	8	4	9
False Negative	7	10	1	1	5	0
Sensitivity (%)	66.67	61.54	**95.45**	**100**	**88.1**	**100**
Specificity (%)	65.38	66.67	**84**	**80**	**80**	42.86
Positive predictive value (%)	60.87	69.57	**84**	**94.87**	**97.37**	68.42
Negative predictive value (%)	70.83	58.33	**95.45**	**100**	44.44	57.14

Within the panel of isolates that were MIC tested there were three isolates that were closely related: js11, js23 and js24. Two of these (js23 and js24) were clinically resistant to pleuromutilins; while js11 was sensitive. All three isolates had the same SNPs in the 23S rRNA and *rplC.* Analysis of the accessory genome by Roary [301] of js23 and js24 identified an ATPase that was absent from js11. The amino acid sequence was investigated using Interpro which indicated this gene contained two AAA+ ATPase domains, and no membrane-spanning domains were identified. It is therefore unlikely to be an efflux pump. Recently a novel pleuromutilin resistance gene *tva(A)* has been identified. BLAST was used to compare the ATPase identified in this study to *tva(A)* (PRJEB24023) and showed 100% coverage and identity. LS-BSR [302] was used to identify *tva(A)* in all sequenced isolates, including all other isolates that had been MIC tested (Figure 4.3.1.2a and b). It was present in all isolates with a tiamulin MIC of 0.25 μg/ml or greater but was only present in one isolate (js27) with a MIC below 0.25 μg/ml (Table 4.3.1.2). In total *tva(A)* was identified in 55.32% of isolates. The tiamulin and valnemulin phenotype and genotype were compared with the presence of *tva(A)* being used to indicate an intermediate or resistant phenotype. As a result, the specificity and

sensitivity for and valnemulin increased from ~60% to 100% and that for tiamulin increased to >80% (sensitivity: 80.77%, specificity: 84%)(Table 4.3.1.3).

Table 4.3.1.3: Comparison of specificity and sensitivity of prediction of sensitivity to tiamulin and valnemulin based on previously published SNPs and *tva(A)*. Previously published SNPs were used to predict genotype. Values above 80% have been shown in bold.

	Tiamulin SNPs	*tva(A)*	Valnemulin SNPs	*tva(A)*
True Positive	14	21	16	26
False Positive	9	0	7	0
True negative	17	21	14	21
False Negative	7	5	10	0
Sensitivity (%)	66.67	**80.77**	61.54	**100**
Specificity (%)	65.38	**100**	66.67	**100**
Positive predictive value (%)	60.87	**100**	69.57	**100**
Negative predictive value (%)	70.83	**80.77**	58.33	**100**

To identify the distribution of *tva(A),* the phylogenetic tree produced previously (Chapter 3, section 3.3.3), was annotated with *tva(A)* presence or absence (Figure 4.3.1.3). It was absent from most of A and B, and was entirely absent from C and D. There were no clades that were composed entirely of isolates containing *tva(A)*. It was present in four clades, and *tva(A)* has become more common over time, in the 2004 to 2009 group there were six isolates with *tva(A)* compared to 20 in the 2010 to 2015 group. This could suggest *tva(A)* is becoming more prevalent, this may be due to clonal expansion of isolates containing *tva(A),* but it could also be due to horizontal gene transfer. Analysis of all previously sequenced *B. hyodysenteriae* isolates identified *tva(A)* in other sequenced isolates; in total 30% of all sequenced *B. hyodysenteriae* isolates contained *tva(A)*. This included two global isolates: the Italian isolates BH718 (with an LS-BSR score of 0.97) and the Australian isolate Q17 (with a score of 0.85). Both of these *tva(A)* genes were compared to *tva(A)* from js24 using BLASTp for BH718 *tva(A)* had an identity of 99%;while for Q17 the *tva(A)* gene had an identity of 95%. BH718 was isolated in 2017 and Q17 was isolated in the 1990's. It is therefore likely that *tva(A)* has been present in *B. hyodysenteriae* for decades. Over this time it is possible that variants of *tva(A)* have developed**.** Few *B. hyodysenteriae* isolates have

been sequenced globally, but, as more isolates are sequenced, it is likely that *tva(A)*, or other AMR genes, will be found more commonly. It is unknown if *tva(A)* can be transferred by horizontal gene transfer; potentially it could be, but there was no evidence of transposons in regions flanking *tva(A)* in sequenced isolates.

Figure 4.3.1.2: Percentage of isolates containing *tva(A)* based on their pleuromutilin resistance phenotype.

The presence of *tva(A)* was identified with LS-BSR [302].

Abbreviations:

a: based on tiamulin resistance phenotype, b: based on valnemulin resistance phenotype.

Percentage of isolates containing *tva(A)* is shown above the bars.

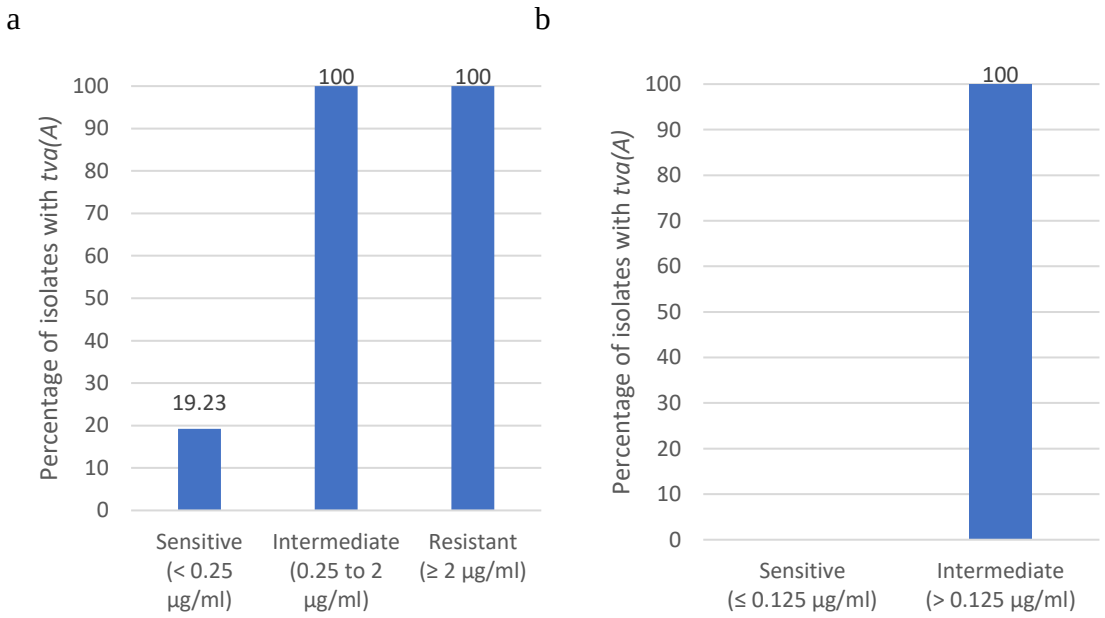
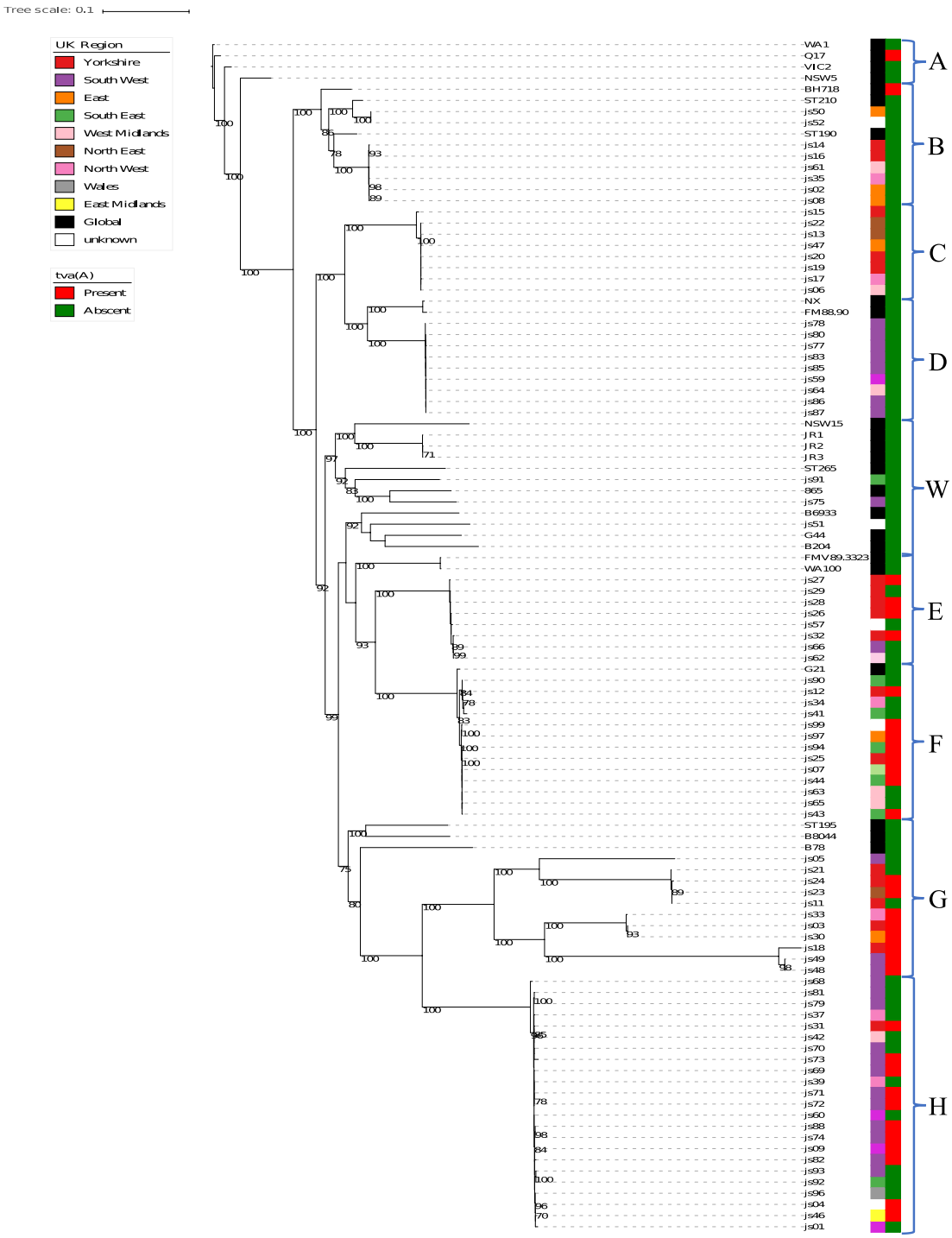


Figure 4.3.1.3: Phylogenetic tree of sequenced *B. hyodysenteriae* isolates (Chapter 3) and isolates from NCBI annotated with presence or absence of *tva(A)*.

RAxML was used for bootstrapping, and 1000 bootstraps were conducted. Bootstrap support above 70 is shown. The first strip shows Region of origin and the second shows the presence or absence of *tva(A)* identified by LS-BSR. The global isolates, and js71 and js51, have been shown with a W.



As it was possible to predict reduced sensitivity to tiamulin, valnemulin, doxycycline, tylvalosin and lincomycin based on genotype with good (higher than 80%) specificity and sensitivity a predicted antibiotic sensitivity was made for all 84 sequenced *B. hyodysenteriae* isolates (Table 4.3.1.5.) The predicted sensitivity to tylosin was also made, but due to the low specificity (42.86%), caution must be taken when drawing any conclusions from this. The most common SNP identified in the isolates was a SNP at A2058T in the 23S rRNA (present in 67 isolates), followed by SNP at G1058C in 16S rRNA (present in 35 isolates). For tylvalosin, tylosin and lincomycin the majority (78.57%) of isolates are predicted to harbour phenotypic resistances above the wild type breakpoint (Table 4.3.1.4). All these antibiotics had the same result as a single SNP was responsible for the reduced sensitivity to these antibiotics. For doxycycline, tiamulin and valnemulin, the levels are lower (41.67%). This was due to a lower number of isolates with the G1058C SNP and *tva(A)* and may suggest these antibiotics would be more suitable for swine dysentery in the UK.

Table 4.3.1.4: Predicted resistance phenotype based on previously published SNPs and *tva(A)* present in all 84 sequenced UK isolates.

	Tiamulin	Valnemulin	Doxycycline	Tylvalosin	Lincomycin	Tylosin
Isolates above wild type breakpoint	35	35	35	66	66	66
Percentage	41.67	41.67	41.67	78.57	78.57	78.57

4.3.2 Mutant selection to tiamulin and valnemulin

It is possible that *tva(A)* may affect tiamulin and valnemulin resistance differently. To investigate this three isolates were grown in increasing sub-lethal concentration of tiamulin or valnemulin. All these isolates had been previously sequenced and contained no SNPs associated with tiamulin resistance. The isolates chosen compromised an isolate phenotypically sensitive to tiamulin that lacked *tva(A)* (js17), a tiamulin intermediate isolate with *tva(A)* (js31) and a tiamulin sensitive isolate js27 with *tva(A)*. None of these isolates had any SNPs previously associated with tiamulin resistance. All isolates were able to grow on disks with higher concentrations of tiamulin or

valnemulin by the end of the experiment. After 20 subcultures, each strain was MIC tested (table 4.3.2.1). The dilution series failed for three daughter isolates (js31t20, js31v20 and js17v20), and it was not possible to repeat these MICs. Therefore, it was not possible to determine if these isolates had developed resistance.

However, MICs were obtained for js17t20 and js27t20 and js27v20. In all these isolates there was a reduction in sensitivity to tiamulin and valnemulin. For js27 there was a clear difference between the js27 isogenic parent and isolates that had been serially subcultured in the presence of tiamulin for 20 passages (js27t20) or valnemulin for 20 passages (js27v20) with both isolates having reduced sensitivity to tiamulin and valnemulin. This was also observed in js17t20, where tiamulin and valnemulin susceptibility also decreased with repeated passage in the presence of the antibiotics. The greatest change occurred in js27, where both js27t20 and js27v20 became clinically resistant to tiamulin from being fully sensitive. This isolate contains the *tva(A)* gene and the greater increase in tiamulin resistance seen in js27 may be due to the presence of this gene. Alternatively, there could have been a mutation in a remote site, for example, a regulator, that resulted in increased expression of *tva(A)* resulting in clinical resistance. In addition, in 17t20 there was a marked increase in resistance to lincomycin and tylosin.

Table 4.3.2.1: MICs of isolates grown in increasing sub-lethal concentrations of tiamulin or valnemulin and the parent strain.

Note MICs have not been shown for js31t20, js31v20 and js17v20 as the dilution series failed for these isolates.

Abbreviations:

t indicated isolates grown in tiamulin while v indicates valnemulin. Tia = tiamulin, Val = valnemulin, Dox = doxycycline, Tylv = tylvalosin, Lin = lincomycin and Tyl = tylosin. None = dilution failed, due to time constraints it was not possible to repeat these isolates.

Isolate	Tiamulin (μg/ml)	Valnemulin (μg/ml)	Doxycycline (μg/ml)	Tylvalosin (μg/ml)	Lincomycin (μg/ml)	Tylosin (μg/ml)
js17-0	0.063	0.031	0.5	0.5	0.5	4
js17t20	1	1	1	2	16	32
js27-0	0.063	0.25	1	1	16	64
js27t20	8	4	0.5	4	32	64
js27v20	4	4	2	4	64	64

All daughter isolates (js17t20, js17v20, js27t20, js27v20, js 31t20 and js31v20) were sequenced after 20 subcultures on a single NextSeq run at the APHA by the Central Sequencing Unit (Table 4.3.2.2). High coverage was obtained for all isolates and is likely due to the greater sequencing power of the NextSeq system. Similar percentage identity to WA1 was observed as for isolates sequenced on the MiSeq; this again highlights the importance of establishing a completely sequenced UK reference strain.

Table 4.3.2.2: Sequencing results of isolates grown in increasing sub-lethal concentration of tiamulin or valnemulin.

Isolates subcultured in the presence of tiamulin are identified with a t, isolates subcultured in the presence of valnemulin are signified with a v, and 20 refers to the number of subcultures.

Isolate	Total reads (bp)	GC content	Total contigs	Largest contig length (bp)	N50	Genome coverage	Kraken (%)
js17t20	3290782	29.5	113	471244	321698	158x	88.29
js17v20	4372734	29.3	149	1157885	396753	212x	91.03
js27t20	7548512	29.8	433	949522	257966	349x	74.73
js27v20	3847642	29.8	168	732489	187840	186x	77.28
js31t20	4414172	29.1	279	485817	280296	213x	78.43
js31v20	4084866	29.2	368	740691	385616	196x	82.24

A core-genome tree was produced and isolates clustered with the parent strain (Appendix 2). Also, isolates were compared to their isogenic parent strain to identify SNPs that could potentially be involved in AMR. Most SNPs identified were in non-coding regions or were non-synonymous. Of the remaining SNPs, most were unlikely to be involved in AMR, for example, SNPs were often identified in variant surface proteins. However, in all isolates that had increased resistance to tiamulin and valnemulin, there were SNPs identified that may have contributed (Table 4.3.2.3). The same potential resistant SNPs were identified in isolates that had been exposed to tiamulin or valnemulin. This may be due to the small sample size, or the short length of exposure (three months), but it may also suggest that the same SNPs can confer resistance to tiamulin and valnemulin.

Table 4.3.2.3: SNPs identified in isolates when compared to the parent strain.
B. hyodysenteriae WA1 locus tags have been given for SNPs in novel genes.

	js17t20	js17v20	js27t20	js27v20	js31t20	js31v20
Total SNPs	237	245	482	411	97	113
Total non-synonymous SNPs	62	60	133	121	30	37
SNPs in 23s rRNA	G2061T	G2061T				
SNPs in *rplC* protein						S149T
SNPs in ABC binding protein (BHWA1_0019)			N503T	N503T		
Non-synonymous SNPs in other genes potentially involved in AMR			Multidrug export protein MepA (BHWA1_01355) (F110L)	Multidrug export protein MepA (BHWA1_01355) (F110L)		
			Multidrug resistance protein MdtC (BHWA1_00506) (S700T)	Multidrug resistance protein MdtC (BHWA1_00506) (S700T)		

There were a variety of SNPs detected with clear differences between isolates originating from js17, js27 and js31. Similar SNPs were detected in both daughter lineages. Although js17t20, js27t20 and js27v20 had reduced tolerance to tiamulin, only js17v20 and js17t20 contained a SNP in the 23S rRNA. The SNP at G2061T (*E. coli* numbering) in the 23S rRNA was the only potential tiamulin resistant SNP identified in js17v20 and js17t20. These isolates also had decreased susceptibility for lincomycin and tylvalosin. This SNP, therefore, might also contribute to the increased resistance to lincomycin and tylvalosin seen in MIC testing. Js27t20 and js27v20 did not contain any new SNPs in the 23SrRNA or *rplC* compared to the isogenic parent. However, for js27t20 and js27v20 there were amino acid substitutions in an ATP binding protein and in potential efflux pumps. It is possible that these SNPs could have increased the efficiency of the efflux pumps leading to increased binding of tiamulin and increasing the efflux out of the cell. There were also SNPs in hypothetical genes, these have not been well categorised in *B. hyodysenteriae* and may include regulatory genes; a

mutation in a regulatory gene could also lead to a reduction in tiamulin and valnemulin susceptibility.

Although the broth dilution susceptibility test failed for both js31t20 and js31v20, there was a SNP identified in js31v20 in *rplC* that has been previously associated with pleuromutilin resistance [159, 167]. To determine if this SNP would have caused an increase in resistance to pleuromutilins it would be necessary to repeat the broth dilution susceptibility test. It is possible that the SNP would result in js31v20 having a different pleuromutilin resistance phenotype compared to js31t20.

4.4 Discussion

This study aimed to investigate the mechanisms behind antibiotic resistance within *B. hyodysenteriae* in the UK. For macrolides, there was a high level of resistance observed, and a high level of reduced susceptibility was predicted from sequenced isolates (78.57%). The same was also found for lincomycin. Resistance to these antibiotics is caused by a SNP in the 23S rRNA: A2058T [144]. Another SNP was identified in this study A2059C; this has been previously identified in Spanish *B. hyodysenteriae* isolates [160]. The high level of resistance observed in this study have been seen in other EU countries including Spain [167], and Italy [122]. High levels of resistance are also seen globally, for example in Brazil [286], the USA [285] and Japan [287]. This supports the decision by the European Medicines Agency to remove tylosin and lincomycin as recommended treatments for swine dysentery [282]. It is likely that the A2058T SNP is common globally. A SNPs at A2058G has also been associated with macrolide resistance in *Mycoplasma smegmatis* and in *M. smegmatis* analysis has suggested that the fitness cost of this SNP is not significant (0.4 to 1.4%) per generation, and this was not due to compensatory mutations [307]. Although this is a different species to *B. hyodysenteriae*, it is possible that the fitness cost of the A2058T SNP for *B. hyodysenteriae* may also be small. If this is the case macrolide and lincomycin resistance in *B. hyodysenteriae* is likely to continue in the UK, despite the removal of tylosin and lincomycin from the recommended list of antibiotics to treat swine dysentery [282].

Resistance to doxycycline was lower than for macrolides and lincomycin. However, the SNP in 16S G1058C associated with doxycycline resistance in *B. hyodysenteriae* was

common in this study. Also, in two doxycycline resistant isolates (js04 and js30) an alternative SNP was detected at G1058T, which has previously been identified in the UK [293]. Global resistance to doxycycline varies considerably in *B. hyodysenteriae*. In the USA 50% of isolates were sensitive [285]; while in Brazil [286] and Belgium [60], most isolates have reduced sensitivity.

In this study, there were low levels of pleuromutilin resistance with only five isolates clinically resistant to tiamulin. This has also been seen in UK surveillance reports, where the majority of isolates were not resistant [292]. However, there were many intermediate isolates, and more than half of the isolates tested were sensitive to tiamulin and valnemulin. When the presence of *tva(A)* was used to predict the resistance phenotype of all sequenced isolates, there was a similar result to resistance detected by MIC testing with 41.67% of isolates having reduced sensitivity to tiamulin and valnemulin. Similar levels have been seen in Belgium, with 48.28% of isolates below the wild type breakpoint when the MIC was obtained by broth dilution (Table 4.4.1) [60]. However, most countries appear to fit into two broad groups: there are countries with very low levels of resistance and countries with higher levels of clinical resistance. The USA [285], Sweden [276] and Switzerland [290] all have low levels of pleuromutilin resistance, with most isolates below the wild type breakpoint. There are likely to be many factors affecting the level of pleuromutilin resistance observed. The low tiamulin resistance seen in the USA could be due to the greater variety of antibiotics that can be used to treat swine dysentery [308]. While Switzerland has little historic exposure to *B. hyodysenteriae* [290] and Sweden has a national control program to reduce SD, which has resulted in the reduction of swine dysentery [276]. Conversely, countries such as Germany [73] and Spain have far higher levels of tiamulin resistance [122]. These countries also produce far more pigs than Sweden or Switzerland, which may lead to more tiamulin use [309].

Although the level of clinical tiamulin resistant *B. hyodysenteriae* in the UK appears low, there was a statistically significant shift towards an intermediate phenotype after 2010 as observed in this study. This shift has also been seen in UK surveillance reports, where the number of sensitive isolates has reduced over time [292]. This likely indicates reduced susceptibility to pleuromutilins in the UK, which potentially could lead to an increase in the number of clinically resistant swine dysentery cases in the future. This

shift was clearly seen in the survival curves, and a similar shift has been seen in survival curves of *B. hyodysenteriae* isolates from Spain, although in Spain there was also an increase in clinical resistance to tiamulin [167]. This shift from tiamulin sensitivity to an intermediate or resistant phenotype has been seen in other countries, for example in Poland all isolates tested had an intermediate phenotype [289]. A decrease in pleuromutilin sensitivity is not restricted to the EU. It has also been observed in Australia, and in the USA [51, 123, 285].

Table 4.4.1: Comparison of tiamulin MICs from this study to previously published data available.

Only the latest published data for each country was used. Studies have not been included if MIC data was not presented, in this case, the most recent study with MIC data was used. The MIC data has been re-interpreted using established breakpoints. For agar dilution the breakpoints suggested by Rønne, H. and J. Szancer have been used while for broth and broth dilution the breakpoints suggested by Pringle *et al.* have been used with one modification: the wild-type breakpoint has been moved to >0.25 μg/ml (Table 1.2.5.1) [109, 110]. For some studies, data could not be obtained, and this is designated with n/a. For Spain raw data was not provided so approximate results were interpreted from the relevant bar charts. Percentages mentioned in the text are given in bold.

Country	Method of determining MIC	Percentage sensitive	Percentage intermediate	Percentage resistant	Reference
Australia	Agar dilution	80.95	4.76	14.29	[123]
Brazil	Broth dilution	9.09	n/a	>40	[286]
Belgium	Broth dilution	48.28	37.93	13.79	[60]
Czech Republic	Agar dilution	n/a	n/a	24.3	[288]
Germany	Broth dilution	34.21	20.18	**45.61**	[73]
Italy	Broth dilution	13.59	33.01	53.4	[122]
Japan	Agar dilution	41.14	41.62	17.24	[287]
Poland	Broth dilution	0	100	0	[289]
Spain	Broth dilution	~22.5	~45	**~32**	[167]
Sweden	Broth dilution	**95**	5	0	[276]
Switzerland	Broth dilution	**100**	0	0	[290]
Taiwan	Agar dilution	n/a	n/a	29.72	[166]
USA	Broth dilution	**70.27**	18.92	10.81	[285]
UK	Broth dilution	47.92	41.67	10.42	This study

The specificity and sensitivity of phenotypic prediction of tiamulin and valnemulin sensitivity based on genotype was poor. The sensitivity was greatly improved when

tva(A) was detected in tiamulin intermediate and resistant isolates [293]. This is the second time this gene has been detected in *B. hyodysenteriae* and was detected independently on a separate panel of isolates using different techniques. This gene appears to be present in multiple distinct clades and may be transferred by horizontal gene transfer. In addition, the likely presence of *tva(A)* in an Australian isolate suggests that *tva(A)* has been present in *B. hyodysenteriae* for decades as no pigs have been imported into Australia since the 1980s [123]. Currently, the best categorised horizontal gene transfer agent in *B. hyodysenteriae* is the gene transfer agent VSH-1 [169]. This transfers a random segments of approximately 7.5 kb of DNA and has previously been shown to transmit resistance to coumermycin A(1) [169, 172]. Recently, a transposon (MTnSag1) has been identified in *B. hyodysenteriae* [165]. However, this transposon was first identified in *S. agalactiae,* and in *S. agalactiae* this transposon requires a co-transposon for transfer between cells [173]. This co-transposon was not found in BH718, and no other mechanism of transfer has been identified in *B. hyodysenteriae* [165]. Further work will be needed to show experimentally that *tva(A)* can be transferred between *B. hyodysenteriae* isolates.

To investigate if *tva(A)* has a differing impact on tiamulin and valnemulin three isolates were repeatedly subcultured in the presence of sublethal concentrations of tiamulin and valnemulin. After 20 subcultures there was less sensitivity to tiamulin and valnemulin sensitivity for sensitive isolates; with the highest increase in resistance occurring in js27. Tiamulin resistance has been selected for previously in *B. hyodysenteriae* [159, 293], *Mycoplasma gallisepticum* [310], *M. bovis* [311], *M. smegmatis* [312] and *S. aureus* [313] by serial passage in sub-lethal concentrations of tiamulin. This is the first study where *B. hyodysenteriae* tolerance to valnemulin has also been selected for. The development of tiamulin resistance does not commonly lead to the development of resistance to other classes of antibiotic, except for valnemulin [293, 313, 314]. In this study exposure to tiamulin or valnemulin also led to reduced sensitivity to the other pleuromutilin. However, with the exception of *M. gallisepticum* (where lincomycin resistance developed), resistance to other classes of antibiotic resistance did not occur [310]. It is likely that most SNPs that result in pleuromutilin resistance do not lead to co-resistance with other classes of antibiotics, but some SNPs in the 23S rRNA may impair binding of multiple drugs classes due to the closeness of their binding sites.

For each isogenic daughter isolate, there was an increase in both tiamulin and pleuromutilin resistance. It is therefore likely that treatment of pigs with swine dysentery caused by tiamulin resistant isolates would not respond to treatment with valnemulin also. It also appears multiple SNPs occurred in the serially passaged strains that increased tolerance to tiamulin and valnemulin. The isolates js17t20 and js17v20 had a SNP at G2061T (*E. coli* numbering) that was not present in the isogenic parent. This has not been associated with tiamulin resistance previously in *B. hyodysenteriae*, but a G2061T has been associated with linezolid resistance in *M. tuberculosis* and a G2061U when *M. galisepticum* tiamulin resistance was selected for [310, 315]. A SNP in the next nucleotide, G2062T, has been linked with tylosin and tiamulin resistance in *M. bovis* [311]. This region of the 23S rRNA is an important binding site for both pleuromutilins and macrolides: tiamulin binds to 2062 (*E. coli* numbering), and macrolides bind to 2058 (*E. coli* numbering) [316, 317]. A SNP could cause a conformational change in the peptidyl transferase centre that could affect binding of both tiamulin and macrolides.

The G2061T SNP was not present in js27, but there were mutations in efflux pumps and in an ATP binding protein. It is possible that these mutations could have increased the efficiency of efflux pumps slightly, resulting in increased resistance to tiamulin and valnemulin. This would be a method for tiamulin resistance that does not rely upon SNPs in the peptidyl transferase centre and may explain the identification of tiamulin resistant isolates that do not contain tiamulin resistant SNPs in the 23S rRNA or *rplC* [159, 167]. Also, other mechanisms could have resulted in tiamulin resistance, for example, a mutation in a regulator that leads to upregulation of efflux pumps. If this regulator was poorly annotated by Prokka, or if the regulator was at a remote site in the genome, it may have been missed in the analysis performed in this study. Further work with RNAseq could identify transcriptional changes and provide valuable information on the role transcription plays in tiamulin resistance.

This study aimed to investigate trends in pleuromutilin sensitivity in the UK and identify the mechanisms behind any trend towards reduced sensitivity. A trend towards reduced pleuromutilin susceptibility was observed with increasing number of pleuromutilin intermediate isolates across the period. Correlation of phenotypic resistance to genotypic resistance (based on previously published SNPs) indicated a

poor match between pleuromutilin resistance phenotype and genotype. This was improved with the identification of a potentially novel tiamulin resistance gene in all tiamulin intermediate and resistant isolates. Further research will be necessary to identify if this gene is transferred between isolates.

Chapter 5. Metagenomic analysis of healthy caecum and swine dysentery positive faecal samples

5.1. Introduction

Diagnosis of swine dysentery is a slow process, due to the time taken to obtain a pure *B. hyodysenteriae* isolate, as this is a slow growing organism [53]. It is possible that by the time a diagnosis has been made, swine dysentery could have spread to connected holdings; this may have occurred in Sweden in 2015 [276]. PCR offers a rapid way of identifying *B. hyodysenteriae*. However, as novel species are becoming more commonly identified as the cause of swine dysentery through microbial cultural methodologies, these might be missed with species-specific PCRs [45]. WGS offers an alternative diagnostic method, and there is increased interest in using WGS for routine surveillance and outbreak analysis [318]. This is likely to be done using pure cultures, but for *B. hyodysenteriae* shotgun metagenomic sequencing might be better as no time is lost purifying samples. This is potentially a quick way of identifying *B.* hyodysenteriae, or novel *Brachyspira* species. It may also provide useful information about ST types and AMR, which could aid in choosing the appropriate treatment. At present, metagenomic sequencing is too expensive for diagnosis; but this could change due to the reduced cost of sequencing in the future because of intense competition between sequencing companies.

Metagenomic shotgun sequencing also enables analysis of the impact that the gut microbiome has upon SD. The pig gut microbiome is composed mainly of *Bacteroides* and *Firmicutes* with the most common species being *Prevotella* [35]. As pigs age, the relative abundance of *Prevotella* declines and is replaced by species such as *Anaerobacter* and *Lactobacilli* species [35]. The microbiome has previously been identified as playing an important role in swine dysentery and may be necessary for the successful colonisation of the pig gut by *B. hyodysenteriae* [89]. A number of species, such as *Campylobacter coli* have been associated with swine dysentery [319]. However,

analysis of clinical cases from commercial farms in the UK might identify other relationships more applicable to the UK pig industrial practices. Although there is movement between the UK and Europe this is mostly movement of pigs from the Republic of Ireland into Northern Ireland, movement from the rest of the EU into the UK was 1673 pigs; this represented 0.00035% of the UK pig herd in 2016 [270]. Within the UK, there is little movement of pigs between different regions, and there are distinct production systems in different regions, with more intensive production in the East of England [21]. In addition, it appears that there is limited travel between regions [21, 269]. This could result in slightly distinct microbiomes developing in different regions of England. It will be necessary to investigate the impact regional production systems has on the pig gut microbiome to ensure erroneous conclusions are not drawn about the impact swine dysentery has on the pig gut microbiome.

5.1.1. Study aims

This study aimed to identify *B. hyodysenteriae* directly from sequenced pig faecal samples and gain useful clinical information. In addition, differences between the microbiome of swine dysentery positive clinical faecal samples, and healthy faecal samples were examined. To assess the impact of farming practices and different geographical locations of farms, the gut microbiome of healthy pigs was analysed by shotgun sequencing faecal samples collected from pigs. Healthy samples are infrequently received by the APHA. However, at the time of this study, there was a research study (VM0518) running, with the aim of developing molecular techniques to rapidly identify AMR present in zoonotic pathogens and indicator species [320]. For this study, caecal samples were collected from pigs at slaughter. These pigs can be defined as healthy, as they have reached slaughter weight, and were from pig farms representing different regions of England and are therefore suitable to use to investigate regional differences in the pig microbiota.

5.2. Methods

5.2.1. Samples

Swine dysentery positive samples came from faecal diagnostic clinical submissions made to the APHA reference laboratory; four were from historic clinical submissions

that had previously been shown to be *B. hyodysenteriae* positive by PCR, and two were clinical submissions from 2016 that were confirmed to be *B. hyodysenteriae* positive by isolation of *B. hyodysenteriae*. Where possible, these samples were age-matched by the age of pig. However, age information was only available for three swine dysentery positive samples: two were 17 weeks, and one was 14 weeks (Table 5.2.1.1). Healthy pigs came from a study investigating the presence of *mcr-1* in the UK; all faecal samples from healthy pigs were taken at 17 weeks [264]. Healthy faecal samples and caecal samples were collected by APHA and frozen at – 80 °C at Weybridge. Historic swine dysentery positive faeces were stored at an APHA regional laboratory before being sent to APHA Weybridge and stored at – 20 °C. Swine dysentery positive faecal samples obtained over the course of this study from the Reference Laboratory at Bury St. Edmunds, were stored at – 80 °C at Weybridge.

Caecal samples were obtained as part of VM0518. These came from Yorkshire, the South West and the Midlands; there were three finisher herds and nine farrow-to-finish herds. For this study, a single 0.25 g sample was taken from each farm of interest, except for MSG54 (Table 5.2.1.2). This was the first farm investigated, and four samples were taken to investigate changes between individuals. The age of the pigs at slaughter was not provided, but the pigs are likely to be older than the pigs that provided the faecal samples.

Table 5.2.1.1: Information on faecal samples used in this study.

Note Yorkshire refers to Yorkshire and the Humber. nk = not known. The original reference for the healthy isolates has been given.

Sample	Region or source	Health	Year	Age (weeks)
14	South West	SD	2015	17
26	South East	SD	2015	14
123	Yorkshire	SD	2010	17
161	Yorkshire	SD	2010	nk
168	South West	SD	2010	nk
174	Yorkshire	SD	2010	nk
8	[264]	Healthy	2016	17
69	[264]	Healthy	2016	17
55	[264]	Healthy	2016	17
100	[264]	Healthy	2016	17
103	[264]	Healthy	2016	17

Table 5.2.1.2: Information on caecal samples used in this study.

Note Yorkshire refers to Yorkshire and the Humber.

Sample	Region	Type
MSG20P2	Breeder-Finisher	Midlands
MSG44P2	Breeder-Finisher	Midlands
MSG54P2	Breeder-Finisher	Midlands
MSG54P3	Breeder-Finisher	Midlands
MSG54P4	Breeder-Finisher	Midlands
MSG54P5	Breeder-Finisher	Midlands
MSG07P2	Breeder-Finisher	Yorkshire
MSG16P2	Breeder-Finisher	Yorkshire
MSG22P2	Breeder-Finisher	Yorkshire
MSG11P2	Breeder-Finisher	South West
MSG14P2	Finisher	South West
MSG12P2	Breeder-Finisher	South West
MSG06P2	Finisher	South West
MSG15P2	Breeder-Finisher	South West
MSG38P2	Finisher	South West

5.2.2. Extraction of DNA from faecal and caecal samples

The PowerSoil Ultra kit (MO Bio) was used for isolation of DNA from caecal and faecal samples. Initially, 0.25 g of the sample was added to a PowerBead tube and gently vortexed to mix. To this 60 µl of Solution C1 was added, and vortexed briefly to mix. PowerBead tubes were then securely placed in a bead beater (MO BIO Vortex Adapter), and vortexed at maximum speed for 10 minutes before being centrifuged for 30 seconds at 10,000 x g. 250 µl of solution C2 was added and vortexed for 5 seconds before being incubated at 4 °C for 5 minutes. This was followed by centrifugation at 10,000 x g for 1 minute. To this 600 µl of the supernatant was then transferred to a 2 ml collection tube, to which 200 µl of solution C3 was added. This was vortexed briefly to mix before being incubated at 4 °C for 5 minutes. This was then centrifuged at 10,000 x g for 1 minute and 750 µl of the supernatant were transferred to a new 2 ml collection

tube. To this 750 µl of Solution C4 was added and vortexed to mix. This was then loaded onto a spin filter and centrifuged at 10,000 x g for 1 minute. The supernatant was discarded and 500 µl of solution C5 was added to the spin filter and centrifuged at 10,000 x g for 30 seconds. The spin filter was then transferred to a new 2 ml collection tube, and the old collection tube and flow through were discarded. To this 100 µl of solution C6 was added to the spin filter and centrifuged at 10,000 x g for 30 seconds. The spin filter was then discarded, and the DNA was stored at –20 °C until needed. Faecal and caecal samples were then sequenced on an Illumina MiSeq as described previously (Materials and methods, section 2.4.1 and 2.4.2).

5.2.3. Identification of antimicrobial resistance genes and plasmids

Fastq files were screened against an in-house AMR database (Seqfinder) [264] using SRST2 (version 0.1.5) [263]. Only genes with a depth of coverage of 15x were used for subsequent analysis, as SRST2 is highly sensitive at 15x coverage [263]. Results were analysed in Excel 2013 (Microsoft) and assembled into a single spreadsheet. AMR genes were normalised to parts per million. This was done by dividing reads mapped to each AMR gene by the total number of reads and multiplying by one million.

5.2.4. Speciation with Sigma

Sigma was used to map raw fastq files against the RefSeq database of sequenced bacterial reference stains [321]. The RefSeq database 2014 was downloaded from NCBI. The 2014 database was used as there was a script to format the database as required by Sigma, this script was part of the Kraken program, later versions of the RefSeq database are incompatible with this script due to differences in the location of the RefSeq database [240]. Relative abundance values were analysed in Excel 2013 (Microsoft) and assembled into a single spreadsheet.

5.2.5. Further analysis of sequenced samples

R was used to make heatmaps using Bray-Curtis clustering [322]. Clustering was done using vegan, and heatmaps were produced using gplots [323, 324]. Principle component analysis (PCA) was conducted using FactoMineR, and PCA plots were made using factoextra [325, 326]. Lefse, on the Galaxy website, was used to conduct LDA [327].

5.2.6. Analysis of *B. hyodysenteriae* directly from sequenced faecal samples

Fastq files were mapped against the *B. hyodysenteriae* reference WA1 (NC_012225.1) using Bowtie2 (version 2.1.0) [328]. Samtools (version 0.1.18) was used to make a SAM file of reads mapping to WA1 using the command *samtools view -f4 –bS*. This was then converted to a BAM file with samtools and BAM files were converted into fastq files with Bam2Fastq (version 1.1) [241, 329].

5.3. Results

To analyse the healthy pig gut microbiome of commercial pigs, 12 caecal samples from healthy pigs were sequenced. All caecal samples were taken at slaughter as part of VM0518. Samples were sequenced on two MiSeq runs. Initially, samples from Yorkshire and the Midlands were sequenced on a single MiSeq run, and subsequent samples from the South West were sequenced on another MiSeq run.

For caecal samples (Table 5.3) less than 4% of reads were mapped against the RefSeq database. The median percentage of reads mapping to the RefSeq database was 0.76%,

with the lowest mapping was 0.34% (MSG12P2), and the highest was 3.23% (MSG06P2). It is likely that most of the DNA in the sample is pig DNA; this would not be detected by the bacteria specific RefSeq database. In addition, low read mapping may also be affected by the database used. Some species diversity is not represented in the database because of a lack of reference genomes. However, the important genera in the pig gut microbiome, for example, *Prevotella*, are represented in the database so it will still be possible to characterise the caecal microbiota.

Table 5.3: Sequencing of caecal samples
Total sequenced reads and reads mapped to the RefSeq database by Sigma from sequenced caecal samples.

Sample	Total Reads	Mapped Reads
MSG20P2	1,137,474	5,066
MSG44P2	4,935,338	49,436
MSG54P2	1,623,570	9,724
MSG54P3	5,157,134	64,372
MSG54P4	1,321,260	10,888
MSG54P5	1,137,474	5066
MSG07P2	4,273,442	25,044
MSG16P2	4,214,036	31,486
MSG22P2	2,934,490	11,828
MSG11P2	2,963,454	36,480
MSG14P2	2,612,186	42,932
MSG12P2	3,823,838	29,712
MSG06P2	3,111,156	100,788
MSG15P2	3,119,832	23,630
MSG38P2	3,823,838	13,122

5.3.1. Speciation of caecal samples

To investigate differences between the microbiome in different regions sequenced caecal samples were speciated (Appendix 3), and the relative abundance predicted was visualised as a heatmap (Figure 5.3.1.1) and used to conduct PCA (Figures 5.3.1.2a and 5.3.1.2b). In total 36 species were identified with most abundant species being *Lactobacillus* species and *Eubacterium rectale*. There also appeared to be a core group of species that were present in most isolates; it is likely that these species, for example, *Faecalibacterium prausnitzii*, are present in all samples but are below the detection limit. The *Lactobacilli* species varied between samples and did not show a difference based on region, or based on production type**.** There was also little variation between samples from the same farm; this was expected as these pigs were grown at the same time and are likely to be fed the same diet. More species were detected in MSG54P3; this is likely to be due to a greater sequencing depth. No large differences were observed between different regions, and this was indicated by PCA where the majority of farms cluster together (Figure 5.3.1.2a). Although there did appear to be some variation between the South West and other regions and was more likely to be due to differences between finisher farms and farrow-to-finisher farms (Figure 5.3.1.2b). There was one outlier MSG15P2; this isolate came from a farm where visitors regularly drove onto the farm. It is possible that this could have resulted in the introduction of bacteria from other farms, for example via vets, visitors or farm workers. LDA analysis did identify species that were differentially abundant in different regions and production systems. *Eubacterium rectale* was associated with the Midlands (with an LDA score of 5.39), this may be due to higher levels seen in samples from MSG54. In addition, *Prevotella melaninogenica* (LDA score of 4.14) was associated with farrow-to-finish farms, and *E. siraeum* (LDA score of 3.96), *C. difficile* (LDA score of 4.08), *Methanobrivibacter smithii* (LDA score of 4.5), and *Streptococcus suis* (LDA score of 4.14) were associated with finisher herds (Figure 5.3.1.3). However, most of these species (*M. smithii*, *C. difficile, E. siraeum, and S. suis)* were only identified in one sample (MSG06P2), this highlights the need for caution when interpreting results from a small sample size. It is likely that these do not reflect significant biological differences between production types or region of origin. *P. melaninogenica* was only found in one finisher herd, this could be due to differences in diet, but it may also be due to the small number of finisher herds sequenced. To investigate further, it would be necessary to

sequence more samples at a greater depth of sequencing. Based on the overlap of species identified, and the similarity of the PCA analysis it can be concluded that differences between production type or region of origin appear only to have a small impact on the caecal microbiome.

Figure 5.3.1.1: Heatmap of species identified in caecal samples.

White indicates that the species was absent. Finisher farms are indicated by *. Samples from Yorkshire and shown in green, samples from the South West are in red, and samples from the West Midlands are shown in blue. Scale represents percentage abundance of mapped reads as detected by Sigma.

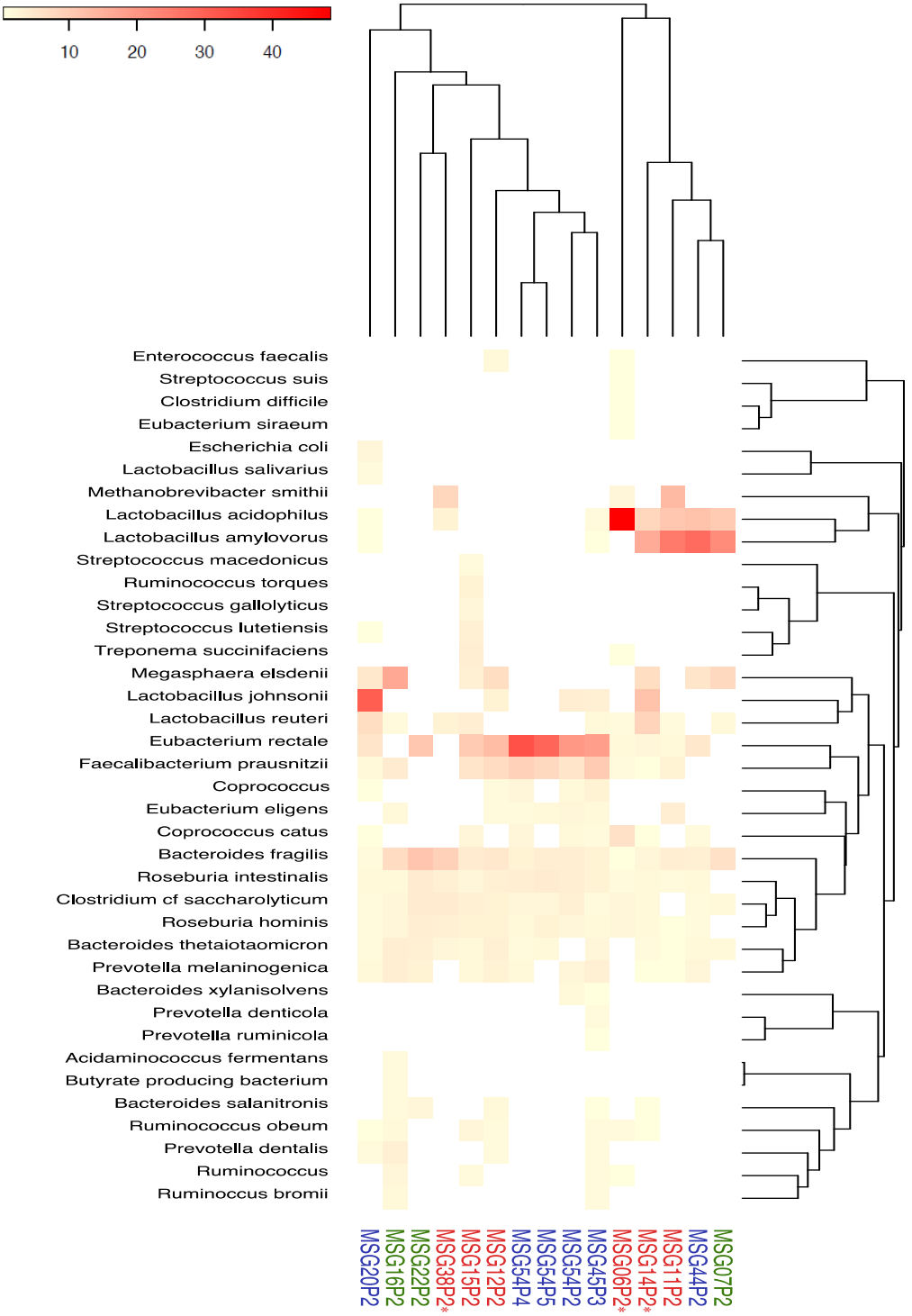
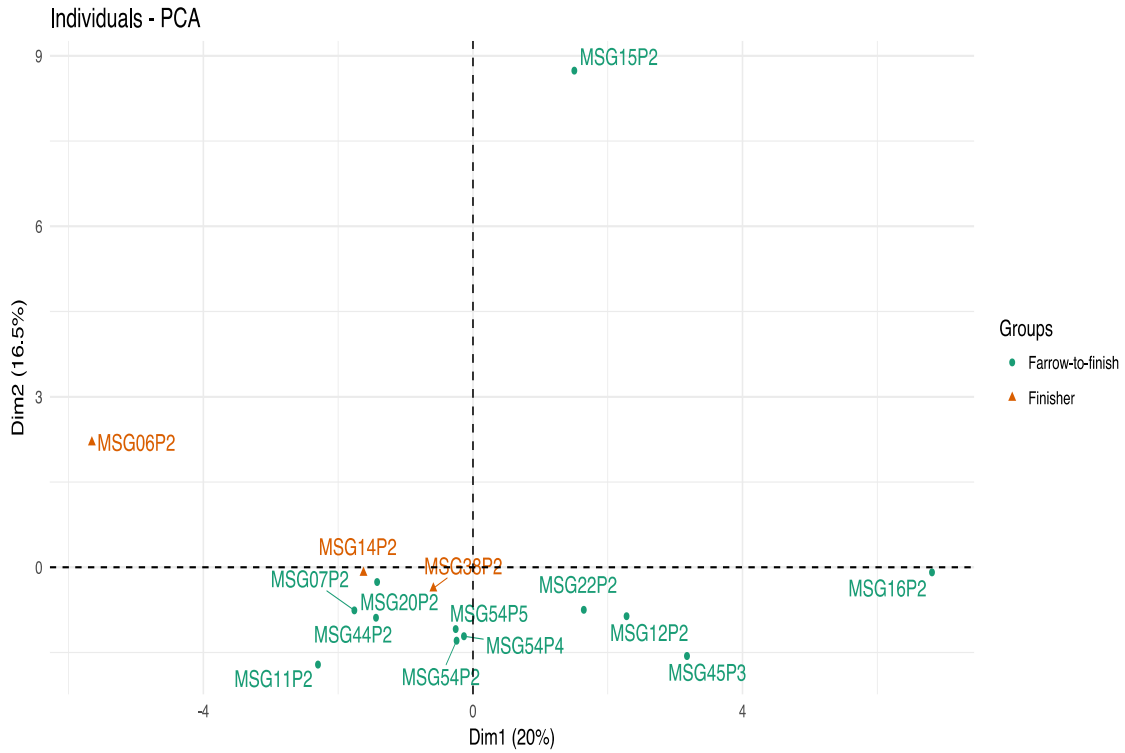


Figure 5.3.1.2: PCA analysis of the relative abundance of species in caecal samples. In both graphs, points are labelled with the name of the sample. Samples are labelled with the sample name. In a, samples are coloured by production type. In b, farms are coloured by region.

a



b

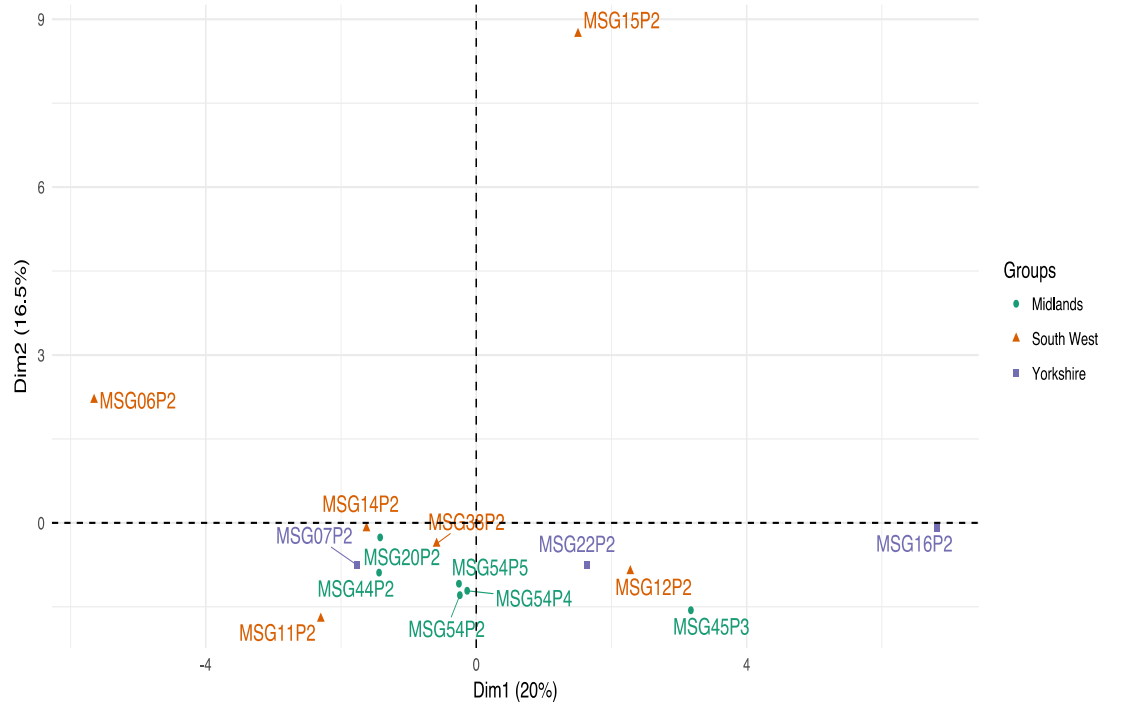
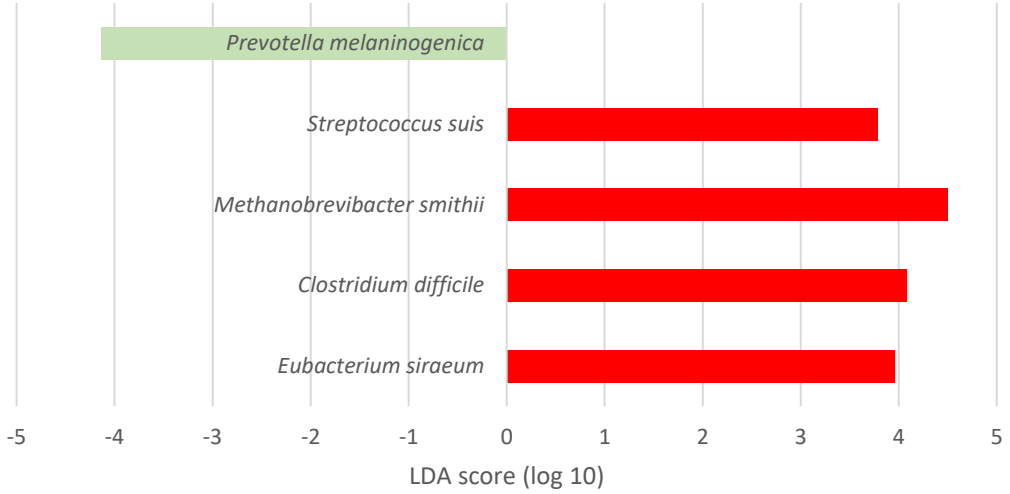


Figure 5.3.1.3: LDA analysis of caecal samples based on production type
Differentially abundant species in caecal samples identified by Lefse when two production types are compared. Species associated with farrow-to-finish has been shown in green, while species associated with finisher samples are shown in red.



5.3.2. Antimicrobial resistance genes in caecal samples

The pig gut microbiome is likely to be a critical area of AMR gene transfer; the SeqFinder database and SRST2 was used to explore AMR gene presence. A small number of AMR genes were detected in caecal samples; this is likely due to using a cut-off of 15x coverage (Table 5.3.2). AMR genes were normalised to parts per million to ensure higher AMR detection was not merely due to a greater read depth. This was used because at lower depths of coverage SRST2 was unable to distinguish between alleles of the same gene. However, it was possible to identify multiple AMR genes, therefore useful information can still be obtained with this cut-off. AMR genes were normalised to parts per million to aid comparisons of samples with different sequencing depths. There was little variation detected between the four samples from MSG54, probably due to the high cut-off used, although two samples had two more AMR genes detected than others. It is likely that if these samples were sequenced at a higher depth more variation in AMR gene carriage would be detected. There was more variation between farms, with the number of AMR genes detected varying between one and five. Tetracycline resistance genes were the most commonly identified AMR genes, with *tet(Q)* present in all samples. *Also, tet(Q)* was the most common tetracycline resistance gene detected in samples from Yorkshire and the Midlands, while *tet(W)* was more common in samples

from the South East. The least common gene detected was *tet(43)*, present in two isolates, one from Yorkshire and the Humber, and one from the South West. In addition, MSG20P2 contained an occurrence of *tet(Q)* genes; this may suggest that multiple copies of this gene are present in the sample. Finisher herds had fewer AMR genes detected than breeder-finisher (with an average of 6 genes detected in farrow-to-finish farms, and 10 genes detected in finisher farms), but it is likely that if more finisher samples were sequenced more AMR genes would have been detected. There was no clear difference between regions of England, or between production types. It is likely that AMR genes detected are affected more by the composition of the microbiome and antibiotic practices on the farm than region or production type.

Table 5.3.2: AMR genes detected in caecal samples by SRST2 using the Seqfinder database.

AMR genes have been normalised to parts per million. This was done for all samples and was done to ensure higher AMR genes detected was not due to a greater depth of sequencing in a sample. Numbers represent the reads per million total reads. Only genes with a depth of coverage of 15 or greater have been included.

Sample	Region	Type	cfxA	lnuC	mefA	tet39	tet43	tetQ	tetW
MSG20P2	Farrow-to-finish	Midlands	18.47	24.58	65.22	0	0	91.77	32.27
MSG44P2	Farrow-to-finish	Midlands	6.56	4.23	19.85	0	0	37.53	7.64
MSG54P2	Farrow-to-finish	Midlands	9.96	0	25.08	0	0	35.83	0
MSG54P3	Farrow-to-finish	Midlands	14.43	3.95	26.16	0	0	41.07	6.82
MSG54P4	Farrow-to-finish	Midlands	15.75	0	21.41	0	0	29.64	0
MSG54P5	Farrow-to-finish	Midlands	14.47	0	22.39	0	0	31.08	0
MSG07P2	Farrow-to-finish	Yorkshire	0	0	0	0	0	10.08	5.45
MSG16P2	Farrow-to-finish	Yorkshire	7.62	0	23.21	0	0	43.06	9.73
MSG22P2	Farrow-to-finish	Yorkshire	9.16	0	27.4	0	6.09	49.58	9.44
MSG11P2	Farrow-to-finish	South West	0	0	0	0	0	12.08	0
MSG15P2	Farrow-to-finish	South West	10.02	0	15.4	0	0	18.46	8.04
MSG12P2	Farrow-to-finish	South West	11.86	6.13	25.81	5.26	0	42.81	6.55
MSG06P2	Finisher	South West	0	0	5.16	9.85	6.33	8.91	18.86
MSG14P2	Finisher	South West	0	0	15.34	0	0	24.05	5.67
MSG38P2	Finisher	South West	0	5.53	15.24	0	0	20.65	0

5.3.3 Sequencing of metagenomic faecal samples from healthy and swine dysentery positive pigs

To identify *B. hyodysenteriae* directly from faeces, six faecal samples from swine dysentery positive pigs were used. In addition, to investigate changes in the microbiome in SD, these were compared to healthy faecal samples collected from pigs of a similar age. The latter samples were provided by the APHA and came from a study from a single farm [264]. All samples were sequenced on a single MiSeq run, as this likely reflects the practices that would occur if metagenomics were used for diagnostics.

Like caecal samples (Table 5.3) few reads in the faecal samples were mapped against the RefSeq database (Table 5.3.3). The median percentage of faecal samples reads mapping to the RefSeq database was 1.33%, the lowest mapping was 103 (0.43%), and the highest was 123 (18.41%). This could be due to high amount pig DNA in the samples, and it may also reflect the database used. If this analysis were to be repeated Sigma would be used with an alternative database, for example, the Kraken database [240] or a pig metagenomic catalogue [33].

Table 5.3.3: Sequencing of faecal samples
Information on reads mapped to the RefSeq database by Sigma for sequenced faecal samples.

Sample	Total Reads	Mapped Reads
14	4549728	217842
26	2908150	60396
123	4836474	890210
161	1475026	12270
168	3029384	155458
174	5082480	218940
8	3425410	36760
69	4275744	56912
55	4782040	27736
100	17191830	115614
103	897996	3880

5.3.4. Identification of *B. hyodysenteriae* directly from faeces.

It was possible to identify *B. hyodysenteriae* in all swine dysentery positive samples using both Sigma and Bowtie2. SRST2 then used to identify the *B. hyodysenteriae* ST type of *B. hyodysenteriae* in faecal samples. MLST alleles could only be identified in sample 14; four alleles were identified: *est* 3, *gdh* 12, *glpK* 11 and *thi* 36. There were three STs that contained these alleles: 8, 69 and 139. To analyse further, *B. hyodysenteriae* reads mapping to the reference strain WA1 from all swine dysentery positive samples were extracted using Samtools (Figure 5.3.4.1) [329]. It was not possible to identify any AMR genes or SNPs associated with chromosomal genes which result in AMR. For one sample, 14, there was enough reads to construct a core-genome based phylogenetic tree with previously sequenced samples and samples from NCBI (Chapter 3, section 3.3.2) (Figure 5.3.4.2). In the tree, there were 1134 SNPs and 10 SNPs per isolate on average. All STs clustered as previously (Chapter 3, section 3.3.2), and 14 clustered in the ST 8 clade, and the main difference was the global cluster W become 2 smaller clusters. It was also possible to identify two sequenced isolates 14 was most closely related to (js97 and js99), this could be useful for root cause analysis in an outbreak investigation. This is far fewer SNPs than were detected previously (Chapter 3, section 3.3.2) and is likely due to the small number of reads in 14. It is, therefore, possible to gain epidemiologically useful information (in this case ST, and closet related strains) by metagenomic sequencing; but this sample contained a high number of reads mapping to WA1 compared to other samples, as 0.95% of total reads mapped to WA1 (Figure 5.3.4.1). It is likely that for most samples a higher sequencing depth would be required to obtain this information. This would be more expensive but the development of new sequencing platforms, for example, the Illumina NextSeq, could make this possible at a reasonable cost and speed.

Figure 5.3.4.1: Percentage of total reads extracted from faecal samples after mapping to *B. hyodysenteriae* WA1 by Bowtie2.

Reads were extracted as described previously (Chapter 5, section 5.2.6). Percentage of total reads extracted are shown, and the total reads have been given in brackets.

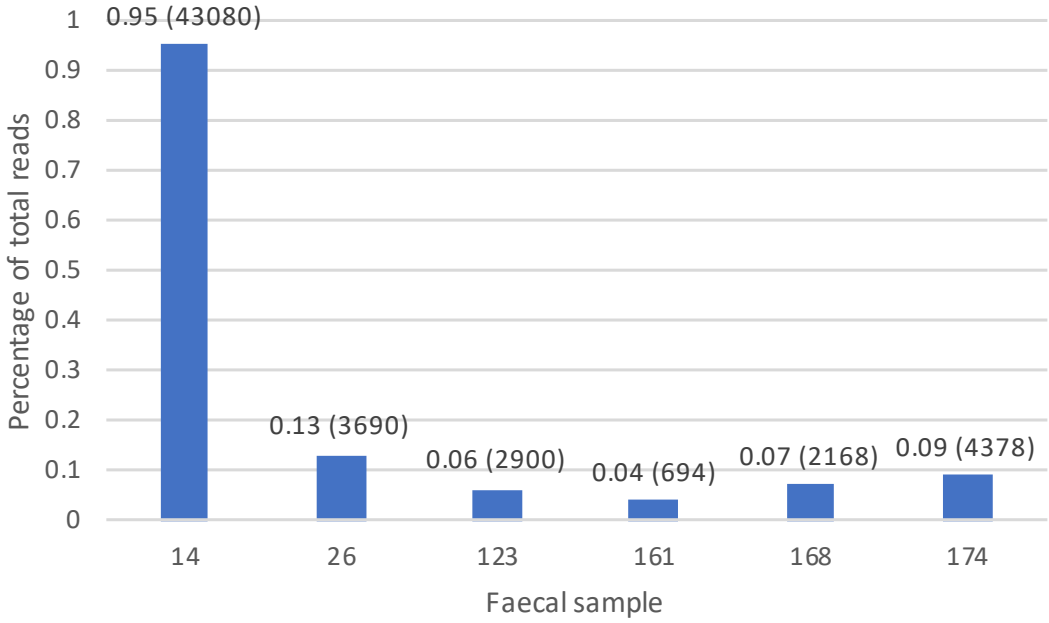
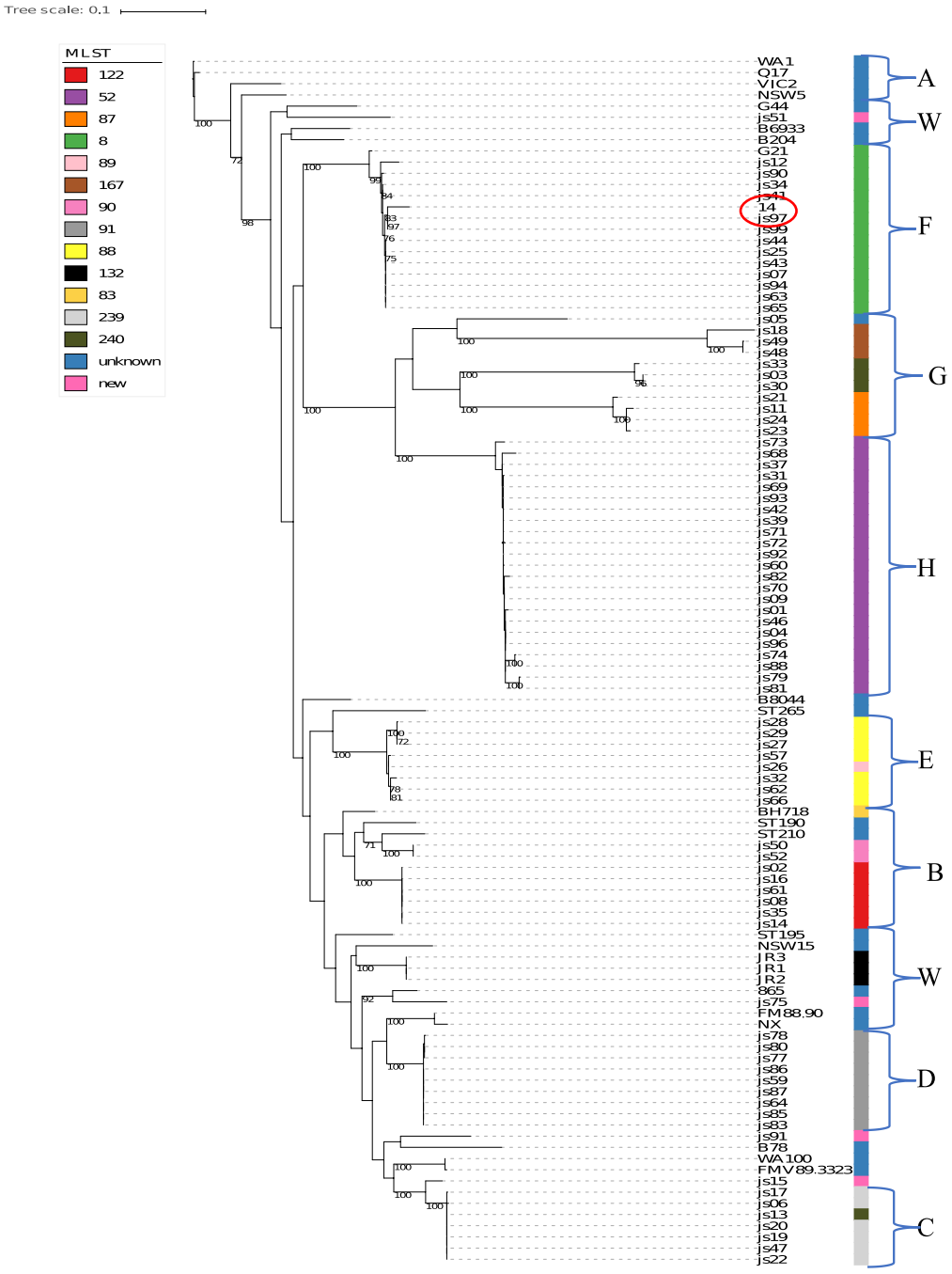


Figure 5.3.4.2: Maximum likelihood core-genome tree of sequenced *B. hyodysenteriae* isolates and *B. hyodysenteriae* genes extracted from faecal sample 14.

Reads were extracted as described previously (Chapter 5, section 5.2.6). Sample 14 has been circled in red. 1000 bootstraps were conducted with RAxML. Bootstrap support is shown when it is above 70. New refers to new ST identified in this study (Chapter 3). Unknown includes global isolates where the MLST was not specified or determined by clustering with UK isolates.



5.3.5. Speciation of faeces

There were 54 species identified in faecal samples. This is higher than the amount identified in caecal samples. This could imply that there a greater diversity of species in the faecal microbiome, and in part may be due to contamination from the ground before collection. The median number of species identified in healthy isolates was 15; while the median species identified in swine dysentery, positive samples was 17. There was some divergence among the healthy faeces; the number of species varied between 7 and 17 for the healthy faeces collected from the same farm. This likely reflects natural variation between individuals and variation in the depth of sequencing between samples. Swine dysentery positive and healthy samples were stored at different temperatures (healthy faecal samples were stored at –80 °C and swine dysentery positive samples were stored at -20 °C). Some swine dysentery positive samples had also been subject to more freeze thawing as they had been used for previous projects and transported between sites. The freeze-thawing could damage the DNA and may have resulted in poorer sequencing, and fewer species being detected. Therefore the diversity of the swine dysentery microbiome is underreported in this study.

In the faecal microbiota, most species were present in both healthy samples and swine dysentery positive samples. However, there were three species that were present only in swine dysentery positive samples (Appendix 4 and Figure 5.3.5.1). *B. hyodysenteriae*, *Campylobacter coli* and *E. coli* were only identified in swine dysentery pigs (Figure 5.3.5.1). *E. coli* was identified in all swine dysentery positive samples at a similar percentage abundance to *B. hyodysenteriae. C. coli* was only identified in four swine dysentery positive isolates and was present at lower percentage abundance. In addition, some unusual species were identified in some samples, in particular, *Pshychobacter articus* and *Shigella sonnei*. *P. articus* is most likely a contaminant, potentially from the soil the faeces were collected from; it is also possible that it could be identified in error due to genes shared with other species, for example, *S. sonnei* is likely to an *E. coli* strain. Removal of these species arbitrarily could introduce greater error into the study, due to research bias, and therefore they were not removed from the analysis. These unusual species only composed a small amount of the total species identified, for example, *S. sonnei*, was only identified in one sample at 0.29% of mapped reads and the

dominant species in the sample were commonly identified in pig metagenomes. Further analysis of the microbiome by PCA appeared to suggest a small difference between the swine dysentery positive samples and the samples from healthy pigs (Figure 5.3.5.2).

To identify if there were any statistical differences between the two groups LDA was used. This identified five species that were differentially abundant in swine dysentery positive and healthy samples. *B. hyodysenteriae, Campylobacter coli* and *E. coli* were associated with SD, with LDA scores of 4.4, 4.04 and 4.8 respectively; while *Ruminococcus bromii* and *Treponema succinifaciens* were associated with healthy pigs with an LDA score of 4.39 and 4.4 respectively (Figure 5.3.5.3). *C. coli* is a known pathogen of pigs. To identify if the *E. coli* was also pathogenic, SRST2 was used to screen the raw reads against Enterotoxigenic *E. coli* (ETEC) specific genes, commonly found in pig ETEC strains. The genes used for the screen were the virulence genes *estA, eltA* and *Stx2e* [330, 331]. These ETEC specific genes were not identified in any samples; it is likely that commensal *E. coli* that have already colonised the swine dysentery positive pigs are able to take advantage of the environment that develops in swine dysentery or that the pigs were colonised by other *E. coli* pathotypes.

Figure 5.3.5.1: Heatmap of species identified in faecal samples.

Swine dysentery positive samples are labelled in red. *E. coli*, *C. coli* and *B. hyodysenteriae* are highlighted in a red box. Numbers in the samples bar represent relative abundance as predicted by Sigma.

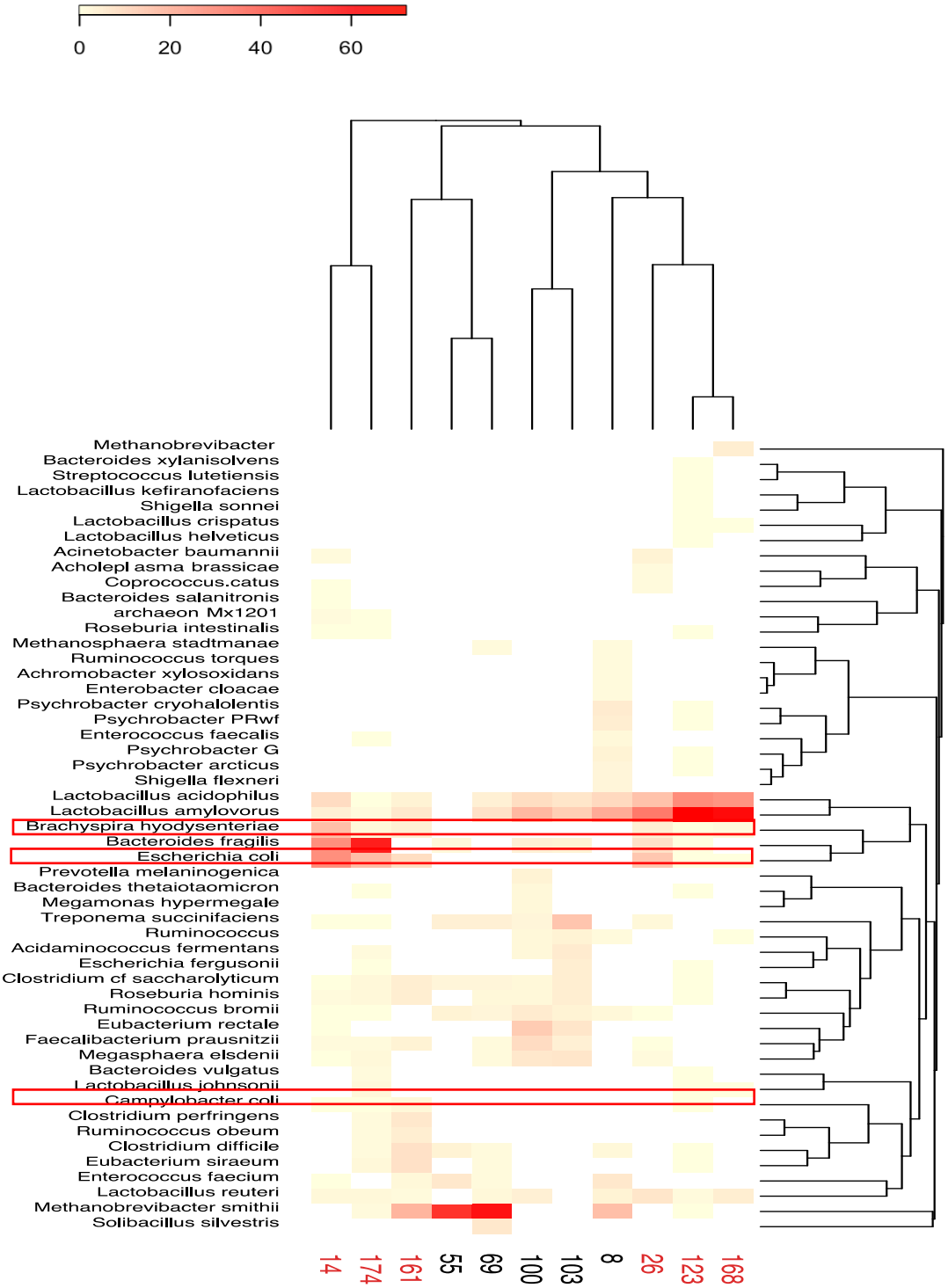


Figure 5.3.5.2: PCA analysis of faecal samples.

Samples are labelled by sample name and are coloured by their health status. SD refers to swine dysentery positive.

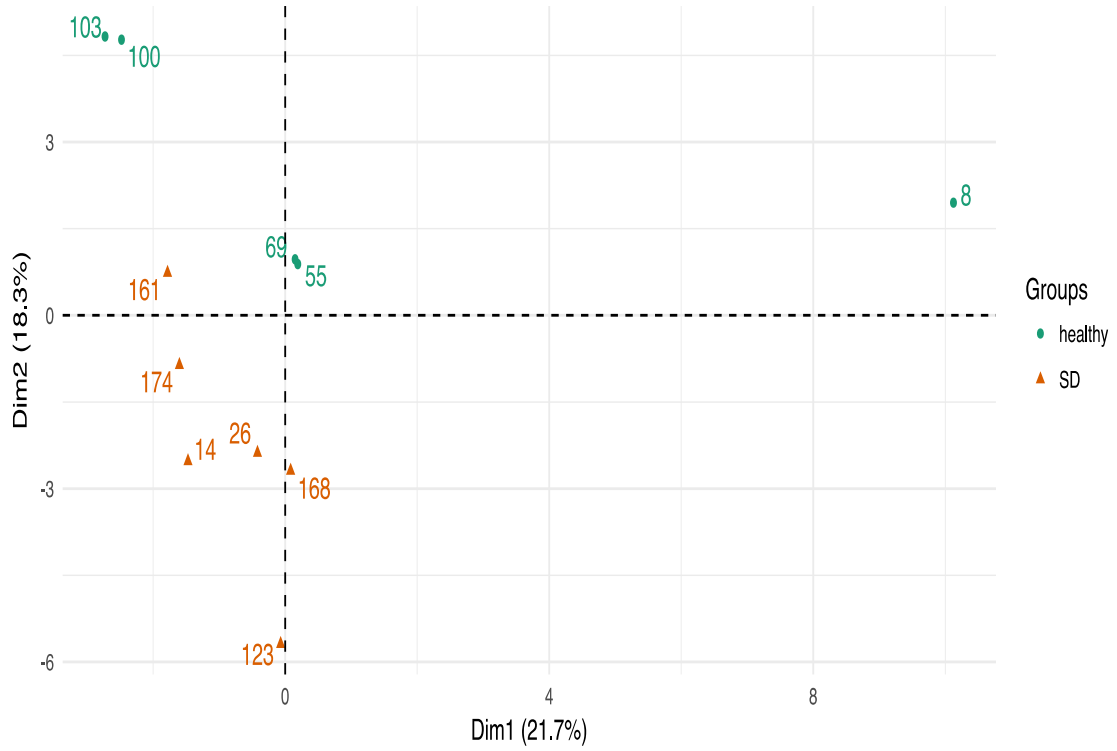
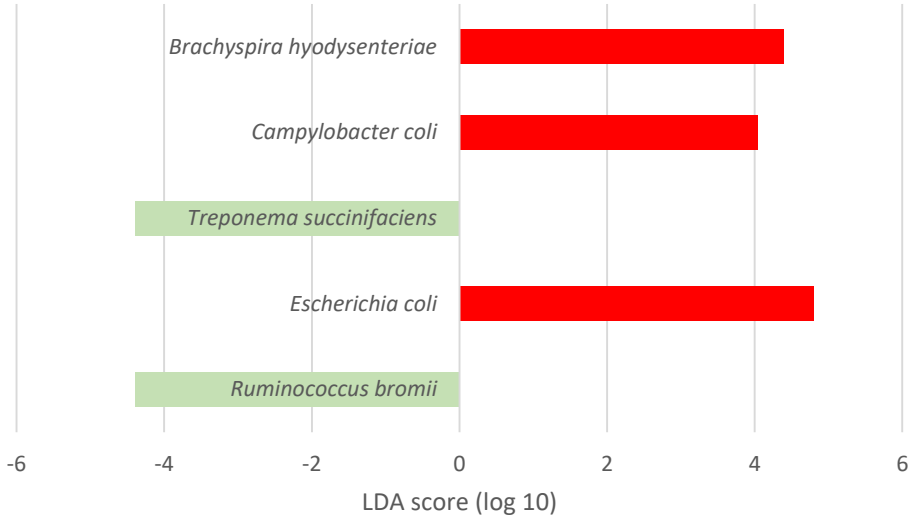


Figure 5.3.5.2: LDA analysis of faecal samples

Differentially abundant species in swine dysentery positive and healthy samples identified by Lefse. Species associated with healthy samples are shown in green; while species associated with swine dysentery samples are shown in red.



5.3.6. Antimicrobial resistance genes in faecal samples

Similar AMR genes were found in the faecal sample as the caecal samples. However, a greater variety of AMR genes from the following classes were detected than in caecal samples sulphonamide, aminoglycoside, streptomycin, and trimethoprim (Table 5.3.5.). Like the caecal samples, the majority of AMR genes are tetracycline resistance genes, with the most common AMR gene being *tet(W),* followed by macrolide resistance genes and the lincomycin resistance gene *lnu(C)* also common. *lnu(C)* has recently been linked to lincomycin resistance in *B. hyodysenteriae,* and the MTnSag1 transposon that has been associated with lincomycin resistance in *B. hyodysenteriae* was detected in all samples that contained *lnu(C).* This transposon appears to be common, so it is possible that in the future more *B. hyodysenteriae* isolates will be identified that contain this transposon [124]. Although all healthy samples originated from the same farm and were collected at the same time, there were variations in the AMR genes detected. This could reflect differences in the total number of reads in each sample, but it may also reflect variation in the microbiome between individual pigs.

There was a lot of variation in AMR gene carriage between swine dysentery samples, probably because swine dysentery samples have come from a wide geographical area and were collected in different years. There was no clear difference between healthy and swine dysentery samples.

Table 5.3.5: AMR genes detected in faecal samples

AMR genes have been normalised to parts per million. This was done for all samples and was done to ensure higher AMR genes detected was not due to a greater depth of sequencing in a sample. Numbers represent the reads per million total reads. Only genes with a depth of coverage of 15 or greater have been included.

Sample	123	14	161	168	174	26	100	103	55	8	69
Health status	SD	SD	SD	SD	SD	SD	H	H	H	H	H
aad6_b	0	0	11.46	0	0	0	0	0	0	0	4.3
aadA1b	0	0	0	0	0	6.07	0	0	0	7.57	0
ant3-1a	0	0	0	0	0	6.38	0	0	0	7.99	0
aph3-Ib	0	0	0	0	0	7.46	0	0	0	7.18	0
aph3-IIIa	0	0	0	0	5.71	0	0	0	0	0	0
aph6-Id	0	0	0	0	0	8.88	0	0	0	9.4	0
cfxA-3	0	0	0	0	5.26	0	3.85	0	0	0	0
ermB	25.2	4.23	0	0	6.47	13	0.42	0	0	0	0
ermF	0	0	0	0	3.5	0	0.32	0	0	0	0
ermG	3.4	0	0	0	4.29	0	0.46	0	0	0	0
lnuA	67.39	0	0	0	0	0	0	0	0	0	0
lnuC	3.51	7.43	20.56	0	20.13	0	4.6	0	5.83	0	5.06
mefA	3.27	4.22	0	0	7.68	5.71	6.23	0	0	0	0
sat4A	0	0	0	0	3.28	0	0	0	0	0	0
sul2	0	0	0	0	0	0	0	0	11.53	22.09	4.3
tet39	0	0	0	0	0	0	0	0	0	0	0
tet43	0	0	25.94	0	6.24	0	1.18	0	10.03	10.31	15.06
tetO	13.56	8.72	0	0	38.77	9.31	28.93	0	6.38	0	5.82
tetQ	13.56	8.72	0	0	38.77	9.31	28.93	0	6.38	0	5.82
tetW	25.86	11.93	22.26	5.18	25.23	6.54	8.73	21.42	11.98	7.6	20.38

5.4. Discussion

In this study, two aspects of the gut microbiome were examined: the bacterial species present in the microbiome and the presence of AMR genes. Similar AMR gene classes were detected in caecal and faecal samples, although there was a greater variety of AMR genes detected in faecal samples. A higher number of species was also detected in faecal samples compared to caecal samples; this has been found previously in two pig breeds (*Bamaxiang* a Chinese micro-pig breed and *Erhualian* another indigenous Chinese pig breed), and may reflect a more varied microbiota in faeces [332]. In both faecal and caecal samples, the most common AMR genes were tetracycline and macrolide resistance genes. This has been found in previous studies investigating pigs, where there was a high prevalence of tetracycline resistance genes [32, 333]. *erm*, genes and *tet* genes have been found in a diverse range of anaerobic bacteria including *Lactobacilli, Prevotella,* and *Bacteroides s*pecies; species that were identified in the faecal and caecal samples. [334]. In *Bacteroides* species, *tet(Q)* was most abundantly, and this may have contributed to the high level of *tet* genes identified [335].

There were several genes found at high prevalence in pigs from France, Denmark, and China that were not detected in this study; for example, cephalosporin resistance genes [33]. It may be that these genes are only present at low levels, and are below the detection limit, or a reduction in the levels of cephalosporin use on farms has led to a reduction in the presence of the cephalosporin resistance genes in the pig microbiota [292]. In a previous study, *E. coli* containing *mcr-1* was isolated from faecal samples from the same holding by microbiological culture and PCR, as the faecal samples used in this study [264]. Neither *E. coli* nor *mcr-1* was detected in this study. It is likely that the level at which they are present are below the detection levels for this study. Although some AMR genes may be below our detection level, it was still possible to identify some important AMR genes, and compare AMR carriage between healthy and swine dysentery positive samples, where there appeared to be little difference.

For speciation of both caecal and faecal samples, few reads were mapped to the RefSeq database when Sigma was used. In part, this may be due to the presence of host DNA in our samples [336]. The database, and the program used, are also likely to have a large impact. Sigma was used because after reads have been mapped to reference sequences

in the database Sigma calculates which species are most likely to be present in the sample [321]. However, unlike Kraken [240] it does not come with a pre-built database. Therefore, one had to be provided; in this study, the RefSeq database (2014) [337] was used as it was a curated database. At the time this study was conducted this appeared to be the most appropriate methodology. Kraken could have been used, but there is no probabilistic analysis after species are identified to determine which species are correctly identified, therefore false positives might be identified. As an aim of this study was to successfully detect *B. hyodysenteriae* in swine dysentery positive faecal samples it was important to avoid false positives, therefore Sigma was used instead of Kraken. However, there are issues with the use of the RefSeq database reference database; previously a low percentage mapping has previously been found in human metagenomic samples where the majority of sequenced reads did not map to the Genbank database [338]. The Genbank and the RefSeq databases are composed of genomes published on NCBI and contain species isolated from a diverse range of environments [274, 339]. In addition, some species are sequenced disproportionally highly due to their importance in human health, for example, the most abundant clade in RefSeq is *Escherichia-Shigella* [337, 340]. If this analysis were repeated a recently produced pig metagenomic catalogue would be used, this might have improved the analysis. In addition, new programs have been produced that would enhance the analysis. Braken uses Bayesian statistics to analyse the Kraken output and reclassifies species based on the species most probably to be there [341]. The use of Kraken either with the pre-existing database, or the pig metagenomic catalogue, would be able to speciate more reads, and Braken would enable correct identification of species.

When the caecum was speciated, genera that had previously been detected in the caecal microbiome were identified, including *Prevotella* and *Lactobacilli* [33, 342-347]. Only a small number of previous studies have investigated the caecal microbiome using shotgun metagenomics [33, 342]. The caecum has been more extensively studied using 16S analysis [343-347]. However, comparison to previous studies is complicated by the different diets used, the breed of pig and the antibiotic use, all of which impact the microbiome [32, 33]. Nevertheless, previously there was an agreement at the phylum level that the caecum was dominated by *Firmicutes* and *Bacteroidetes*, and this was also found in our study [342-347]. At the genus and species level, some differences arose between all studies, and between individuals in the same study [342]. *Prevotella* is a

genus that is often found to be present at high levels in the caecum [346]. The samples used in this study were collected at slaughter and are older than many pigs investigated. The abundance of *Prevotella* has been found to decline with age and is present at lower levels in older pigs [35, 342]. An increase in *Lactobacilli* has been observed with age [35]. As species identified in this study using older pigs were similar to previous research, it is likely that they accurately reflect a healthy ceacal microbiome.

Further analysis by PCA did not indicate any difference between the microbiome of caecal samples from pigs from different regions of England. There did appear to be a small difference between different production types. This could be due to the inflow of new weaner pigs onto a farm, introducing new strains of different species, which could lead to a more dynamic microbiome, especially if weaner pigs are supplied from multiple herds, than seen on breeder-finish herds, which are closed systems [14]. MSG07P2 is a farrow-to-finish farm that appeared as an outlier. This farm had seven vehicles coming into the farm per week, and it is possible that they may introduce new species onto the farm in a similar way to new pigs. Further investigation with LDA identified a number of species that were differentially abundant between groups, but only one that could reflect a difference between the groups. This was *P. melaninogenica,* this species is typically found in the oral microbiome and is likely a related *Prevtella* species [348]. An increase in *Prevotella* may reflect the feeder diet used, or it may reflect the small number of finisher herds samples. It would be necessary to sequence more caecal samples from finisher herds to investigate this further. Few significant differences were detected between regions or production types and may reflect a shared microbiome between pig herds. This appears consistent with previously published studies where there appeared to be a core microbiome, with the same phylum and genus evident in multiple studies, albeit at differing abundances [33, 342-347].

It does not appear likely that differences between healthy and swine dysentery positive pigs are due to these factors, and may be due to environmental changes that occur during SD. When swine dysentery positive faeces were sequenced, it was possible to identify *B. hyodysenteriae* in all samples. For faecal sample 14, it was also possible to identify the ST and the most closely related isolates. However, this was only possible as *B. hyodysenteriae* was present in high abundance in this sample. Typical abundance can be as low as 0.006% in colonic luminal contents [319], and the median abundance in

our study was 2.02%. Therefore, it is likely that to get clinically relevant data from metagenomics it would be necessary to either enrich the sample, or sequence to a higher depth. Enrichment in pyruvate media and the addition of acriflavin, cefsulodin and vancomycin were previously used to enrich spinach spiked with Shiga toxin-producing *E. coli*. Reads mapping to *E.coli* in spinach spiked with 10 CFU/ml rose from 0.01% pre-enrichment to 49.73% after a 23-hour enrichment [349]; a similar method might be useful for *B. hyodysenteriae* enrichment. Although this would increase the time taken it would still likely be faster than culturing *B. hyodysenteriae* and performing biochemical tests.

A variety of other species were also detected in sequenced faecal samples, with most species being present in both groups. One of the main species detected in both groups was *Lactobacillus* species. Previously, *Lactobacillus* has been associated with healthy samples; differences in the current study may be due to differences in the experimental design. In the previous study, pigs were grown at the Swine Nutrition Farm at Iowa State University and euthanised at nine weeks of age [226, 319]. The swine dysentery positive samples in our study came from clinical submissions to the APHA from different farms, and, although for three samples the age was unknown the other samples were aged between 14 and 17 weeks. Healthy samples were also 17 weeks of age. Variations in diet were also likely to have an impact on *Lactobacilli* abundance as has age, where there is an increase in abundance as pigs age [35, 350]. This could have resulted in the higher levels of *Lactobacilli* being observed in our samples.

Faecal samples from swine dysentery positive pigs and healthy pigs appeared to cluster separately by PCA. This was supported by LDA, where four species that were associated with different disease states were detected. *E. coli, C. coli* and *B. hyodysenteriae* were associated with swine dysentery isolates, while *R. bromii* and *T. succinifaciens* was associated with healthy pigs. However, the samples from healthy pigs came from one farm, and therefore the difference could be due to variation between farms. *T. succinifaciens* has previously identified in pigs and appears to be commensal, and has not previously identified in metagenomic analysis of swine dysentery pigs [319, 351, 352]. *R. bromii* is important in the degradation of resistant starch in humans; resistant starch along with non-starch polysaccharides have been previously linked with

an increased risk of swine dysentery [219, 353]. It is possible that the degradation of resistant starch by *R. bromii* might have a protective effect. Previous analysis of swine dysentery microbiome also found the *Ruminococcus* genus to be associated with healthy pigs [319].

C. coli is commonly identified in pigs and has previously been associated with swine dysentery [319, 354, 355]. It is likely that there is an important relationship between these two species as *C. coli* has had a long association with SD, but this has not been explored in detail [355]. Unlike *C. coli* an increase of *E. coli* in swine dysentery has not previously been identified. *E. coli* is a part of the healthy gut microbiota and are present throughout the life of the pig, although there is a turnover of *E. coli* strains present [356]. As no ETEC genes were identified it is possible the *E. coli* are commensal strains that can take advantage of the conditions created by *B. hyodysenteriae* or belong to other pathotypes. Commensal *E. coli* are non-motile and have been found to be associated with the mucosa in the large intestine where they multiply rapidly in excess of the turnover rate of mucin [357-359]. In the mucosa, there can be increased expression of ferric iron uptake genes by *E. coli* and increased production of enterobactin, compared to the lumen [359]. This suggests that a limiting factor for *E. coli* is ferric iron in the mucosa. During swine dysentery, bloody diarrhoea is produced which would lead to a more plentiful supply of ferric iron in the gut lumen. This could result in increased growth of *E. coli.* In previous studies, other commensal species have been found at higher relative abundance in swine dysentery including *Mogibacterium* and a potential opportunistic pathogen *Anaerotruncus* [319, 360, 361]. It is likely that there are a range of different commensals that can multiply in the environment created during SD.

In this study, it was demonstrated that it is possible to sequence *B. hyodysenteriae* directly from clinical samples. Whilst this may not be a practical diagnostic technique currently, with continued decreases in the cost of sequencing, and enrichment of samples, it may become one in the future. The time for diagnosis would also go down as expertise develops in this area. In addition, an association between *B. hyodysenteriae* and *E. coli* during infection, which has not been reported previously, was identified. Further research will be needed to elucidate the details of this.

Chapter 6. Conclusion

To our knowledge, this has been the largest study performed to date using WGS data for analysis of *B. hyodysenteriae* an important pig pathogen. The data provides a valuable set of sequenced samples for subsequent *B. hyodysenteriae* surveillance in the UK, which can be used in future enabling rapid detection of clonal expansion of an isolate across regions or farms and the acquisition of SNPs or AMR genes which results in antimicrobial resistance. The phylogenetic analysis of *B. hyodysenteriae* (Chapter 3) has indicated differences between the core genome present in *B. hyodysenteriae* globally. In addition, potential differences between pig production systems in different NUTS 1 regions in England were revealed that may have led to different populations of *B. hyodysenteriae* persisting in these regions. The sequencing of 84 clinical isolates of *B. hyodysenteriae* will enable more in-depth swine dysentery outbreak investigation, as it will be possible to trace isolates to specific regions, or potentially holdings, in the UK where the sequence type may have persisted.

Antimicrobial susceptibility testing of 47 isolates using MICs (Chapter 4) indicated a low level of clinical resistance in the UK, but a significant increase in the number of intermediate isolates from 2004 to 2010. This may reflect a long-term trend toward decreased pleuromutilin sensitivity and is likely to have been caused by historic, and current, antibiotic usage. Also, a newly identified gene *tva(A)*, was detected in isolates with an intermediate and resistant tiamulin phenotype. This improved the correlation between phenotype (identified by broth dilution susceptibility testing) and genotype (based on the presence of *tva(A)*) substantially, allowing prediction of *B. hyodysenteriae* isolates with decreased sensitivity to tiamulin or valnemulin. *tva(A)* was further analysed by induction of clinical tiamulin and valnemulin resistance in a sensitive isolate that contained *tva(A)* (js27), and a sensitive isolate without *tva(A)* (js17), by repeat subculturing in sublethal concentrations of tiamulin or valnemulin. Tiamulin and valnemulin sensitivity of both isolates had decreased after 20 subcultures, but clinical resistance only occurred in js27. It appears *tva(A)* does contribute to pleuromutilin resistance and confers resistance to both tiamulin and valnemulin. It is possible that multiple genes, including *tva(A)*, could be involved in pleuromutilin resistance in

clinical isolates, whole genome sequencing provides a mechanism of identifying these new resistance genes.

Using shotgun metagenomic sequencing of swine dysentery positive samples, it was possible to gather epidemiologically useful information on *B. hyodysenteriae* (Chapter 5). A comparison of the microbiome of healthy and swine dysentery positive faecal samples indicated that a number of species may be associated with swine dysentery. These were *E. coli* and *C. coli;* these species may cause secondary infections complicating the treatment of swine dysentery and requires further verification in future. Secondary infections could be further exasperated by the acquisition of AMR genes, making treatment more difficult, for example in *E. coli* commensal isolates have been found to readily accept some antibiotic resistance plasmids [362].

This study has highlighted the value of WGS for the study of *B. hyodysenteriae.* At present there are few published *B. hyodysenteriae* genomes limiting the potential of WGS for the study of *B. hyodysenteriae* but the numbers are likely to increase as the technique becomes more widely adopted, costs decrease, and analysis methods become more standardised. WGS has advantages over existing techniques currently used to study *B. hyodysenteriae.* Currently, MLST is used to investigate the population structure. This has enabled quick comparison to other studies and increased the understanding of the global population structure of *B. hyodysenteriae* [52, 60, 73, 93-95, 121-125]. As demonstrated in this study WGS has the potential to enhance the investigation of the population structure of *B. hyodysenteriae* globally by providing a greater depth of resolution that enables identification of differences between STs. In addition, the bioinformatics tools available for the analysis of sequences are developing rapidly. This has led to the development of powerful analytical programs that have enabled new avenues of investigation to be conducted. For example, in this study, SCOTTI was used to predict the transmission of *B. hyodysenteriae* between regions of England and Wales. The results generated provide a hypothesis of how *B. hyodysenteriae* has spread, which highlighted regional differences. Although this is only a prediction and may not completely reflect the historical transmission events that occurred, it may provide new tools for the analysis of outbreaks increasing the accuracy of the analysis this may provide valuable information useful for biosecurity and control. As well as powerful analytical programs, there has also been a trend in bioinformatics

towards more intuitive programs. For example, Nullabor provides a pipeline designed for WGS diagnostics that automates assembly, annotation and analysis of clinical sequences and provides an easily understandable output [237].

WGS has enhanced the analysis of antibiotic resistance in *B. hyodysenteriae* by enabling the analysis of all genes in the genome. Previously, tiamulin resistant SNPs were detected by sequencing the 23S rRNA gene and *rplC* [159, 163-165, 167]. This identified a number of SNPs that may have contributed to tiamulin resistance; but there have been discrepancies between a tiamulin resistance phenotype and the genotype, with resistant isolates not always harbouring SNPs conferring tiamulin resistance [159, 167]. In this study, examination of the whole genome confirmed the recent identification of *tva(A)* in a larger set of UK isolates. Sequencing of several Italian isolates also revealed a lincomycin resistance gene (*lnu(C)*) that had previously not been reported in *B. hyodysenteriae* [165]. Previously, only a SNP at position 2058 in the 23S rRNA gene was associated with lincomycin resistance in *B. hyodysenteriae* [165]. In this study another SNP was identified in the 23S rRNA G2062T, that may have caused lincomycin and pleuromutilin resistance. It is likely that as more samples are sequenced more resistance genes, and new SNP variants will be identified. In addition, the recent identification of a transposon (MTnSag1) by WGS, suggests that horizontal gene transfer in *B. hyodysenteriae* might be more common than previously expected [165].

Diagnosis of swine dysentery is a slow process complicated by the need to culture *B. hyodysenteriae*. Although WGS provides valuable comparison of clonal isolates identifying potentially new virulence and AMR genes, it would not be considerably faster than existing techniques for rapid clinical diagnosis, due to the need to purify *B. hyodysenteriae* from clinical specimens. Direct sequencing of faeces by shotgun sequencing would be more rapid for diagnosis, and it was demonstrated in this study to identify the closest related sequenced clinical isolates. The techniques used to extract reads, and identify the most closely related clinical isolates could be automated into a pipeline similar to Nullabor that would allow replication by non-experts [237]. This could also be used to detect other important pathogens quickly. The results obtained could be improved by using an alternative database that includes species more representative of the pig; this would result in more reads mapping and better identification of species. However, for routine diagnostic, it would be necessary to

curate the database to avoid false positive identification of pathogenic species. Metagenomic sequencing is currently too expensive for routine diagnostics, but this study has shown it is a possible alternative that can be used for rapid, and as sequencing costs continue to decline it may become a technique that be used for routine diagnosis of swine dysentery.

Metagenomic sequencing is also likely to prove useful for surveillance of AMR genes. Increased surveillance of is a key priority of government antibiotic resistance strategy and has also been included in highly influential reviews [149]. The use of genetic techniques has been suggested as an important component of antibiotic surveillance in the UK government 5-year antibiotic resistance strategy, and metagenomics can complement other genetic based techniques [150]. For example, PCR enables rapid identification of AMR genes, but it cannot provide the total number of AMR genes in a sample. With metagenomic sequencing, this could be possible.

There were a number of results from this study that could be continued. There are other *B. hyodysenteriae* isolates have been sequenced at the APHA, some of these have recently published [293]. Analysis of all *B. hyodysenteriae* isolates sequenced by the APHA would provide greater information on the population structure of *B. hyodysenteriae* in the UK. It would also be possible to predict the spread of *B. hyodysenteriae* between regions of the UK, this may reveal more regional differences or could highlight important nodes where *B. hyodysenteriae* eradication programs would be most effective. Also, the metagenomic analysis presented here could be used to investigate speciation in other bacterial infections. However, it would be necessary to first improve the percentage of reads identified; this would improve species identification resulting in greater quantification of the microbiome.

The pig industry is undergoing substantial structural changes with increased industrialisation of pig farming [13], this could lead to an increased need for antibiotics as more pigs are kept on the same holding [9]. In addition, growing resistance to specific antibiotics, for example, tylosin, has led to a reduction in the variety antibiotics available to treat swine dysentery, and if AMR continues to develop treatment options could be reduced further [282]. The continued need for antibiotic treatment, and the continued acquisition of resistance to new antibiotics are likely to have an impact on the

development of *B. hyodysenteriae* in the future. This could result in tiamulin and valnemulin being used more frequently. This, in turn, could result in increased resistance, creating a significant challenge to the pig industry that will be difficult to resolve. In future, surveillance of *B. hyodysenteriae* could enable rapid detection of the spread of multi-drug resistance *B. hyodysenteriae*, whereupon control measures could be used to limit the spread. WGS offers a useful technique that will strengthen surveillance by enabling more in-depth analysis of clinical isolates. For WGS to be implemented as effectively as possible, it will be important for data to be shared publically, this will enable rapid identification of outbreak strains and their location of origin; especially between trading partners or regions across which animals travel freely.

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Appendix

Appendix 1

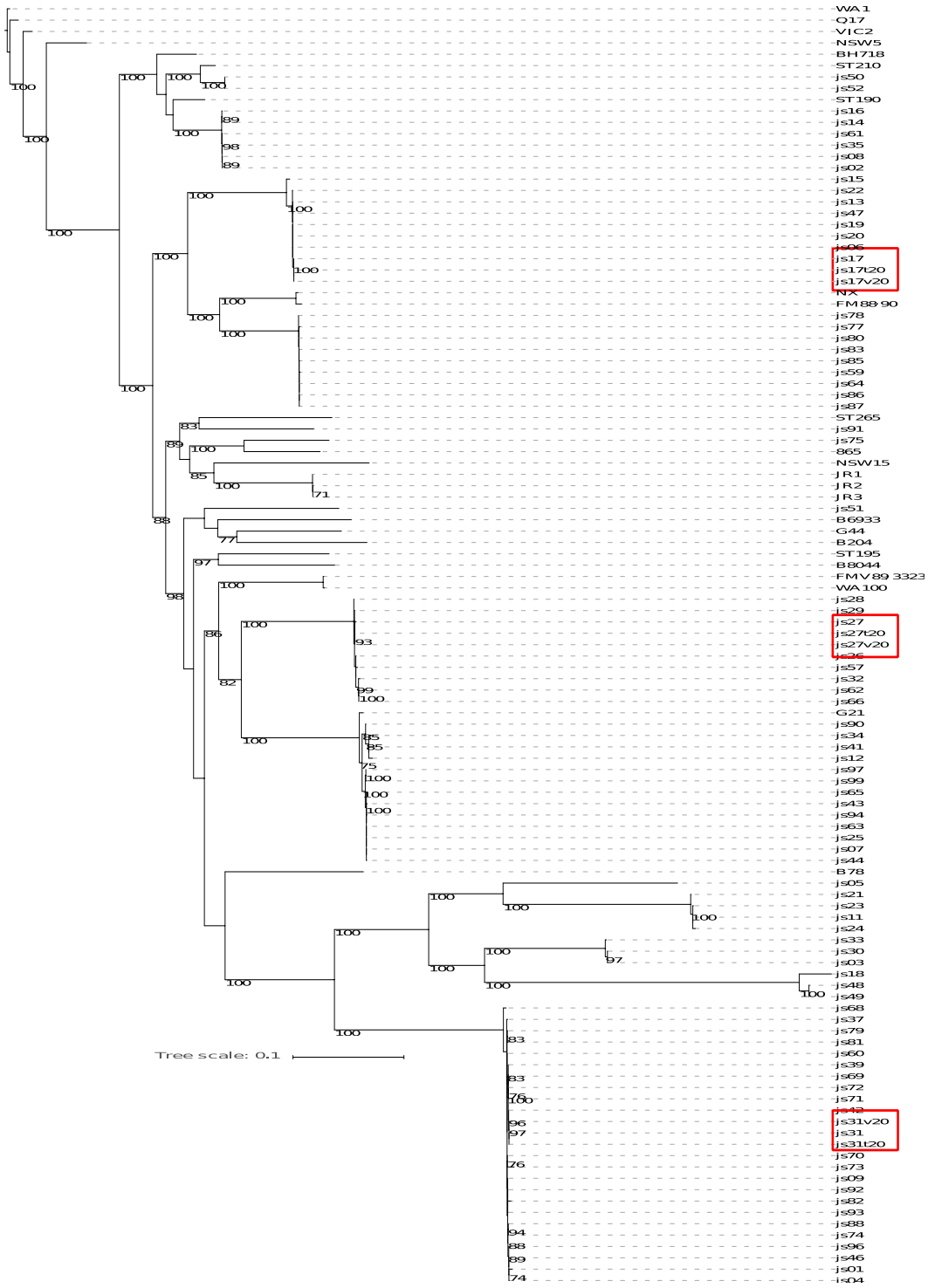
Table of uncertainties in MLST assignment by SRST2.
Edge refers to coverage over the first, or last 2 bases of the gene.

Isolate	SNP	Edge
js02		gdh-5/edge2.0
js05	glpK-15/51snp,2indel,37holes; thi-19/62snp	thi-19/edge2.0
js15	est-7/1snp; glpK-23/1snp	
js16		gdh-5/edge1.0
js43		gdh-12/edge2.0
js48		gdh-1/edge2.0
js59		gdh-4/edge2.0

Appendix 2

Phylogenetic tree of *B. hyodysenteriae* isolates grown in sub-lethal concentrations of tiamulin and valnemulin sequenced in this study and other isolates previously sequenced (chapter 3).

Isogenic parent strain and the isolates after 20 subcultures of tiamulin and valnemulin are shown in red boxes. 1000 bootstraps were conducted, and bootstrap support above 70 is shown.



Appendix 3

Relative abundance of sequenced caecal samples.

Abbreviations: BG: Farrow-to-finish, F: Finisher, M: Midlands, Y: Yorkshire, SW: South West, 44: MSG44P2, 07: MSG07P2, 16: MSG16P2, 20: MSG20P2, 54.2: MSG54P2, 54.3: MSG54P3, 54.4: MSG54P4, 54.5: MSG54P5, 15: MSG15P2, 38: MSG38P2, 11: MSG11P2, 12: MSG12P2,06: MSG06P2,14: MSG14P2

Sample	44	7	16	20	22	54.2	45.3	54.4	54.5	15	38	11	12	6	14
Farm Type	BF	BF	BF	BF	BF	BF	BF	BF	BF	BF	F	BF	BF	F	F
Location	M	Y	Y	M	Y	M	M	M	M	SW	SW	SW	SW	SW	SW
Acidaminococcus fermentans	0	0	1.37	0	0	0	0	0	0	0	0	0	0	0	0
Bacteroides fragilis	4.03	8.11	9.7	1.43	15.48	4.8	3.47	3.44	4.47	5.7	12.68	4.73	6.17	0.67	2.46
Bacteroides salanitronis	0	0	1.55	0	2.96	0	0.99	0	0	0	0	0	1.77	0	0.76
Bacteroides thetaiotaomicron	2.31	1.99	4.65	1.11	4.04	0	2.89	1.61	2.28	2.03	3.01	0.86	4.11	0	1.05
Bacteroides xylanisolvens	0	0	0	0	0	2.2	0.99	0	0	0	0	0	0	0	0
Butyrate producing bacterium	0	0	1.4	0	0	0	0	0	0	0	0	0	0	0	0
Clostridium cf saccharolyticum	1.48	2.07	2.95	1.25	5.42	3.98	1.69	2.75	2.59	4.04	5.37		3.56	3.06	2.11
Clostridium difficile	0	0	0	0	0	0	0	0	0	0	0	0	0	0.45	0
Coprococcus ART55	0	0	0	0.72	0	1.94	3.73	2.54	0	0	0	0	1.66	0	0

Sample	44	7	16	20	22	54.2	45.3	54.4	54.5	15	38	11	12	6	14
Farm Type	BF	BF	BF	BF	BF	BF	BF	BF	BF	BF	F	BF	BF	F	F
Location	M	Y	Y	M	Y	M	M	M	M	SW	SW	SW	SW	SW	SW
Coprococcus catus	1.15	0	0	0.63	0	2.07	1.64	2.39	0	2.72	0	0	0	8.46	0.98
Enterococcus faecalis	0	0	0	0	0	0	0	0	0	0	0	0	2.25	0.42	0
Escherichia coli	0	0	0	2.76	0	0	0	0	0	0	0	0	0	0	0
Eubacterium eligens	0	0	1.84	0	0	2.56	1.81	1.51	2.37	0	0	4.53	1.44	0	0
Eubacterium rectale	5.97	0	0	6.87	14.89	27.27	25.97	46.66	40.95	13.44	0	1.77	17.97	1.8	2.94
Eubacterium siraeum	0	0	0	0	0	0	0	0	0	0	0	0	0	0.55	0
Faecalibacterium prausnitzii	0	0	5.37	2.21	0	6.62	13.39	12.37	10.16	7.01	0	3.23	9.85	1.52	0.88
Lactobacillus acidophilus	16.67	13.6	0	0.69	0	0	1.34	0	0	0	3.13	15.24	0	69.08	10.83
Lactobacillus amylovorus	39.59	30.91	0	0.57	0	0	0.85	0	0	0	0	35.87	0	0	23
Lactobacillus johnsonii	0	0	0	43.09	0	4.75	3.99	0	0	0	0	0	3.11	0	16.33
Lactobacillus reuteri	0	1.71	1.5	8.81	0	0	2.23	0	0	4.9	3.13	1.15	0	1.04	11.84
Lactobacillus salivarius	0	0	0	1.15	0	0	0	0	0	0	0	0	0	0	0
Megasphaera elsdenii	6.98	10.28	23.1	6.12	0	0	0	0	0	4.09	0	0	8.9	0	8.76
Methanobrevibacter smithii	0	0	0	0	0	0	0	0	0	0	10.92	18.43	0	2.41	0

Sample	44	7	16	20	22	54.2	45.3	54.4	54.5	15	38	11	12	6	14
Farm Type	BF	BF	BF	BF	BF	BF	BF	BF	BF	BF	F	BF	BF	F	F
Location	M	Y	Y	M	Y	M	M	M	M	SW	SW	SW	SW	SW	SW
Prevotella dentalis	0	0	3.76	1.31	0	0	1.34	0	0	0	0	0	1.35	0	0
Prevotella denticola	0	0	0	0	0	0	1.29	0	0	0	0	0	0	0	0
Prevotella melaninogenica	2.39	0	4.74	1.81	3.2	2.73	4.11	1.98	0	1.92	0	0.97	3.33	0	0.8
Prevotella ruminicola	0	0	0	0	0	0	0.98	0	0	0	0	0	0	0	0
Roseburia hominis	1.1	0	2.47	1.15	4.71	2.9	1.87	1.78	3.27	3.39	3.88	0.99	3.36	2.39	1.73
Roseburia intestinalis	2.19	0	1.77	2.21	5.3	4.55	3.17	4.95	5.38	2.78	4.01	1.22	4.31	1.13	1.81
Ruminococcus	0	0	3.06	0	0	0	1.01	0	0	1.61	0	0	0	0.32	0
Ruminoccus bromii	0	0	2.23	0	0	0	1.51	0	0	0	0	0	0	0	0
Ruminococcus obeum	0	0	1.7	0.55	0	0	1.48	0	0	3.01	0	0	1.3	1.09	0.69
Ruminococcus torques	0	0	0	0	0	0	0	0	0	3.14	0	0	0	0	0
Streptococcus gallolyticus	0	0	0	0	0	0	0	0	0	2.59	0	0	0	0	0
Streptococcus lutetiensis	0	0	0	0.87	0	0	0	0	0	4.17	0	0	0	0	0
Streptococcus macedonicus	0	0	0	0	0	0	0	0	0	1.42	0	0	0	0	0
Streptococcus suis	0	0	0	0	0	0	0	0	0	0	0	0	0	0.35	0
Treponema succinifaciens	0	0	0	0	0	0	0	0	0	4.78	0	0	0	0.36	0

Appendix 4

Relative abundance of sequenced faecal samples.

Abbreviations: H: Healthy, SD: swine dysentery positive.

Isolate	100	103	55	69	8	123	14	174	26	168	161
Health	H	H	H	H	H	SD	SD	SD	SD	SD	SD
archaeon Mx1201	0	0	0	0	0	0	0.99	0.27	0	0	0
Achromobacter xylosoxidans	0	0	0	0	0.95	0	0	0	0	0	0
Acholeplasma brassicae	0	0	0	0	0	0	0	0	0.71	0	0
Acidaminococcus fermentans	1.86	4.74	0	0	0	0	0	1.09	0	0	0
Acinetobacter baumannii	0	0	0	0	0	0	0.8	0	2.92	0	0
Bacteroides fragilis	3.78	2.89	1.87	0	0	0.23	27.5	55.17	7.22	0	0
Bacteroides salanitronis	0	0	0	0	0	0	0.23	0	0	0	0
Bacteroides thetaiotaomicron	1.81	0	0	0	0	0.1	0	0.31	0	0	0
Bacteroides vulgatus	0	0	0	0	0	0.3	0	1	0	0	0
Bacteroides xylanisolvens	0	0	0	0	0	0.05	0	0	0	0	0
Brachyspira hyodysenteriae	0	0	0	0	0	0.15	15.89	1.51	3.87	0.77	3.81
Campylobacter coli	0	0	0	0	0	0.46	0.58	0.39	0	0	1.94

Isolate	100	103	55	69	8	123	14	174	26	168	161
Health	H	H	H	H	H	SD	SD	SD	SD	SD	SD
Clostridium cf saccharolyticum	1.84	4.36	2.37	1.98	0	0.12	0.48	1.53	0	0	4.05
Clostridium difficile	0	0	3.07	1.21	1.85	0.06	0	0.95	0	0	7.47
Clostridium perfringens	0	0	0	0	0	0	0	1.04	0	0	5.31
Coprococcus catus	0	0	0	0	0	0	0.29	0	1.1	0	0
Enterobacter cloacae	0	0	0	0	1.02	0	0	0	0	0	0
Enterococcus faecalis	0	0	0	0	1.75	0	0	0.4	0	0	0
Enterococcus faecium	0	0	5.48	1.24	5.17	0	0.59	0	0	0	1.69
Escherichia coli	0	0	0	0	0	0.19	27.77	16.34	14	0.22	7.84
Escherichia fergusonii	0	3.98	0	0	0	0.21	0	0.41	0	0	0
Eubacterium rectale	12.44	6.05	0	0	0	0	0.23	0	0	0	0
Eubacterium siraeum	0	0	0	1.08	0	0.13	0	1.79	0	0	7.17
Faecalibacterium prausnitzii	8.5	3.46	0	0.83	0	0	0.7	1.12	0.55	0	2.67
Lactobacillus acidophilus	8.07	6.01	0	3.48	8.98	28.51	8.41	0.54	15.1	26.79	2.99
Lactobacillus amylovorus	17.28	11.91	0	7.11	20.64	63.77	3.65	0.73	32.95	60.43	5.34
Lactobacillus crispatus	0	0	0	0	0	0.23	0	0	0	0.24	0
Lactobacillus helveticus	0	0	0	0	0	0.55	0	0	0	0	0

Isolate	100	103	55	69	8	123	14	174	26	168	161
Health	H	H	H	H	H	SD	SD	SD	SD	SD	SD
Lactobacillus johnsonii	0	0	0	0	0	0.23	0	1.85	0	0.35	0
Lactobacillus kefiranofaciens	0	0	0	0	0	0.18	0	0	0	0	0
Lactobacillus reuteri	3.48	0	0	1.57	3.16	1.3	1.89	1.95	5.82	4.26	1.2
Megamonas hypermegale	1.92	0	0	0	0	0	0	0	0	0	0
Megasphaera elsdenii	5.71	6.38	0	0.94	0	0	0.27	1.53	0.83	0	0
Methanobrevibacter AbM4	0	0	0	0	0	0	0	0	0	3.97	0
Methanobrevibacter smithii	0	0	50.48	58.13	15.74	0.64	0	0.83	0	0	18.14
Methanosphaera stadtmanae	0	0	0	0.77	1.14	0	0	0	0	0	0
Prevotella melaninogenica	3.08	0	0	0	0	0	0	0	0	0	0
Psychrobacter arcticus	0	0	0	0	2.04	0.07	0	0	0	0	0
Psychrobacter cryohalolentis	0	0	0	0	4.82	0.21	0	0	0	0	0
Psychrobacter G	0	0	0	0	2.63	0.13	0	0	0	0	0
Psychrobacter PRwf	0	0	0	0	3.92	0.13	0	0	0	0	0
Roseburia hominis	1.71	3.92	0	1.43	0	0.12	0.9	1.42	0	0	4.22
Roseburia intestinalis	0	0	0	0	0	0.08	0.45	0.3	0	0	0
Ruminococcus	1.68	3.13	0	0	0.99	0	0	0	0	0.28	0
Ruminococcus bromii	4.83	2.85	2.78	2.2	1.85	0	0.23	0.29	0.52	0	0
Ruminococcus obeum	0	0	0	0	0	0	0	1.09	0	0	3.9

Isolate	100	103	55	69	8	123	14	174	26	168	161
Health	H	H	H	H	H	SD	SD	SD	SD	SD	SD
Ruminococcus torques	0	0	0	0	1.14	0	0	0	0	0	0
Shigella flexneri	0	0	0	0	2.22	0	0	0	0	0	0
Shigella sonnei	0	0	0	0	0	0.12	0	0	0	0	0
Solibacillus silvestris	0	0	0	5.25	0	0	0	0	0	0	0
Streptococcus lutetiensis	0	0	0	0	0	0	0	0	0	0	0
Streptococcus lutetiensis	0	0	0	0	0	0.06	0	0	0	0	0
Treponema succinifaciens	2.1	14.63	3.42	3.48	0	0	0.62	0.24	1.74	0	0