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# ESCHERICHIA COLI CONTAMINATION OF PORK CARCASSES IN UK SLAUGHTERHOUSES

**By**

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## ABSTRACT

Despite the HACCP systems which have been introduced to the pork industry, cross-contamination which occurs within pork slaughterlines remains an important concern for food safety of the final carcass. The aim of this work was to understand the dissemination and cross-contamination of enteric bacteria during slaughter processing by investigating Escherichia coli populations. E. coli is widely used as an indicator of faecal or enteric pathogen contamination, and a strong correlation between the presence of Salmonella and E. coli levels was seen in this study. With microbiological counts and molecular typing of E. coli, changes of contamination levels as well as of the bacterial communities was observed during processing. The results demonstrated that temperature variation at different carcass sites during the singeing process allowed strains to survive on the cooler sites of the carcass and be present in the subsequent processing stages. The polishing process was recognised as an important site of cross-contamination not only because of an increase in contamination levels but also because a high variety of sources contributed contamination at this site, including strains surviving through singeing, strains that persist in the polisher overnight and strains from faecal leakage during the polishing process. A high percentage of virulence factor-carrying E. coli were present on the slaughtered carcasses and recombination between virulence genes from different pathovars was observed. These findings suggest carcasses slaughtered from a healthy pig herd may still be a potential source for E. coli pathovars in the food chain.

# CHAPTER 1 INTRODUCTION

## 1.1 THE SAFETY OF MEATS

According to a report of the European Food Safety Authority and the European Centre for Disease Prevention and Control (EFSA & ECDC), in 2010, a total of 99,020 human cases of food borne disease caused by food borne pathogens were reported in 27 European Union Member States (EFSA and ECDC, 2012). Food borne disease costs an estimated £ 0.75 billion per annum to the UK (Milnes et al., 2009). Food borne diseases occur by consumption of food contaminated with microorganisms, including undercooked meat products, unpasteurised milk and ready-to-eat products, and raw vegetables (Willshaw et al., 2001). The bacterial contamination of raw meats continues to represent a major food safety issue. When meat is contaminated with Salmonella, Campylobacter jejuni, or Escherichia coli it will still appear normal but could potentially cause food borne illness in humans (Ha and Pham, 2006). Little et al. (2008) determined the prevalence of Campylobacter and Salmonella in 3959 samples of raw red meats in the UK during 2003-2005. Meats were contaminated with Campylobacter (7.2%) and Salmonella (2.4%). Offal samples (36.6%) were more frequently contaminated with Campylobacter or Salmonella than muscle tissue (7.0%). Pork had the highest contamination with Salmonella (3.9%), followed by lamb (2.0%), other meats (2.0%) and beef (1.3%). In 2010, Germany reported three human salmonellosis outbreaks cause by S. Typhimurium involving 45 cases with 10 hospitalisations and one death, and these outbreaks were all caused by pig meat or pork meals (EFSA and ECDC, 2012). This demonstrates that pork is a key food safety issue and

reduction of enteric pathogen contamination of pork meat should contribute to a reduction of food borne disease..

## 1.2 PRODUCTION OF PORK

Many steps are involved in the production of pork meat from farm to product such as breeding, animal transporting, slaughtering, cutting, and packing and each step can be a source of bacterial contamination (Sheridan, 1998; Warriner et al., 2002; Botteldoorn et al., 2003b; Pearce et al., 2004; Pearce et al., 2006). This is summarised in Figure 1.1 (Giovannacci et al., 2001). Slaughter is a key contamination step in the conversion of animals from livestock to food stuff. During this process bacteria can be transferred to the meat surfaces of swine carcasses from the skin or gastrointestinal tract of pigs, from the workers, or from equipment during slaughtering (Carr et al., 1998; Yu and Palumbo, 2000) A wide range of potential pathogens have been found in the slaughter process, such as E. coli (Gill and Jones, 1998; Warriner et al., 2002; Pearce and Bolton, 2005), Salmonella (Borch et al., 1996; Davies et al., 2004) and Listeria monocytogenes (Gill et al., 1995; Autio et al., 2000; Giovannacci et al., 2001). This indicates that the slaughter process may potentially introduce these pathogens onto the final products and cause food safety problems hence improved control of this process is important.

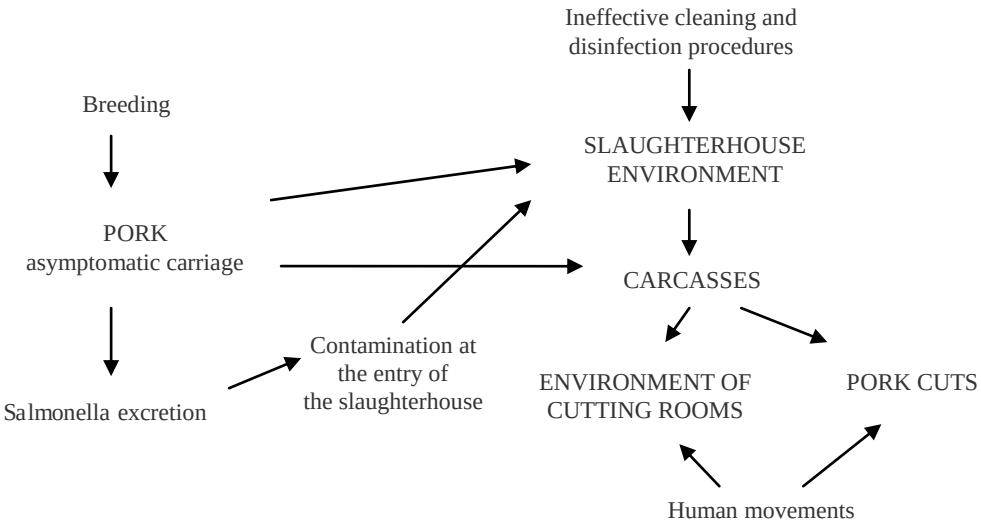



Figure 1.1. Possible events involved in the contamination of pork by Salmonella (Adopted from Giovannacci et al., 2001).

## 1.3 HYGIENE DURING PORK SLAUGHTERING

According to the annual statistics from the Department of Environment Food and Rural Affairs (DEFRA), in the UK the total number of slaughtered pigs was 8,979,800 head and pork production was 712,100 tonnes in 2010 (DEFRA, 2011b). To process such a large number of pigs, the hygiene conditions of the slaughterhouses are relatively important. The overall quality of meat and edible by-products depends heavily on its microbiological quality (WHO, 1990). Although most of the muscle tissues are considered sterile in the living animal,

microorganisms may cause spoilage and food borne illness (Gill et al., 1978). Slaughter of animals for meat provides an opportunity for bacterial colonisation of the surface of animal carcasses (Gill et al., 1978; Roberts et al., 1980). Pork products may be contaminated with a range of human pathogens, including Salmonella (Berends et al., 1997), Campylobacter (Malakauskas, et al., 2006), Escherichia coli (Bouvet et al., 2001; Bonardi et al., 2003; Namvar and Warriner, 2006; Wong et al., 2009), Yersinia (Bonardi et al., 2003; Fredriksson-Ahomaa et al., 2009), Staphylococcus (Nitzsche et al., 2007) and Listeria (Sheridan et al., 1994; Borch et al., 1996). Such pathogens enter the slaughter environment on or in live animals. Salmonella, Campylobacter, E. coli and Yersinia are carried into the plant in the pig intestinal tract or in faeces adhering to the skin, while Yersinia can also be carried in the porcine tongue and tonsils (Bolton et al., 2002). Salmonella contamination is the problem of most concern during pork slaughter as pork products are recognized as one of the significant sources of human salmonellosis (Berends et al., 1997; Rostagno et al., 2005; Vieira-Pinto et al., 2006).

## 1.4 COMMERCIAL PIG SLAUGHTER LINE

The generic stages of a commercial pig slaughter process are shown in Figure 1.2 (Botteldoorn et al., 2003), including lairage, stunning/bleeding, scalding, scraping, dry polishing, singeing, wet polishing, evisceration, carcass splitting, washing, and chilling. The features and the microbiological impacts of each process stage will be described in the following subsections.

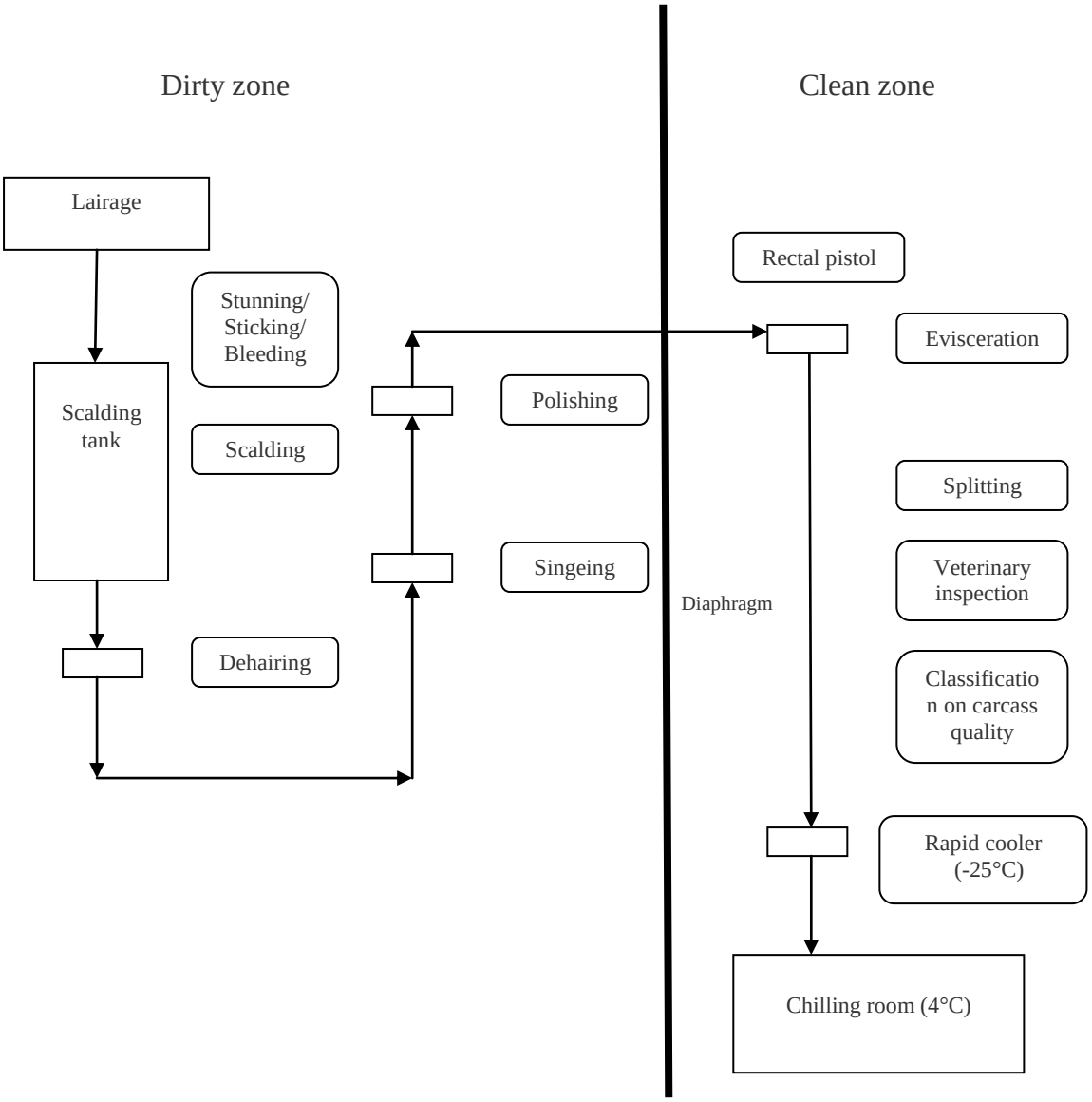



Figure 1.2. Schematic representation of a pork slaughter line (Botteldoorn et al., 2003)

### 1.4.1 Stunning/ Sticking/ Bleeding

Stunning is a step to render an animal unconscious before it is slaughtered in order to eliminate pain and stress from the process. Three stunning methods are applied to pig slaughter including gas, percussive, and electrical stunning. Effective stunning should result in: head being limp and floppy, no blinking, no rhythmic breathing, no response to ear or nose pinching, no arched backs, and no vocalization (Grandin, 1998). The animals should be rendered insensible immediately on application, with the insensibility persisting such that the pig does not recover before it dies from bleeding out, or exsanguination.

Stunning and bleeding are important for meat quality because improper stunning/bleeding will lead to blood-splashing, which is the rupture of capillaries in muscle, and occurrence of blood spots in the meat (Belk et al., 2002). According to the Humane Slaughter Association (HSA), bleeding needs to be done within 15 seconds of stunning so there is insufficient time for recovery to take place before there is irreversible loss of brain function from lack of oxygen (HSA, 2008). The blood volume is approximately 6 L for a 100 kg pig presented for slaughter (Dickson et al., 2002). Dressing procedures must not be started until at least 20 seconds after bleeding has commenced.

According to the USDA-Food Safety and Inspection Service (USDA-FSIS) generic HACCP module for pork slaughter, the sticking operation is not a critical control point because the site of the sticking itself is normally trimmed

out at a later point in the process, removing any bacteria which may have adhered to the tissue (USDA, 1999). However, the microbiological surface contamination during stunning/ bleeding operations is of concern because of the high number and variety of bacteria which can be found on the surface of the skin. Bolton et al. (2002) indicated that stunning and bleeding led to a considerable increase in bacterial contamination to the carcasses and a 50% incidence of Salmonella on carcasses. This suggests that the stunning/ bleeding processes may pose potential risks at the initial stage of slaughter and an effective control of contamination needs to be considered.

### 1.4.2 Scalding

Pig carcasses are dressed with the skin still on hence initial processing of the carcass has the primary purpose of the removal of hair. During a typical operation, pigs are scalded in a hot water tank to facilitate the hair removal. An alternative method is condensation scald operation, which uses steam for scalding. This lather system offers a numbers of advantages, such as being easy to ventilate during emergency stops, no damages to carcass surfaces occurs, and less water consumption (approximate 1.5L per carcass) (Gosansa, 2004). In terms of the characteristics of hygiene, the use of steam for scalding allows a constant supply of clean water and prevents an increase in water organic load (Delhalle et al., 2008). In the United States, operating parameters for scald operations range from 57.7 – 61 ˚C (136 ˚F– 142 ˚F) for 3 to 8 min and a typical scald condition would be 58.8 ˚C for 6 min (Dickson et al., 2002). In the UK, scalding temperatures and durations have been reported as between 58

- 64 ˚C and 4 - 6.75 min (Tinker et al., 2007).

Although not the main reason for the process, the combination of time and temperature means scalding is an important antimicrobial process which reduces the bacteria on the surface of carcasses. Bolton et al. (2003) determined D-values (time for a log reduction of bacterial numbers) for Salmonella isolates from pigs in scald tank water of different temperatures. The D-value for samples treated at 60˚C ($D_{60^{\circ}C}$) was 1.4 min; $D_{55^{\circ}C}$ increased to 7.3 min, and $D_{50^{\circ}C}$ was 83.2 min. This indicates that failure to maintain the temperature of scalding water can significantly reduce the decontamination effects of the scalding process. Pearce et al, (2004) measured population changes on the surfaces of carcasses after scalding for approximately 8 min using a liner scald tank at 61±1 ˚C. After scalding, the aerobic counts from surface were significantly reduced by approximately 3.7 $\log_{10}$ cfu $cm^{-2}$ ($D_{61^{\circ}C} \approx$ 2.2 min) with an approximately 3.5 $\log_{10}$ cfu $cm^{-2}$ reduction in coliform counts ($D_{61^{\circ}C} \approx$ 2.3 min).

Scalding may not kill all bacteria by the heat of the scald water because they could be embedded in dirt, faeces, or ingesta carried by the pigs (Bolton et al., 2003). If so, the contamination of scald water needs consideration as this could act as a route of cross-contamination between carcasses. Therefore, despite being considered as a decontamination process, proper operations and well controlled temperatures of scalding are still important to minimise cross-contamination.

### 1.4.3 Dehairing

The dehairing process normally takes place in a roller with steel scrapers and high-pressure water, or water with chemical spray (sodium sulfate or oxidative agents), used to remove hair from the carcasses. In some types of pull-through scalding tanks, dehairing and scalding may be combined in one operation. As the hair is loosened by the scalding water it is removed by the rubbing effect of the paddles against the skin (Sheridan et al., 1991). Thus, the dehairing process results in visually cleaner carcasses and reduces the requirement for trimming due to faecal contamination.

The potential for cross-contamination during the dehairing process was studied by Gill and Bryant (1992) who showed that the mesophilic bacterial populations on pig carcasses increased after the dehairing operation when compared to the populations before dehairing. During dehairing, faecal matter may be spread onto the carcasses surface. Pearce et al. (2004) observed that after dehairing, coliform counts and total aerobic mesophilic counts on the ham, belly, and neck areas were significantly higher, by 2 $\log_{10}$ cfu $cm^{-2}$, than after scalding. The frequency of Salmonella positive carcasses also increased from 1% to 7% throughout the same stages. This confirmed that the dehairing process could be a major source of carcass contamination.

### 1.4.4 Singeing

The singeing or flaming process is used to remove any remaining hairs, and it shrinks and sets the skin to leave an attractive clean appearance and develop a rind. It may be done with a hand-held gas torch but more commonly automated systems transport the pig into a furnace and leave it long enough for an effective singe. Singeing normally exposes the carcasses to temperatures between 900 ˚C and 1500˚C for less than 20 sec. Thus, singeing has been identified by many studies as the most important operation for reducing microbial contamination (Berends et al., 1997; Rivas et al., 2000; Bolton et al., 2002; Pearce et al., 2004). Bolton et al. (2002) showed significant reductions (3 $\log_{10}$ cfu $cm^{-2}$) in TACs during singeing whilst Pearce et al. (2004) also showed a significant reduction in aerobic mesophilic counts (2.2- 2.9 $\log_{10}$ cfu $cm^{-2}$) and coliform counts a 2-2.5 $\log_{10}$ cfu $cm^{-2}$after singeing. Alban and Stärk (2005) noted that singeing is the only step where Salmonella can actually be killed in the production process, and modelling showed that increasing singeing efficacy is a relatively cheap way of reducing Salmonella prevalence. However, when singeing is carried out incorrectly and/or when very large amounts of Salmonella are located in the deeper layers of the skin, the hair follicles, the base and orifices of the ear, or the deeper skin folds, a reduction of only 5-30% in positive carcasses may be achieved (Berends et al., 1997) .

Although singeing for 10 seconds can raise the surface temperature of the carcass to approximately 100˚C (Borch et al., 1996), it may be still insufficient to fully decontaminate carcasses. It has been observed that the singeing

temperature is not uniform over the whole length of the carcass (Tinker et al., 2007). A study of the efficacy of singeing may allow more understanding of its limitations and would be helpful to improve its overall decontamination effectiveness.

### 1.4.5 Polishing

The polishing operation is conducted by machines which brush carcasses with stainless steel scrapers or nylon brushes for intensive cleaning of heads and hind feet. The polishing process provides a visible cleaning of carcasses by removing singed hair and skin debris.

Unlike the scalding and singeing processes which result in a considerable decrease in numbers of microorganisms on carcass surfaces, polishing appears to contribute to the spread of bacteria which surviving singeing (Borch et al., 1996). The polishing process has been identified as the most important operation for the microbial recontamination of pork carcasses following the significant reduction during singeing (Bolton et al., 2002; James et al., 2007). Pearce et al. (2004) observed that after polishing, the coliform counts and total aerobic mesophilic counts on the ham, belly, and neck areas were 1.5 $\log_{10}$ cfu $cm^{-2}$ higher than after singeing. Berends et al. (1997) indicated that dehairing and polishing are steps which result in a considerable increase in total viable counts and Enterobacteriaceae counts based on the finding that these two steps lead to significantly higher counts than after scalding and singeing.

### 1.4.6 Evisceration

Evisceration is the step to remove the digestive tract by manual operators or with automated evisceration systems. Polished carcasses are moved into a separate evisceration area and carcasses debunged by cutting around the rectum. In some slaughterhouses, the detached rectum is sealed with a plastic bag ("bagging") to prevent faecal contamination of carcasses during subsequent processing (Berends et al., 1997; Pearce et al., 2004). Damage occurring to the internal organs during normal removal of the viscera has the potential to distribute stomach, intestinal or faecal contents throughout the peritoneal and pleural cavities (Dickson et al., 2002).

Yu et al. (1999) demonstrated that the routine evisceration operations could contribute to 55 and 90% of total carcass contaminated with Salmonella without rupture of gut. Rivas et al. (2000) also observed that in the evisceration stage, as faecal contamination indicators, the Enterobacteriaceae and E. coli count increased significantly, reaching values of 1.2 and 1.1 $\log_{10}$ cfu $cm^{-2}$ on average respectively, and thus suggested that contamination of faecal origin occurred at this stage. However, Dickson et al. (2002) suggests that contamination is of a random nature and typically would affect only the specific carcass in which the gut rupture occurred. There is a risk of making holes in the intestinal tract so that gut contents are spread over the carcass. However, with well-trained operators this occurs infrequently (Borch et al., 1996).

### 1.4.7 Chilling

There are currently three commonly used chilling systems in use for pig processing: conventional forced air chilling, spray chilling, and blast chilling (Dickson et al., 2002). The conventional chill system uses standard refrigeration techniques and air movement to remove heat from the carcasses. Spray chilling combines conventional refrigeration with a system that sprays cold water on the carcasses. The principal of spray chilling is that evaporative cooling of spray chilling results in a more rapid removal of the heat from the warm carcass. Blast chilling involves moving the carcasses through a blast chiller (essentially a freezer) to chill the external surfaces of the carcasses rapidly, and then moving the carcasses into a conventional chiller to allow them to equilibrate. The chilling step reduces the activity of histaminases in the meat, which affects meat quality and shelf life (Xu et al., 2012).

The chilling process prevents the proliferation of bacteria on warm carcass surfaces and often results in reduction of overall microbial populations. Ingram and Roberts (1976) reported that the populations of coliform and E. coli were significantly reduced after chilling. In addition to the temperature changing, the dehydration effects of these air-chilling may result in insufficient moisture to support microbial growth on the skin surface (Yu et al., 2001).

A study of chilling effects on the survival of bacteria conducted by Gill and

Bryant (1992) showed that some growth (approx. 1 $\log_{10}$ cfu increase) was apparent at one plant, where carcass surfaces were at first cooled slowly. However, no growth was apparent at another plant, where carcass surfaces were rapidly cooled at the beginning of the chilling operation by a blast of freezing air. These observations suggest that during the cooling of carcasses, the contaminating flora may proliferate if the chiller conditions allow carcass surfaces to remain moist and relatively warm for extended periods.

### 1.4.8 Specific interventions on the slaughter line

As serious contamination during evisceration may not be a frequent event, pre-evisceration washing has been considered as an intervention to remove the surface contamination and to enhance the safety of final products. In some countries, such as US, Canada, Japan, Denmark, and Ireland, pre-evisceration washing has been applied in commercial slaughterlines. Bolton et al. (2002) observed that pre-evisceration power-washing (with 40°C water) producing visibly clean carcasses. However, rather than achieving reductions in bacteria, the washing was associated with an increase of 2.5 $\log_{10}$ cfu $cm^{-2}$ total aerobic counts. In contrast, a reduction of microbial contamination using pre-evisceration hot water pasteurization was observed by Gill et al. (1995). In that study hot water treatment of carcasses with water at 85°C for 20 seconds gave a significant reduction in numbers of bacteria. That treatment reduced the numbers of bacteria recovered from the relatively heavily contaminated back and front leg regions by 2 $\log_{10}$ cfu $cm^{-2}$and the numbers of aerobic bacteria recovered from the less contaminated waist and belly regions were reduced by

2.5 $\log_{10}$ cfu $cm^{-2}$.

Since the polishing operation leads to recontamination following a singeing operation, additional singeing systems have been installed post polishing in commercial slaughter lines in some countries to minimise recontamination in the following stages (Yu et al., 1999; De Montzey and Minvielle, 2002; Minvielle et al., 2005; Namvar and Warriner, 2006). Minvielle et al. (2005) compared the effectiveness of the double singe with single singe operations. Their results demonstrated that the dual singe systems improved the control of microbial contamination by a showing higher reduction in total aerobic counts (approximate 2 log) and in Enterobacteriaceae (approximate 1 log) than the single singe systems. Namvar and Warriner (2006) studied the number and genotype distribution of E. coli in a Canadian slaughter line with a triple singeing / polishing facility employed. According to the genotyping of E. coli isolates recovered, the study suggested that the triple singeing step could minimise the deposition of E. coli on the polisher blades thereby reducing the accumulation of enteric organisms.

## 1.5 MICROBIOLOGICAL CONTROL SYSTEMS IN PORK SLAUGHTER

Microbiological contamination during meat production is undesirable but unavoidable in the conversion of live animals to meat for consumption (Eggenberger-Solorzano et al., 2002). However, uncontrolled microbiological

contamination is a principal hazard and is unacceptable. Pig slaughter is an open process with many opportunities for the contamination of the carcasses with potential pathogens and no point within a slaughter line is free from hazards (Borch et al., 1996). Thus, control systems, such as Hazard Analysis Critical Control Point (HACCP) and Good Manufacturing Practice (GMP), play an important role as an intervention of pathogenic contamination within pork processing plants.

In the UK, the presence of a HACCP system is now a requirement for a pork slaughter plant under EU Regulation 852/2004. An overview of the slaughter process (Borch et al., 1996) is presented in Table 1.1. HACCP-based processing gives a systematic approach to process control, and is generally accepted as an effective means of minimizing the levels of contamination. Pearce et al. (2004) made comparisons between the HACCP system in the US and in the EU, the US system focuses on reducing the incidence of pathogens and verifies process control by a pathogen reduction programme. However, the EU system at present just requires hygiene standards to verify process control and this may not be sufficient. The prevention or reduction of pathogenic contamination is a major objective of HACCP systems. The use of microbiological testing for assuring the safety of meats is considered necessary for the implementation and maintenance of effective HACCP and GMP systems (Brown et al., 2000).

Table 1.1. Hygienic aspects and preventive actions with respect to bacterial hazards during swine slaughter (Borch et al., 1996).

| Process step | Hygienic aspect | Preventive actions | CP/CCP* |
|---|---|---|---|
| **Lairage** ↓ | Contamination between animals | Cleaning & disinfection | CP |
| **Stunning** ↓ | | | CP |
| **Killing** ↓ | Contamination from tools | Cleaning & disinfection | CP |
| **Scalding** ↓ | Reduction of bacterial levels<br>Contamination of lungs | Time/temperature | CP |
| **Dehairing** ↓ | Contamination from machines | Cleaning &disinfection | CP |
| **Singeing** ↓ | Reduction of bacterial levels | Time/temperature | CP |
| **Polishing** | Contamination from machines | Cleaning & disinfection | CP |
| **Evisceration** ↓ | Contamination from intestines<br>Contamination from tongue, pharynx and tonsils<br>Contamination from tools | Enclosure of rectum<br>Working instructions<br>Disinfection of tools | CCP |
| **Splitting** ↓ | Contamination via splitter | Line-speed<br>Water temperature | CP |
| **Meat inspection** ↓ | Contamination from inspection | Disinfection of tools | CCP |
| **Deboning of head** | Contamination from head | Working instructions<br>Disinfection of tools | CCP |

*CP: control point; step in a process whereby biological, chemical, or physical factors may be controlled

CCP: critical control point; the point in a specific food system where loss of control may result in a high probability of a health risk

## 1.6 ESCHERICHIA COLI IN PIG SLAUGHTER

### 1.6.1 E. coli

E. coli is a Gram negative bacterium which is commonly found in the lower intestine of animals. E. coli is also abundant in human and animal faeces and not usually found in other niches (Hitchins et al., 1992). Hence, the presence of E. coli in food or water has become accepted as indicative of recent faecal contamination (Tutenel et al., 2003). In a Belgian study (Ghafir et al., 2008), the E. coli counts for pork and beef samples taken during the meat slaughtering process were significantly higher in samples contaminated with Salmonella, hence E. coli counts were considered as a good indicator for enteric zoonotic agents. The direct identification of the contamination of the enteric pathogens, such as Salmonella, would be problematic due to their low level and sporadic occurrence (Warriner et al., 2002). E. coli is widely distributed in the environment and can be used to indicate faecal contamination and potential transfer of enteric pathogens (Warriner et al., 2002; Eblen et al., 2005).

### 1.6.2 Pathogenic E. coli on pork

Although most E. coli strains are harmless, some strains of E. coli are capable of causing disease, if the genes conferring pathogenicity have been acquired. Depending on the categories, pathogenic E. coli can cause clinical infection with colonisation of a mucosal site, and is associated with evasion of host

defences, toxin production (Hartl and Dykhuizen, 1984; Nataro and Kaper, 1998). Pathogenic E. coli strains have been found in pork (Botteldoorn et al., 2003a; Ojo et al., 2010; Xia et al., 2010; Martins et al., 2011) and pigs at slaughter (Bouvet et al., 2002a; Kaufmann et al., 2006; Zweifel et al., 2006). This indicates that healthy pigs can be a carrier of pathogenic E. coli. Verotoxigenic E. coli (VTEC) are some of the most studied strains in slaughtered pigs. In investigations carried out in different countries, the detection of VTEC O157 in slaughtered healthy pigs ranged from 0.1 to 2.0% (Kaufmann et al., 2006). In a French study conducted by Bouvet et al. (2002a) , the overall contamination rates of pork carcass samples from three abattoirs with VTEC were 46% after bleeding, 16% after dressing, and 15% for samples after chilling. At three French cutting plants Bouvet et al. (2002b) showed 12% and 19% of positive samples for VTEC on carcasses and untrimmed cuts, respectively. Furthermore, no plant was free of positive environmental samples. In the UK, a 1-year study of the carriage of VTEC O157 in pigs showed that the prevalence of VTEC O157 was 0.3% (Milnes et al., 2008). In pork meat, the studies indicated that the E. coli O157:H7 prevalence in fresh raw meat was lower than 2% in European countries (Bouvet et al., 2002b; Chahed et al., 2005). Although the detection rates of pathogenic E. coli are normally low, pork may be a vehicle for pathogenic strains and healthy pigs cannot be excluded as a potential source of human infection (Zweifel et al., 2006).

#### 1.6.2.1 Enterotoxigenic E. coli

Enterotoxigenic E. coli (ETEC) refers to E. coli which adhere to the microvilli

of small intestinal epithelial cells without inducing morphological lesions; they produce enterotoxins that act locally on enterocytes (Nagy and Fekete, 1999). Disease caused by ETEC follows ingestion of contaminated food or water and is characterized by profuse watery diarrhoea lasting for several days that often leads to dehydration and malnutrition in young children (WHO, 2009). Wenneras and Erling (2004) reported that the carriage of ETEC was primarily associated with diarrhoea in children less than five years old based on data reported between 1970 and 1999. ETEC is the most common cause of diarrhoea in the developing world, causing annually 280-400 million diarrhoeal episodes in children aged less than five years and an additional 100 million episodes in children aged 5 -14 years (WHO, 2006).

The pathogenesis of ETEC starts with non-destructive attachment to the intestinal microvilli by one or more adhesive fimbriae. Following colonization of the microvilli, plasmid-encoded heat stable (ST1 or ST2) and heat labile (LT) enterotoxins induce hypersecretory diarrhoea (Scheutz and Strockbine, 2005). The enterotoxin genes are encoded on plasmids. The plasmids associated with enterotoxin production may code for LT only, ST only, or both LT and ST (Hartl and Dykhuizen, 1984). The fimbrial genes are also usually encoded on plasmids, which typically encode the enterotoxins ST and/or LT (Nataro and Kaper, 1998). Pigs are known as reservoirs of ETEC; diarrhoea induced by ETEC is one of the most important diseases of suckling and post-weaning pigs, and causes negative economic implications in the pig industry due to reduced growth rate and high mortality (Do et al., 2005; Zhang et al., 2007; Madoroba

et al., 2009) .

### 1.6.2.2 Enteropathogenic E. coli

Enteropathogenic E. coli (EPEC) was found to be frequently associated with human diarrhoeal symptoms in the late 1940s (Hartl and Dykhuizen, 1984). Central to the pathogenesis of EPEC is the formation of attaching and effacing (A/E) lesions: "attaching" indicates the intimate attachment of bacteria to the enterocyte; "effacing" indicates the localized effacement of brush border microvilli (China et al., 1998). Intimin, an outer membrane protein encoded by the eae gene, is the key component of the attachment to the surface of epithelial cells in the gastrointestinal tract (Nataro and Kaper, 1998). The eae gene and other genes for the production of A/E lesions are located on the locus of enterocyte effacement (LEE), a pathogenicity island on the EPEC chromosome. EPEC has become rare in industrialized countries, although it is occasionally reported in child care settings. However, it persists as an important cause of infantile diarrhoea in many developing countries (Fasano, 2008). The common clinical features of EPEC illness include fever (60%), diarrhoea (> 90%), respiratory symptoms (50%), dehydration (30%, especially in infants under one year of age), and paralytic ileus (10%) (DuPont and Mathewson, 1998). The most typical transmission of EPEC is via the faecal-oral route.

EPEC have also been associated with diarrhoea in post-weaning pigs (Zhu et

al., 1994; Kim et al., 2010). Predisposing factors, such as a weaner diet containing soybean and field peas or porcine reproductive and respiratory syndrome (PRRS) virus infection, can enhance bacterial colonisation and development of A/E lesions (Gyles and Fairbrother, 2011). In a Hungarian study, Malik et al. (2006) reported that eae-positive E. coli strains were detected on 12% of diarrhoeal weaned pigs (n = 137), and the Beta type eae gene was the most frequent type found in porcine ileum and colon.

#### 1.6.2.3 Enteroaggregative E. coli

Enteroaggregative E. coli (EAggEC) are E. coli strains that demonstrate a characteristic "stacked-brick" aggregative adherence when cultured with human epithelial Hep-2 cells (Nataro et al., 1987). In some regions of the developing world, EAggEC are the second most common cause of traveller's diarrhoea, only surpassed by ETEC (Veilleux and Dubreuil, 2006). The aggregative adherence fimbriae (AAFs), plasmid-encoded toxin (Pet), and enteroaggregative heat- stable toxin 1 (EAST1) are the three known virulence factors of EAggEC. All of these virulence genes are encoded on the plasmids and may have relevance in human colonization and disease. EAggEC pathogenesis is thought to involve three primary steps (Johnson and Nolan, 2009). First, the bacteria adhere to the intestinal mucosa and to each other with AAF. Second, the bacteria produce a mucus-mediated biofilm on the enterocyte surface which is believed to enhance their persistence. Finally, the bacteria release Enteroaggregative heat-stable toxin 1 (EAST1). EAST1 is produced by the expression of the astA gene, which can be found on the

chromosome and/or plasmids (Harrington et al., 2006). The astA gene is found amongst different categories of diarrhoeagenic E. coli. The strains belonging to EAggEC, EHEC, and atypical EPEC have been shown to be able to carry the astA gene on plasmids (Paiva de Sousa and Dubreuil, 2001).

Strains of E. coli carrying EAST1 have been associated with diarrhoea in pigs. In a US study (Zhang et al., 2007), astA was detected in 35% of the E. coli (n = 304) isolated from post-weaning diarrhoeal pigs. Kim et al. (2010) sampled 122 diarrhoeal piglets from 55 pig farms in Korea and showed that the astA gene was found on 41% of the E. coli isolates ( n = 191), which was the most prevalent diarrhoeal-associated gene in this study.

#### 1.6.2.4 Verotoxin-producing E. coli

Verotoxigenic E. coli (VTEC) strains may produce two types of verotoxins (VT1 and VT2) (Levine, 1987). VT1 and VT2 are also termed Shiga-like toxins (STX1 and STX2). VTEC strains were first linked with human disease in the early 1980s. Since then, infections with VTEC have been reported with increasing frequency (Heuvelink et al., 1999). VTEC may cause severe disease in humans, such as watery or bloody diarrhoea, haemorrhagic colitis (HC) and haemolytic ureamic syndrome (HUS) (Besser et al., 1999). The virulence factors causing HUS may vary with the serotypes of VTEC, such as O148 (stx2c or stx2d), O26 (stx1, eae, ehxA), and O157 (stx2c, eae, ehxA) (Haus-Cheymol et al., 2006). Those VTEC strains which are able to induce HC

and HUS are called enterohaemorrhagic E. coli (EHEC) (Levine, 1987). In May of 2011, a big outbreak of EHEC O104:H4 in Germany caused 50 major deaths, 852 HUS cases and 3,496 cases of infection worldwide (Rosner et al., 2011). This EHEC O104:H4 was demonstrated as a new hybrid strain expressing genes for verotoxin 2A (stx2a), AAF/I fimbriae and extended spectrum β-lactamase resistance (Aurass et al., 2011). The most predominant EHEC serotype associated with human infection and death is O157:H7 (Orden et al., 2002).

Cattle are regarded as the principal reservoir of VTEC; however, pigs may be a vehicle for pathogenic VTEC strains because shiga-like toxin-positive bacteria have been isolated from healthy and diseased pigs (Botteldoorn et al., 2001; Kim et al., 2010; Rivera et al., 2012). Porcine VTEC strains produce the shiga-toxin 2e (Stx2e), which can cause edema disease, and is associated with post-weaning diarrhoea (PWD) with the presence of enterotoxins (Macleod et al., 1991; Blanco et al., 1997). A Cuban study of diarrhoeal pigs demonstrated that 53% of the diarrhoeal E. coli strains (n = 36) were carrying Stx2e with the PCR detection (Blanco et al., 2006). Kim et al. (2010) reported that 30% of E. coli isolates (n = 191) from piglets with diarrhoea in Korea were carrying VTEC associated genes (stx1, stx2, and stx2e).

## 1.7 BIO-MOLECULAR TECHNIQUES FOR EXAMINATION OF BACTERIAL POPULATIONS DURING SLAUGHTER

With the development of genetic fingerprinting methods, the genetic diversity of strains in a population can be studied and the sources of contamination of a species can be more precisely traced (Aslam et al., 2003). DNA fingerprinting based methods have been utilized to examine food safety controls (Botteldoorn et al., 2004; Dixit et al., 2004; Wu et al., 2009; Thakur and Gebreyes, 2010). These techniques are applied to offer a sensitive, efficient, and reproducible database to characterise large numbers of bacteria isolates.

Enterobacterial repetitive intergenic consensus (ERIC) sequences are highly conserved 127-base pair noncoding regions that are repeated multiple times through the enterobacterial genome (Ni Chulain et al., 2006). ERIC-PCR is a molecular technique which is based on the primers binding to the ends of ERIC sequences and which then will amplify the DNA between the ERIC sequences (Versalovic et al., 1991). This results in reproducible and unique banding patterns for different genomes. ERIC-PCR has been widely used for genetic typing of bacteria providing strain-specific fingerprinting. For food safety ERIC-PCR has been applied to determine the genotypes of enterobacteria, such as E. coli (Warriner et al., 2002; Namvar and Warriner, 2006) and Salmonella (Cao et al., 2008). Wan et al. (2011) conducted a study in the Southwest of China to examine the genetic diversity of E coli in commercial swine farms using ERIC-PCR and repetitive extragenic palindrome-PCR (REP-PCR). They found a high diversity of E. coli strains from five pig farms in this area by both

methods, and the ERIC-PCR profiles provided higher discrimination power than REP-PCR. Yuan et al.(2010) studied the genotypes of airborne E. coli isolates using ERIC-PCR in four Chinese pig houses, and demonstrated that the airborne strains were mainly (90%) from faeces due to the identical ERIC-PCR profiles. Warriner et al. (2002) studied cross-contamination of pig carcasses and slaughter equipment with ERIC-PCR genotyping of E. coli, and showed that ERIC-PCR was useful for tracing the movements and distributions of genotypes from carcasses to environmental contact surfaces.

## 1.8 AIMS AND OBJECTIVES OF THIS STUDY

There is much evidence that pig carcasses may be contaminated with pathogens during slaughtering. Although HACCP have been applied to reduce or eliminate such contamination, however, as a preventative measure HACCP has just focused on final microorganism counts of end products being of a hygienic standard. Previous studies have shown the power of examining the microbial community changes which occurs during slaughter processing stages rather than examining the final microbiological status only. It is important to study the microbial community changes because it helps to identify cross-contamination points, which could lead to improving the hygiene of certain processing stages, and also provides a detailed view of the cross-contamination events, such as the introduction of new bacterial strains. Uncontrolled bacterial contamination and/or cross-contamination during the whole slaughter process is a principal hazard that must be prevented, eliminated, or reduced. This information can also verify a HACCP system by

determining the effectiveness of controls.

The aims of the study was to examine the microbiological contamination of pork carcasses and the working environments during slaughter, through identification of the changes in the E. coli community during the slaughter process with a view to determine the sources of cross-contamination, and therefore the major sites of faecal pathogen transfer.

# CHAPTER 2 GENERAL MATERIALS AND METHODS

## 2.1 CARCASS SAMPLING

Carcasses were selected at random and labelled (tagged using black cable ties before dehairing, and labelled using Castell meat marking pencils on the head, after dehairing) so that the same carcasses could be followed along the processing line. Polywipe sponge swabs (50 $cm^2$, pre-moistened with peptone-saline; Medical Wire & Equipment, UK) were used for carcass surface sampling. The whole carcass swab samples were collected by the FSA-recommended sponge-sampling method for the assessment of microbial carcass contamination in slaughterhouses (FSA, 2006). This procedure used agitated sponging (moving the sponge by a few centimetres from side-to-side as the carcass is swabbed) down the whole length of one side of the carcass. The area sponged was then calculated by estimating the length of the carcass swabbed by the width of the path contacted by the sponge. The sampled area was approximate 1500 $cm^2$.

## 2.2 EQUIPMENT SURFACE SAMPLING

Equipment surface samples were taken from the dehairer and the polisher by sponge swabbing. The swabbing areas in the polisher were as shown in Figure 2.1. Because it was difficult to swab an exact area, the team decided to swab the same zone on the inner surface and single brushes of the dehairer or polisher at each sampling.

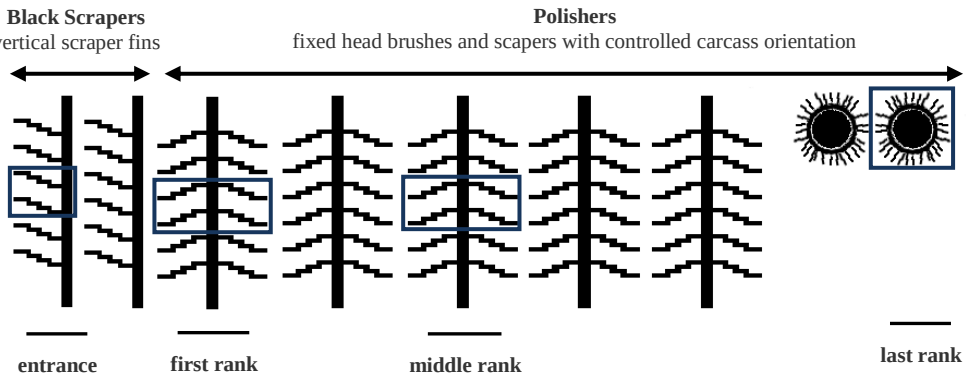



Figure 2.1. Schematic diagram of the polisher apparatus. The areas swabbed were marked in the boxes.

## 2.3 SAMPLE PREPARATION FOR MICROBIOLOGICAL EXAMINATION

In the laboratory sponge swabs were transferred to sterile stomacher bags (A. J. Steward and Co, UK) using sterile forceps. Bacteria were released from the sponges by the addition of 30 ml 0.1% buffered peptone water (BPW; CM0509, Oxoid, UK) and stomaching for 2 min at 230 rpm using a Model 400 stomacher (A. J. Steward and Co, UK). For each sample, 10 ml of the stomached bacterial suspension was transferred to a sterile universal and a series of 10-fold serial dilutions was made in maximum recovery diluent (MRD; CM0773, Oxoid, UK).

## 2.4 ENUMERATION OF MICROORGANISMS

### 2.4.1 Total aerobic count

The determination of total aerobic count (TAC) utilised the spread plate method on plate count agar (PCA; CM0463, Oxoid, UK), using a decimal serial dilution series prepared in MRD. One hundred µl aliquots of the relevant

dilutions were spread in duplicate on the surface of PCA plates. The plates were incubated at 30˚C for 48 hours. After incubation, the number of colonies was counted using plates in the range of 30-300 colonies and the viable counts of the sampled carcass surfaces were estimated.

### 2.4.2 E. coli count

One ml of serially diluted sample was inoculated into tryptone bile X-glucuronide (TBX) agar (116122, Merck, Germany) by pouring in duplicate plates for enumeration of E. coli (excluding E. coli O157). Plates were incubated at 42˚C for 48 hours. E. coli present as blue colonies were enumerated.

### 2.4.3 Statistical analysis

Plate counts were converted to cfu per $cm^2$ by multiplying by dilution factors and dividing by the size of the area swabbed. For the whole carcass swab, the area factor is 1500 $cm^2$. The logarithmic count results ($log_{10}$ $cfu.cm^{-2}$) were used for statistical analysis. Student's t test was used to determine the statistical significance between two data populations. The significance (P value) under Student's t test, the geometric means, and the standard deviation of the sample sets were processed in Microsoft® Excel 2007. For those sample sets for which the statistical relationships were examined, the Pearson's correlation coefficients and significances were calculated in IBM® SPSS Statistics V20.

## 2.5 ISOLATION OF E. COLI

Isolation of E. coli was carried out under ACDP2 conditions. To examine the populations of E. coli, up to five representative E. coli colonies from each sample (a carcass swab sampled at a designated sampling point) were picked from a TBX agar plate. Isolates were grown overnight on Luria Bertani (LB; Difco, US) agar at 37 ˚C for subsequent examination.

### 2.5.1 Confirmation of E. coli using indole production test

A well-isolated colony from LB agar was inoculated into 10 ml of tryptone water (Oxoid, UK) and then incubated (37˚C, 48h). Kovac's Reagent (500 µl; Pro-lab Diagnostics, UK) was added and mixed by rubbing the bottle between the hands. A pink to red colour formed in the upper layer within 10 min was identified as indole-positive.

### 2.5.2 Confirmation of E. coli using oxidase test

The oxidase test was carried out using Oxidase identification strips (Oxoid, UK). A small amount of a colony on LB agar was spread on the test strip with a sterile plastic loop. A positive result was the development of a purple colour within 5 seconds and a negative showed no colour change.

## 2.6 GENOTYPING OF ISOLATES

### 2.6.1 DNA extraction

For bacterial DNA extraction, one colony of E. coli or Salmonella from an

overnight culture on LB agar was dispersed into 100 µl of 1x TE buffer (10 mM Tris-Cl, 1mM EDTA buffer, pH7.6). The suspension was boiled for 30 min to rupture bacterial cells, and then centrifuged at 13,000 g for 15 min. Supernatants containing crude DNA were carefully transferred into new eppendorf tubes and stored at -20 ˚C before use.

### 2.6.2 ERIC-PCR

Genotypes were analysed using the ERIC-PCR method presented by Versalovic et al. (1991). The PCR mixture (25 µl) contained 1 µl DNA and 24 µl of reaction buffer (Thermal Scientific, UK), containing 0.2 mmol $l^{-1}$ each of the deoxribonucleotide triphosphates (Promega, US), 4 mmol $l^{-1}$ $MgCl_2$ (Thermal Scientific, UK), and 100 pmol $l^{-1}$ of primer ERIC1 (forward) 5'–ATGTAAGCTCCTGGGGATTCAC–3' and ERIC2 (reverse) 5'–AAGTAAGTGACTGGGGTGAGCG–3' (MWG Oligo Synthesis, UK), and 1 U of Taq DNA polymerase (Thermal Scientific, UK). The reactions were carried out in a programmable thermocycler TC-512 (Techne, US) at the following temperatures: one cycle for 3 min at 94˚C, then 35 cycles comprising 30 s at 94˚C, 1 min at 52˚C, 4 min at 65˚C. The final cycle was for 8 min at 65 ˚C. The PCR product (5 µl) was mixed with loading buffer (1 µl) (Promega, US) and electrophoresed in a 2% w/v agarose gel, containing ethidium bromide (0.5 µg $ml^{-1}$) in 1x TAE running buffer (40mM Tris-acetate, 1mM EDTA) at 70 V for 2 h. The gel images were obtained by image-capture using the Gel-Doc XR system (Bio-Rad, UK). Electrophoretic patterns of the selected isolates were analysed by FPQuest (Bio-Rad, UK) gel analysis software. A normalization step was included in the analysis to make each entry

(information of a lane) an equal length for gel-to-gel comparisons. For the normalization, a 100 base-pair DNA size marker (Invitrogen, UK) was applied on every electrophoretic gel to allow gel-to-gel variation to be accounted for. ERIC-PCR profiles were compared using Dice's coefficient factor and clustered by unweighted group pair method with arithmetic averages (UPGMA) with 1.5% of optimization and 1.0% of tolerance to display the dendrogram.

### 2.6.3 Confirmation of clusters and statistical examination

Clusters in a dendrogram were defined at a selected similarity level. The confidence of the selected similarity threshold and the significance of clusters were tested using the analysis of molecular variance framework (AMOVA) presented by Excoffier et al. (1992). The significance was examined with the calculation of $\Phi_{PT}$, a measure of population differentiation that suppresses intra-individual variation. In the case of AMOVA, the null hypothesis (H0; $\Phi_{PT} = 0$) means there is no genetic difference amongst the populations and the alternative hypothesis (H1; $\Phi_{PT} > 0$) means genetic differences exist amongst the populations. The calculation was performed in the software GenAlEx v6.41 as described by Peakall and Smouse (2006).

# CHAPTER 3 E. COLI AND SALMONELLA CONTAMINATION ON PORK CARCASSES

## 3.1 INTRODUCTION

As discussed in the introduction, slaughterhouse processes can contribute to the presence of enteric pathogens on pork carcasses. This investigation was to examine the changes in E. coli populations on pig carcasses from the entry of the slaughterline to the end of process as a way of determining sources of contamination on the final carcass. Comparison of sources with those contributing to Salmonella found on the carcasses would allow the appropriateness of E. coli as an indicator for Salmonella to be examined. Warriner et al. (2002) previously used this approach to examine carcass contamination in pork processing but in their study ten carcasses were sampled randomly at each processing stage. In the current study it was intended that ten tagged carcasses would be sampled so that flora changes on individual carcasses could be monitored through the whole process. The objective was to investigate carcass contamination levels at different slaughter stages by using microbiological enumeration and population changes in E. coli, and compare this with the detection of Salmonella on the carcasses. In this chapter the first stage of this work is presented based on the count data for E. coli and presence of Salmonella through the process. In addition, aerobic count data are used for comparison as these are a standard hygiene indicator typically used in slaughterhouses to determine process efficacy.

Slaughterhouse A, chosen for this initial study, had a vertical condensing steam

scalding system, which is unusual in UK plants. This system had been introduced in a recent refit to the slaughterhouse. This scald system also included a prewash feature using cold water sprayed on the carcasses and the carcasses were brushed. There is a limited literature on such systems and so the impact of the prewash on carcass contamination was evaluated as part of this study. Carcasses were swabbed by the Food Standard Agency recommended method as the microbiological assessment criteria for this method had been well established in previous studies (Snijders and Collins, 2004). In this study, E. coli was used for the investigation of enteric bacterial contamination during slaughter processing.

The sampling in this study was done in association with FSA study MO1040. Samples at the slaughterhouse were collected by a team of four researchers. E. coli enumeration from the samples was carried out solely by S.H. Wei; the aerobic counts and Salmonella data were provided by researchers on the MO1040 project and are included for comparison.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Slaughterhouse

The in-plant sampling of this study was conducted in Slaughterhouse A. Sites sampled are shown in the schematic flow diagram of the slaughter line (Figure 3.1). The normal throughput in slaughterhouse A was 210 carcasses per hour (approximate 1800 carcasses/day). The type of scalding was a vertical condensing steam scalding module at 60-61 ˚C on the transport line; the singer

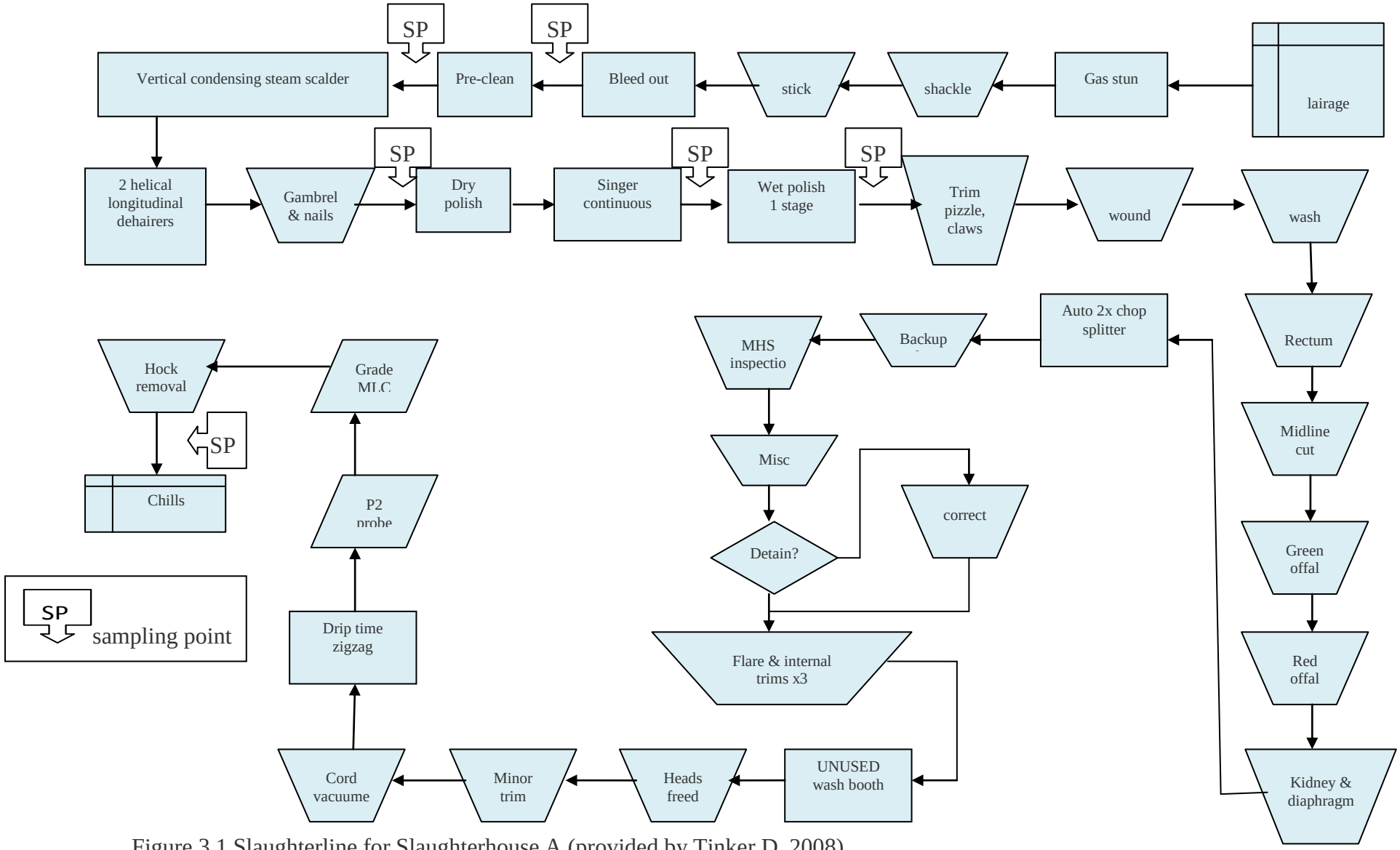



Figure 3.1 Slaughterline for Slaughterhouse A (provided by Tinker D, 2008)

SP: the designated sampling point; rectangles: automatic operations; trapezium: manual operations; parallelograms: inspections; rhombus: detaining area

was an intermittent gas flaming with two ranks of gas burners, and a singeing residence time of approximate 16 seconds. More detailed information of this abattoir's processes is listed in Appendix 3.1. The flow diagram and the table were reproduced by kind permission of Dave Tinker & Associates complied as part by project MO1040.

### 3.2.2 Carcass sampling

Carcasses sampling at this slaughterhouse was carried out as given in Section 2.1.

### 3.2.3 Bacterial sample preparation

Bacterial samples were prepared from sponge swabs as described in Section 2.3

### 3.2.4 Enumeration of microorganisms

Samples were examined for total aerobic counts and E. coli counts as given in Section 2.4.1 and Section 2.4.2, respectively. The statistical significances between two data populations were examine using Student's t test as described in Section 2.4.3.

### 3.2.5 Limit of detection

The limit of detection (LOD) is the least number of live cells of microorganism that can be derived from a specimen based on the sampling and enumeration method used. For the whole carcass swabbing, the LOD of total aerobic counts is - 1.00 $\log_{10}$ cfu $cm^{-2}$ and is - 2.00 $\log_{10}$ cfu $cm^{-2}$for E. coli counts.

### 3.2.6 Detection of Salmonella

The bacterial sample BPW prepared as previously described was incubated at 37˚C for 18h for the detection of Salmonella. For enrichment of Salmonella, 100 µl of the incubated BPW was inoculated into 10 ml Rappaport-Vassiliadis broth (RV; Oxoid, UK) and incubated at 42˚C for 24h. A full loop (1µl) of enriched RV broth was streaked on XLD agar (Oxoid, UK) for single colonies and incubated at 37˚C for 18h. Salmonellae were typically shown as red colonies with black centres on XLD. The pure cultures of the presumptive Salmonella colonies were made on brain heart infusion agar (BHI; Oxoid, UK). Colonies were confirmed by Salmonella polyvalent somatic O antiserum (poly-O; Pro-Lab Diagnostics, UK). The Salmonella antiserum was used as given by the manufacturer's instructions. Colonies from each confirmed Salmonella isolate were suspended in 1 ml 10% glycerol (v/v) in a freezing tube and stored at -80˚C until required.

## 3.3 RESULTS

### 3.3.1 Enumeration and detection of microorganisms

#### 3.3.1.1 Total Aerobic Counts and E. coli counts

The results of mean total aerobic counts (TAC) and E. coli counts (ECC) enumerated for each sampling point are presented in Figure 3.2. Sampling at the post-bleeding stage enumerated the initial microflora which was attached on the surface of un-washed pig carcasses. The counts were the highest ($4.95 \pm 0.43$ $\log_{10}$ cfu $cm^{-2}$) of the whole slaughter procedure. The second sampling point, pre-scalding, involved a cold water wash removing soil, faeces,

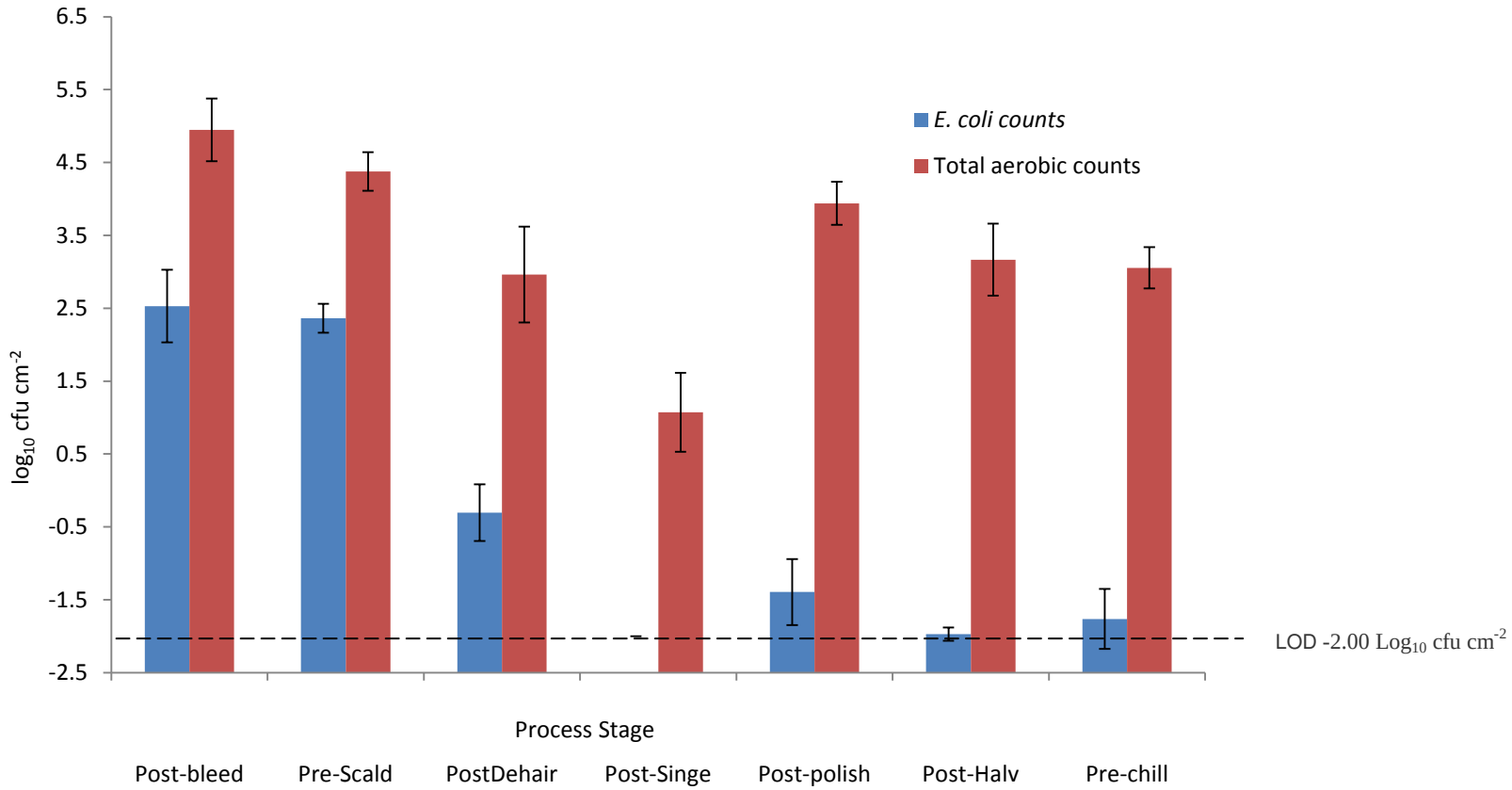
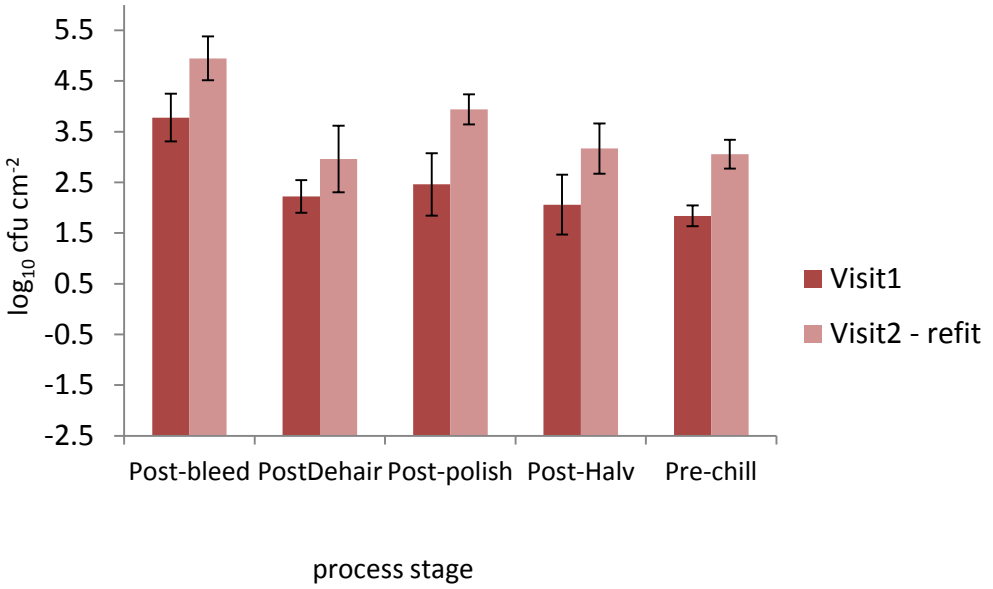



Figure 3.2. Mean total aerobic counts and E. coli counts of carcasses (n =10) across slaughter process.

skin attachments, and blood on the surface of the carcasses. However, the TAC was not significantly changed (P=0.2). The combined processes of scalding and dehairing led to a significant (P<0.01) decrease of TAC which was obtained at post-dehairing. This value combined the presumptive decrease associated with scalding and the potential increase through dehairing as the design of the process meant no sample could be taken in between. However, it would be expected that the scalding process decreased the microbiological contamination. The singeing process showed a decontaminating effect as the TAC was significantly (P<0.01) decreased, to the lowest level obtained (1.07±0.54 $\log_{10}$ cfu $cm^{-2}$). However, a significant increase (P<0.01) of TAC at the post-polishing stage suggested the recontamination of the carcasses by this operation. The TAC at the subsequent stages (post-halving and pre-chilling) with counts remained at > 3 $\log_{10}$ cfu $cm^{-2}$ on the final carcasses. This indicates that the evisceration process did not significantly increase microbial contamination.

The ECCs were highest on carcasses post-bleeding and pre-scalding (Figure 3.2), and were not significantly different between these two stages (P=0.09). A significant reduction (P<0.01) of E. coli numbers post-dehairing was observed. No E. coli were found at the post-singeing stage (< -2.00 $\log_{10}$ cfu $cm^{-2}$), again indicated the decontamination of the singeing process. In the following stages, E. coli was found on 8 carcasses post-polishing and only 1 and 2 carcasses post-halving and pre-chilling, respectively. These results showed that E. coli counts had the same trends of increase and decrease as the total aerobic counts.

(a)




(b)

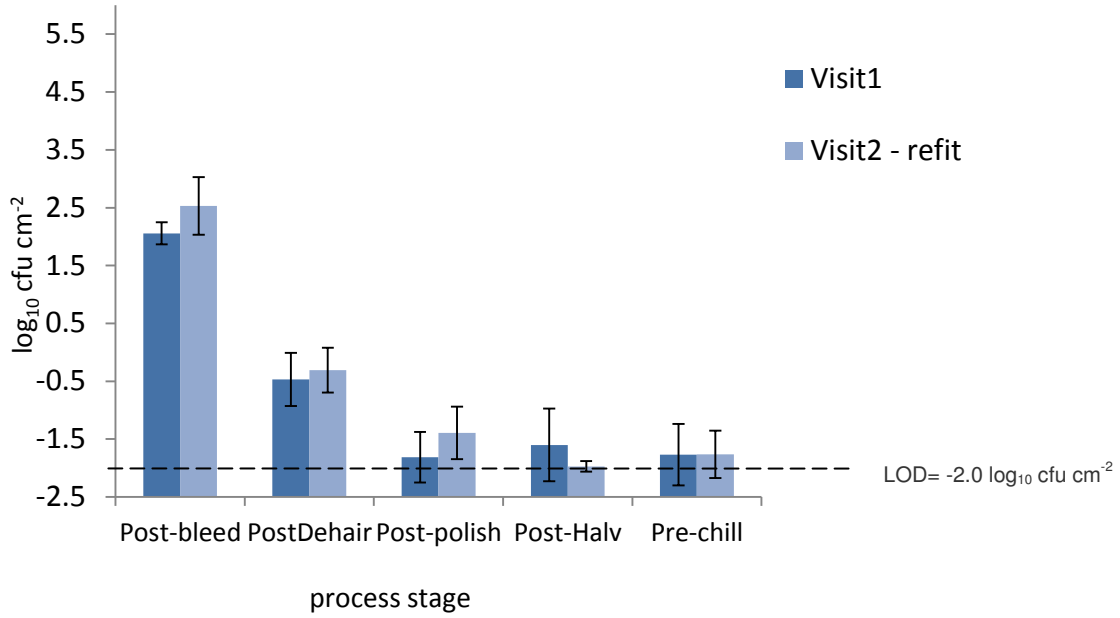



Figure 3.3. Comparisons of (a) TAC and (b) ECC on pork carcasses (n=10 for both visits) before and after slaughterline refits. Visit 1 data provided from FSA study MO1040.

In FSA study MO1040, which took place in parallel with the current work, Slaughterhouse A was visited prior to the refit and a similar determination of bacteria on the surface of carcasses conducted. Comparisons of TAC and ECC recovery between the two visits are shown in Figures 3.3a and b, respectively (diagrams were reproduced based on the original data provided by Richards, P.J. and Dodd, C.E.R.). The comparisons were made at the five sampling points which were sampled in both trials and by the same methods. TAC observed with Visit II (post-refit) were higher than with Visit I ($P=0.05$) at all five sampling points, but both trials showed the same trend ($r=0.949$) during the slaughter process. Plus, a non-significant difference ($P=0.90$) between the ECCs of the two visits indicated that the refit did not provide positive effects on the reduction of bacterial contamination.

#### 3.3.1.2 Prevalence of Salmonella

The swabs taken from the ten carcasses at the seven sampling points were subjected to the detection of Salmonella. Of these samples (n=70), nineteen (27.1 %) tested were confirmed as positive (Table 3.1). Salmonella contaminated samples represented 70 % of post-bleeding and 60 % of pre-scalding samples, but it was only found relatively sporadically thereafter. The correlations between E. coli counts and Salmonella prevalence were examined using Pearson's correlation coefficient. A positive correlation between the presence of E. coli and Salmonella detection ($r=0.555$; $P<0.001$) was found on individual pigs. Furthermore, a strong positive correlation ($r=0.942$; $P=0.005$) was observed between the mean of ECCs and the

Table 3.1.The detections of (a) Salmonella and (b) E. coli on pig carcasses (n=10).

**(a)**

| | Processing stages | | | | | | |
|---|---|---|---|---|---|---|---|
| Pig no. | Post-bleed | Pre-scald | Post-dehair | Post-singe | Post-polish | Post-halv | Pre-chill |
| 1 | X* | X | | | | | |
| 2 | X | X | | | X | | |
| 3 | X | X | | | | | |
| 4 | X | X | | | | | X |
| 5 | | | | | | | |
| 6 | X | | | | | | |
| 7 | | | | | | | |
| 8 | | | X | | X | | |
| 9 | X | X | X | | X | | |
| 10 | X | X | | | | | |
| Sum of positive pigs | 7 | 6 | 2 | 0 | 3 | 0 | 1 |

* Samples with Salmonella detected were labelled 'X'. Data were provided from FAS study MO1040

**(b)**

| | Processing stages | | | | | | |
|---|---|---|---|---|---|---|---|
| Pig no. | Post-bleed | Pre-scald | Post-dehair | Post-singe | Post-polish | Post-halv | Pre-chill |
| 1 | X* | X | X | | | | X |
| 2 | X | X | X | | X | | |
| 3 | X | X | X | | X | | |
| 4 | X | X | X | | X | | |
| 5 | X | X | X | | X | | |
| 6 | X | X | X | | | | X |
| 7 | X | X | X | | X | X | |
| 8 | X | X | X | | X | | |
| 9 | X | X | X | | X | | |
| 10 | X | X | X | | X | | X |
| mean of ECC ($log_{10}$ cfu $cm^{-2}$) | 4.53 | 4.36 | 1.69 | ND† | 0.61 | 0.03 | 0.24 |

* Samples with E. coli detected were labelled 'X'.
† ND: E. coli was not detected.

percentage of Salmonella detection at each sampling point. This indicates that the factors affecting the change in ECCs during the slaughter process had a similar effect on the presence of Salmonella.

## 3.4 DISCUSSION

In this study, the microbiological reduction effects of scalding and singeing were observed although it was not possible to sample directly after scalding because of the contained design of the scalding-dehairing process. Post-dehairing carcasses showed a 1.42 $\log_{10}$ cfu $cm^{-2}$ reduction in TAC and a 2.67 $\log_{10}$ cfu $cm^{-2}$ reduction in ECC compared with pre-scalding, even though the dehairing process may in some cases, cause re-contamination of carcasses after scalding (Gill and Bryant, 1993; Berends et al., 1997). Such overall reductions are probably associated with the steam (approximate 61-66˚C) treatments in this process. Similar to the findings in the present study, the positive effects of scalding on pork hygiene have been observed in other studies. Dockerty et al. (1970) demonstrated an approx. 1.7 log decrease of TACs due to scalding of carcass (at 58.5˚C for 6 min). Bolton et al. (2002) reported that the TACs after scalding-dehairing (at 62-70˚C for 2-3 min) were approximate 1 $\log_{10}$ cfu $com^{-2}$ lower than after bleeding. However, in the present study the refit of the condensed steam scalder may not enhance microbiological benefits as no significant difference was seen between the bacterial counts of Visit 1 and Visit 2, although the Institute for Prospective Technological Studies (IPTS) has reported that condensed scalding system offers advantages of the reduction of energy and water consumption (ECJRC-IPTS, 2005).

Singeing showed a significant decontamination ability during the slaughter process and reduced aerobic bacteria to approximately 1 $\log_{10}$ cfu $cm^{-2}$, and the E. coli on carcass surfaces was undetectable. E. coli at this stage may be injured and unable to recover on selective media. Such reductions are typical; Bolton et al. (2002) showed that the average TACs obtained after singeing were approximate 3 $\log_{10}$ cfu $cm^{-2}$ lower than the TACs after dehairing. Pearce et al. (2004) showed that singeing resulted in a 2.5 $\log_{10}$ cfu $cm^{-2}$ reduction in coliform counts.

According to the Food Standards Agency, the acceptable number of Enterobacteriaceae on pork carcasses using the swab method is < 1.3 $\log_{10}$ cfu $cm^{-2}$, and it is unacceptable if > 2.3 $\log_{10}$ cfu $cm^{-2}$ (FSA, 2002). Although FSA do not provide specified criteria for E. coli, it can be seen that the level of E. coli contamination on the carcasses before scalding was unacceptable as they were > 2.3 $\log_{10}$ cfu $cm^{-2}$ and Enterobacteriaceae would be higher than this. The E. coli counts at the stages after the singeing process were all < -1.5 $\log_{10}$ cfu $cm^{-2}$, which suggested the final products would be verified as safe from Enterobacteriaceae counts.

During pork processing, the decontamination steps, such as scalding and singeing, showed an effective bacterial reducing effect as seen in other studies (Pearce et al., 2004; Delhalle et al., 2008). On the other hand, a relatively high TAC was recovered from carcasses post-polishing, which is generally seen as a cross-contamination step (Berends et al., 1997; Bolton et al., 2002; James et al.,

2007). This slaughterline therefore showed a very typical pattern of cross contamination as seen in other studies. The increases of bacterial counts during polishing as a source of carcass contamination has been discussed (Gill and Bryant, 1993; Berends et al., 1997; Pearce et al., 2004), and the results also supported this viewpoint. Gill et al. (1997) suggested that bacteria persist on areas of the skin protected from the singeing flames and are redistributed over the carcass during the polishing operation. This may explain the significant increases of TAC and ECC, and the detection of Salmonella after the polishing process.

Sponge-swabbing is a frequently-used method for bacteriological sampling of animal carcasses which is non-destructive and less labour intensive than excision methods (Korsak et al., 1998; Capita et al., 2004; Pearce and Bolton, 2005). Using the Food Standard Agency recommended sponge swabbing method, the swabbed area of 1500 $cm^2$ was an estimated size. The different sampling sizes estimated by the workers can be a possible factor resulting in changes in counts seen in different visits. With regards to the variation in counts seen between Visit 1 and 2, although all of the mean TACs were higher after the refit compared with the first visit, the patterns of increase and decrease of mean TACs of both visits were similar, and so were the mean ECCs. These results indicate that the sampling method provides a stable level of reproducibility. Thus, the higher level of microbial detection in Visit 2 suggested that the refit may have resulted in the changes of route and level of contamination among the operation stages. The different origins of the slaughtered animals could be another factor which may affect the level of

contamination.

This study has demonstrated that Salmonella was frequently present along a slaughterline, especially at the stage where the carcasses entered the slaughterline. The presence of Salmonella at early processing stages indicates that Salmonella-carrying pigs have been introduced into the plant. Salmonella infected pigs entering slaughterhouses have been demonstrated as an important source of Salmonella at slaughter (Bahnson et al., 2006). Berends et al. (1997) demonstrated that about 70% of carcass contamination results from the animals themselves being carriers, and 30% because of Salmonella-free pigs being cross-contaminated from infected pigs. In a national survey in the UK, a wide range of prevalence of Salmonella on carcasses was found between abattoirs, with a mean of 5.3% positive, with 8.7% prevalence found in the Midlands, 3.5% in the South, and 4.4% in the North of England and Scotland (Davies et al., 2004). In a survey conducted with four Northern Ireland slaughterhouses, 40% of swabs taken from the surface of carcasses post-evisceration were Salmonella positive (McDowell et al., 2007). Botteldoorn et al. (2003b) sampled five commercial abattoirs in Belgium and found Salmonella on 37% of pig carcasses which ranged from 0% to 70%. In another study conducted in Belgium (Delhalle et al., 2008), the variability of Salmonella prevalence was found ranging from 2.6 to 34.3% among 10 large pig slaughterhouses, according to the area of origin. Various environmental factors might affect the prevalence, such as the control of lairage pens, the status of the scald water, and the cleanliness of the pigs (Letellier et al., 2009). Although eight out of the ten carcasses were positive for Salmonella in the present study, only one

pre-chilling carcass was identified as Salmonella-positive which may indicate that the slaughter processes reduced the final carcass prevalence of this slaughterhouse.

The post-bleeding stage was the most prevalent stage of Salmonella amongst the slaughter process, and the number detected was then reduced by the scalding and singeing processes. The occurrence of Salmonella on carcasses correlated with the average counts of E. coli suggesting that these two bacteria were transmitted through similar contamination routes. Delhalle et al. (2008) have also demonstrated a significant positive correlation between Salmonella prevalence and the E. coli colony counts on carcasses. Although Salmonella is of primary importance, the low numbers and problems of sampling and detection require a more numerous indicator organism to be used (Ingram and Roberts, 1976). From the findings in the present study, E. coli has shown its suitability for indicating the control of contamination of Salmonella across the slaughter process.

# CHAPTER 4 CHANGES IN CARCASS CONTAMINATION DURING PORK PROCESSING EXAMINED BY GENETIC DIVERSITY OF E. COLI AND SALMONELLA

## 4.1 INTRODUCTION

In the previous chapter, viable counting demonstrated the prevalence of microorganisms at different stages of the slaughter process. However, with only those methods, limited details of cross-contamination or dynamic changes of populations can be provided (Namvar and Warriner, 2006).

With the development of bacterial molecular typing methods, this scenario has been addressed. Many of these methods exhibit a high discriminatory power and have been applied in the investigations of bacterial contamination during pork production (Giovannacci et al., 2001; Warriner et al., 2002; Laukkanen et al., 2008; Piras et al., 2011).

In the present study, the genetic community changes of E. coli across the pork processing stages were investigated using ERIC-PCR. This typing method was used because it was quick and sensitive for genotyping a large number of Enterobacteriaceae and has been used in similar investigations (Warriner et al., 2002; Namvar and Warriner, 2006; Yuan et al., 2010). After the enumeration of E. coli described in the previous chapter, typical E. coli colonies recovered from each sampling point in Slaughterhouse A were isolated and the population diversity was further analysed. As well as E. coli isolates, the 29 Salmonella isolates obtained previously were genotyped by ERIC-PCR, and

the correlation between changes in this pathogen populations and E. coli as its indicator were also compared.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Isolation of E. coli

After the enumeration of E. coli as described in Section 2.4.2, E. coli colonies were subsequently isolated from the TBX plates as described in Section 2.5. E. coli isolates were confirmed using indole production test as described in Section 2.5.1.

### 4.2.2 Preparation of Salmonella isolates

The Salmonella isolates prepared as described in Section 3.2.6 were thawed from -80 ˚C and streaked on LB agar, and then incubated (37˚C, 24h). These Salmonella isolates were characterized with the monovalent somatic O antisera (Pro-Lab Diagnostics, UK) for specific O antigen groups. The Salmonella antisera were used as described by the manufacturer's instructions.

### 4.2.3 Antimicrobial susceptibility testing of Salmonella

The antimicrobial susceptibility of Salmonella isolates was tested using the disc diffusion method described by Mayrhofer et al. (2004) based on the National Committee for Clinical Laboratory Standards (NCCLS-M2-A7). Briefly, Salmonella colonies were inoculated in normal saline (0.9% NaCl) to a turbidity equivalent to 0.5 McFarland standard. The adjusted suspension (100

μl) was spread on Mueller-Hinton agar (CM0337, Oxoid, UK). The plates were allowed to dry for less than 15 min, and the standard discs (Oxoid antimicrobial susceptibility test discs) were applied using a disc dispenser and the plates were incubated at 37˚C for 24h immediately. After incubation the size of the inhibition zone was determined according to disc diffusion supplemental tables (http://www.oxoid.com/pdf/uk/2011-CLSI&FDA - table-update.pdf). Six antibiotic discs, including ampicillin (A, 10 μg; CT0003B), chloramphenicol (C, 10 μg; CT0012B), penicillin (P, 5 units; CT0124B), streptomycin (S, 25 μg; CT0048B), sulphonamide compound ($S_3$, 300 μg; CT0059B), and tetracycline (Te, 30 μg; CT0054B).

### 4.2.4 Genotyping of isolates

Genotyping of the E. coli and Salmonella isolates was undertaken using the ERIC-PCR method as described in Section 2.6.

## 4.3 RESULTS

### 4.3.1 Preliminary genotyping

PCR based typing methods are often reported as showing poor reproducibility. Therefore, a preliminary study was conducted before a large number of isolates was analysed, to determine the percentage similarity (%S) which was found between ERIC profiles of replicate strains (Figure 4.1). More than ninety percent (90.5% - 100%) similarity was seen for the replicate samples on the same gel or on different gels, and less than 2% similarity was observed for the same strain compared with Salmonella. The high reproducibility and the

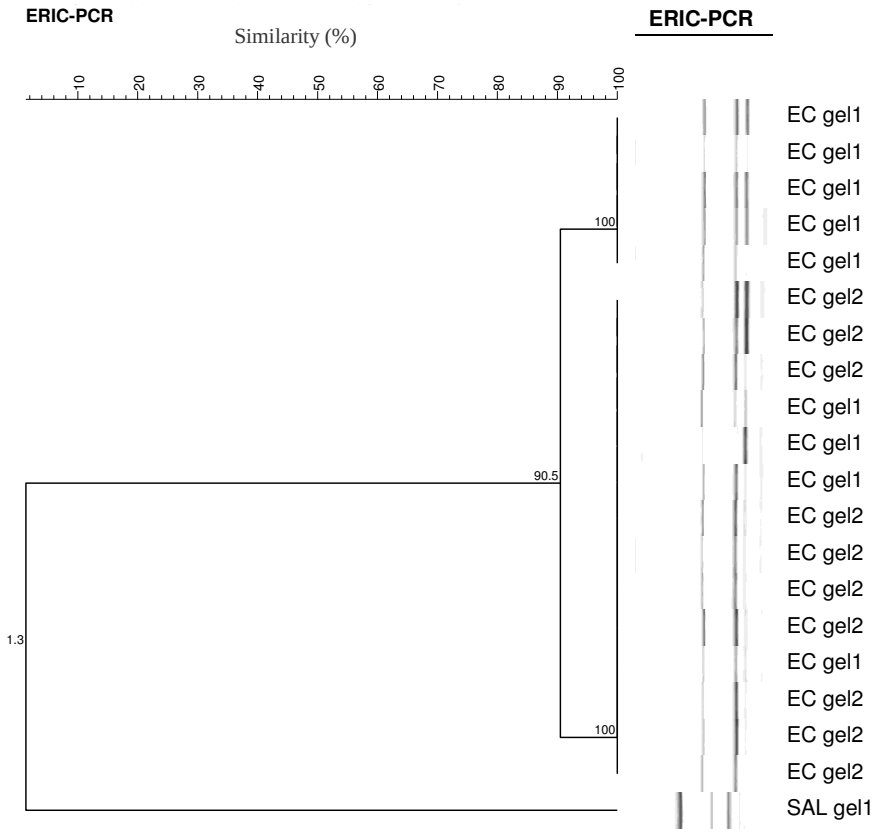



Figure 4.1. Preliminary trial of ERIC-PCR genotyping. The ERIC-PCR amplicons (n=19) of a pig carcass E. coli isolate were resolved by two agarose gels [2% TAE gel, 80V for 2h; 9 replicates in gel 1 (EC gel1) and 10 replicates in gel 2 (EC gel2)]. The comparison was performed with an outliner strain (pig Salmonella, SAL gel1). This dendrogram was produced by FPQuest v5.1 with the UPGMA algorithm based on a Dice similarity coefficient with a 1.5% band position tolerance.

discrimination ability suggested that ERIC-PCR was a suitable tool for the evaluation of E. coli diversity in this study.

### 4.3.2 Genotyping of E. coli isolates

One hundred and thirty five E. coli colonies were isolated from TBX plates of the ten carcasses at different processing stages as representative isolates. All of these isolates were confirmed as E. coli by indole testing. The similarity of the ERIC-PCR profiles of these E. coli isolates from processing stages, post-bleeding (n=44), pre-scalding (n=49), post-dehairing (n=35) and post-polishing (n=7) were analysed and the clustering analysis is shown as a dendrogram (Figure 4.2). The ERIC-PCR profiles were divided into eight groups, and one unrelated isolate, at a 40% similarity threshold. This threshold was chosen due to the cluster significance ($\Phi_{PT}$=0.295 ; P<0.01); AMOVA statistical analysis using a higher threshold (>40%S) demonstrated non-significant branches amongst the groups. Group I (n=8) was a small group composed of pre-scalding, post-dehairing, and post-polishing isolates. In this group, seven isolates from pig 3 (post-dehairing and post-polishing), pig 6 (pre-scalding and post-dehairing), and pig 10 (pre-scalding and post-dehairing) indicated survival of E. coli on the same carcass along the processing line. Most of the pre-scalding and post-dehairing isolates (n=80) were clustered into Group III suggesting the same strains were present over these two stages and strains survived the scalding process. The post-bleeding isolates were mainly clustered in group IV (n=24) at 49.2% similarity level suggesting a related population. The majority (57%) of post-bleeding E. coli isolates clustered together as a subgroup (group IV, Figure 4.2) but showed a significant

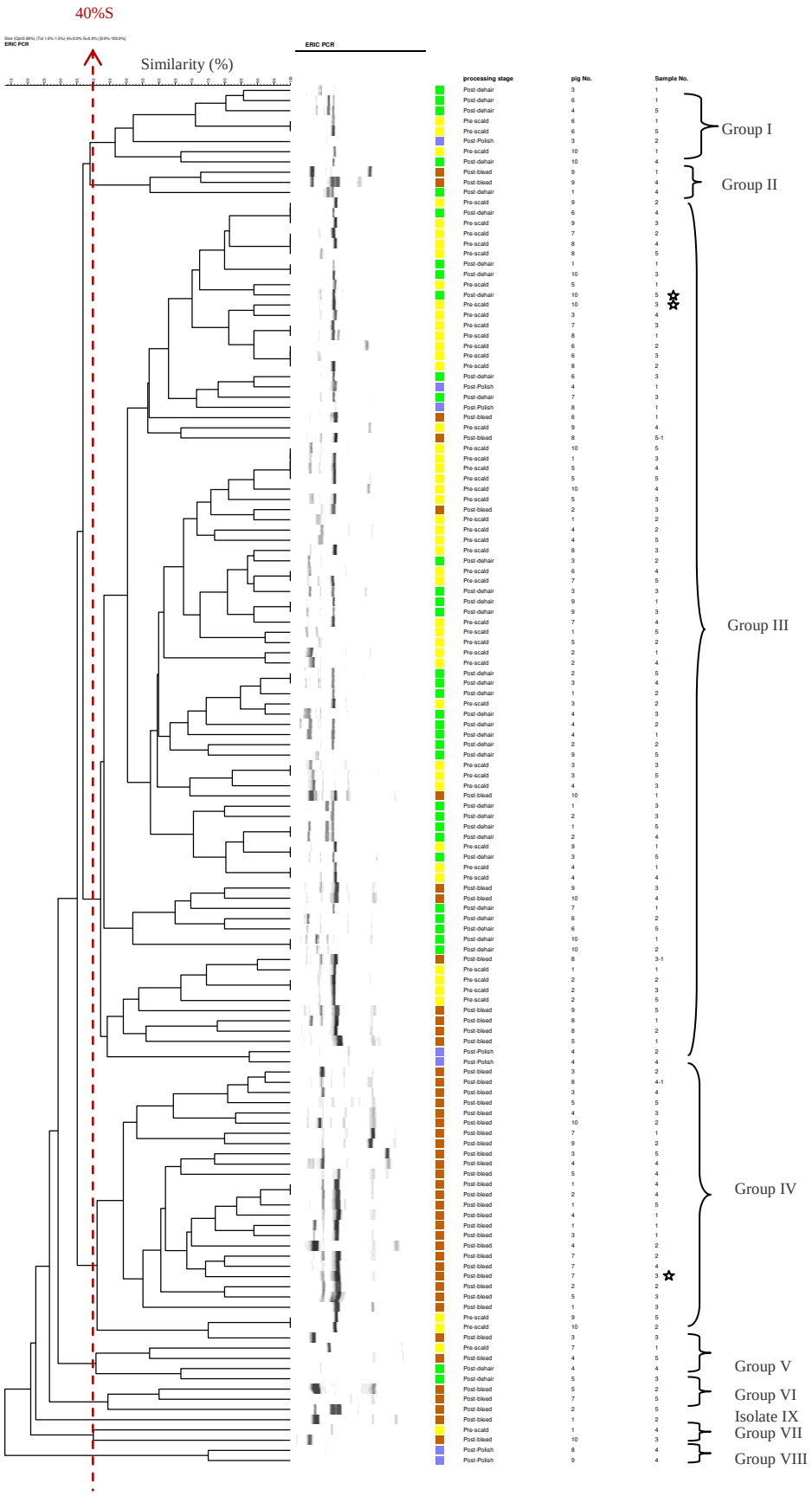



Figure 4.2. ERIC-PCR genotyping of 135 E. coli isolates from pig carcass samples (similarity≧ 40%). The dendrogram was produced by UPGMA algorithm based on a Dice similarity coefficient with a 1.5% band position tolerance.

(brown: post-bleeding; yellow: pre-scalding; green: post-dehairing; purple: post-polishing; asterisks: selected isolates for further characterisation)

difference ($\Phi_{PT}$=0.264) from the majority genogroup (86%) of pre-scalding samples (group III). As the difference between these two stages is a wash stage, it does suggest that this stage is important in changing the E. coli populations present. Cluster analysis of strains isolated at each stage of processing showed different levels of overall similarity of the populations at each stage (in Appendix.4.1). After bleeding the overall population diversity was 21.1%S suggesting a very diverse population. At the pre-scalding stage this was raised to 28.9%S indicating a change in population structure by loss of diversity, supporting the previous analysis that a change in population occurred at this stage. The number of isolates recovered after polishing was low (n=7) but the population was very diverse (18.9%S) which could suggest the introduction of new strains rather than an increase in the number of those already on the carcasses.

Isolates with 100% similarity were considered genotypically identical. Amongst the identical isolates (n=41), 44% were from the same pig at the same processing stage. However, identical isolates were more frequently (51%) found from different pigs at the same stage (eg., pigs 1, 5 and 10 pre-scalding; pigs 2 and 3 post-dehairing) , indicating cross contamination during processing.

#### 4.3.2.1 Changes in strains through processing

A comparison of the E. coli counts with the genogroups detected at each stage of processing is shown in Figure 4.3. The post-bleeding samples showed both the highest counts and the most strain diversity (six genogroups detected) amongst the stages. The pre-scalding isolates were in five genogroups.

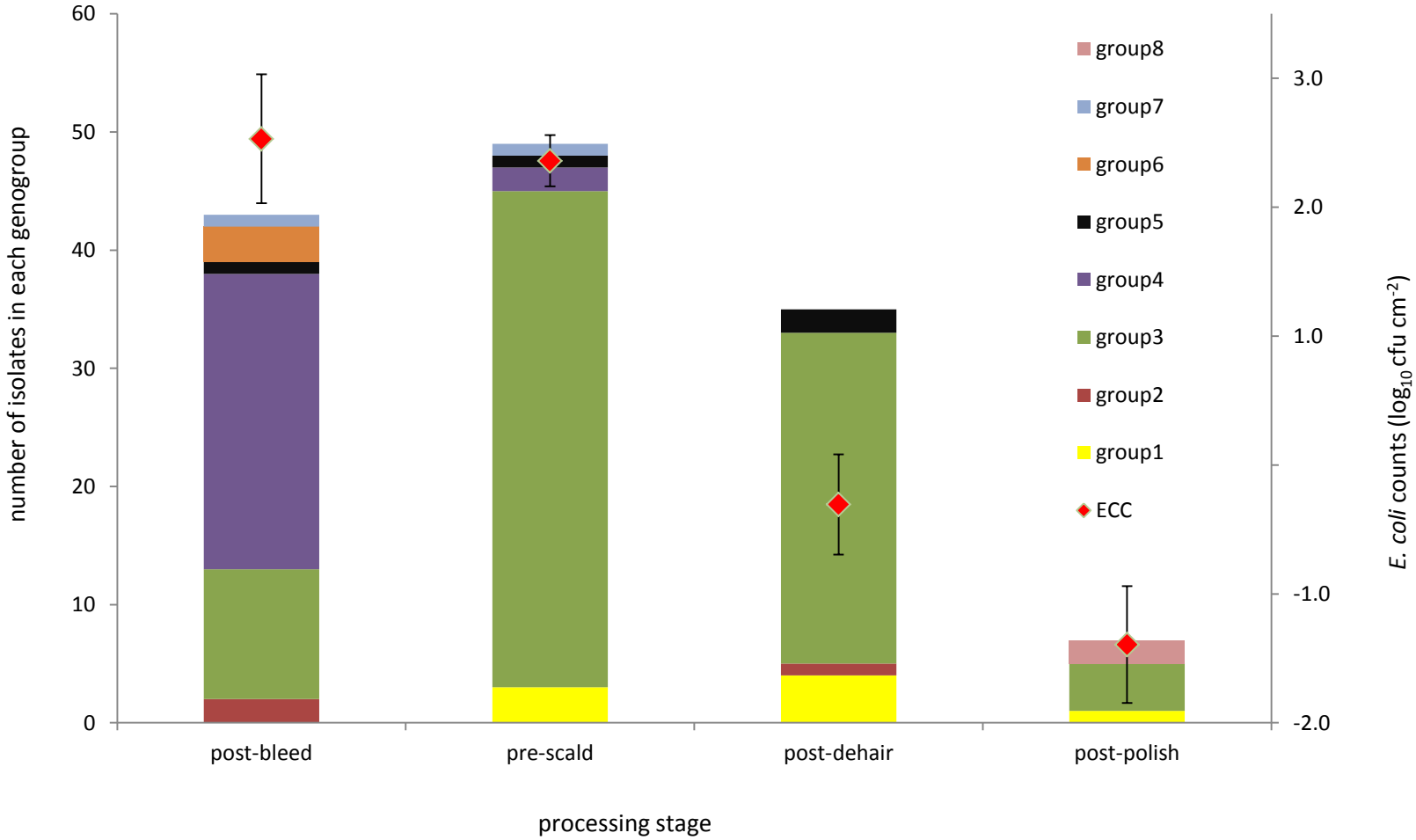



Figure 4.3. Evaluation of population changes during processing by the counts and genotyping of E. coli isolates ( n=135).

However, the population of pre-scalding samples was different from the post-bleeding samples whilst the ECC had barely changed. The majority of the post-bleeding samples were in Group IV (57%) whereas the pre-scalding samples was Group III (86%). This indicates that the washing step between the bleeding and scalding processes performed a selection effect on genotypes. Group VIII showed a low similarity (13%) with other isolates and was only detected on the post-polishing carcasses suggesting that this genogroup may be introduced from other sources.

#### 4.3.2.2 Multi-drug resistance and ERIC-PCR profiles of Salmonella isolates

The Salmonella isolates (n=29) obtained from this slaughterline were subjected to serogrouping, genotyping, and antimicrobial susceptibility testing. Serogrouping of the Salmonella isolates from the positive pigs showed that all of those isolates belonged to serogroup O4 or serogroup O23 with both found on pigs at the start of processing (Figure 4.4). It was found on three animals (pig 2, 4, and 9) that the Salmonella serotype at initial stages (post-bleeding and pre-scalding) was different from the serogroup found at later processing stages. The change of serogroups could be a consequence of cross-contamination during processing, and in two of the three examples was detected post-polishing when recontamination with TAC and ECC was seen.

Amongst the 29 isolates, six multi-drug resistant (MDR; defined as resistance to three or more classes of antimicrobials) isolates were identified (Table 4.1). The isolate Sal-post-dehair 9A was the most resistant strain and showed

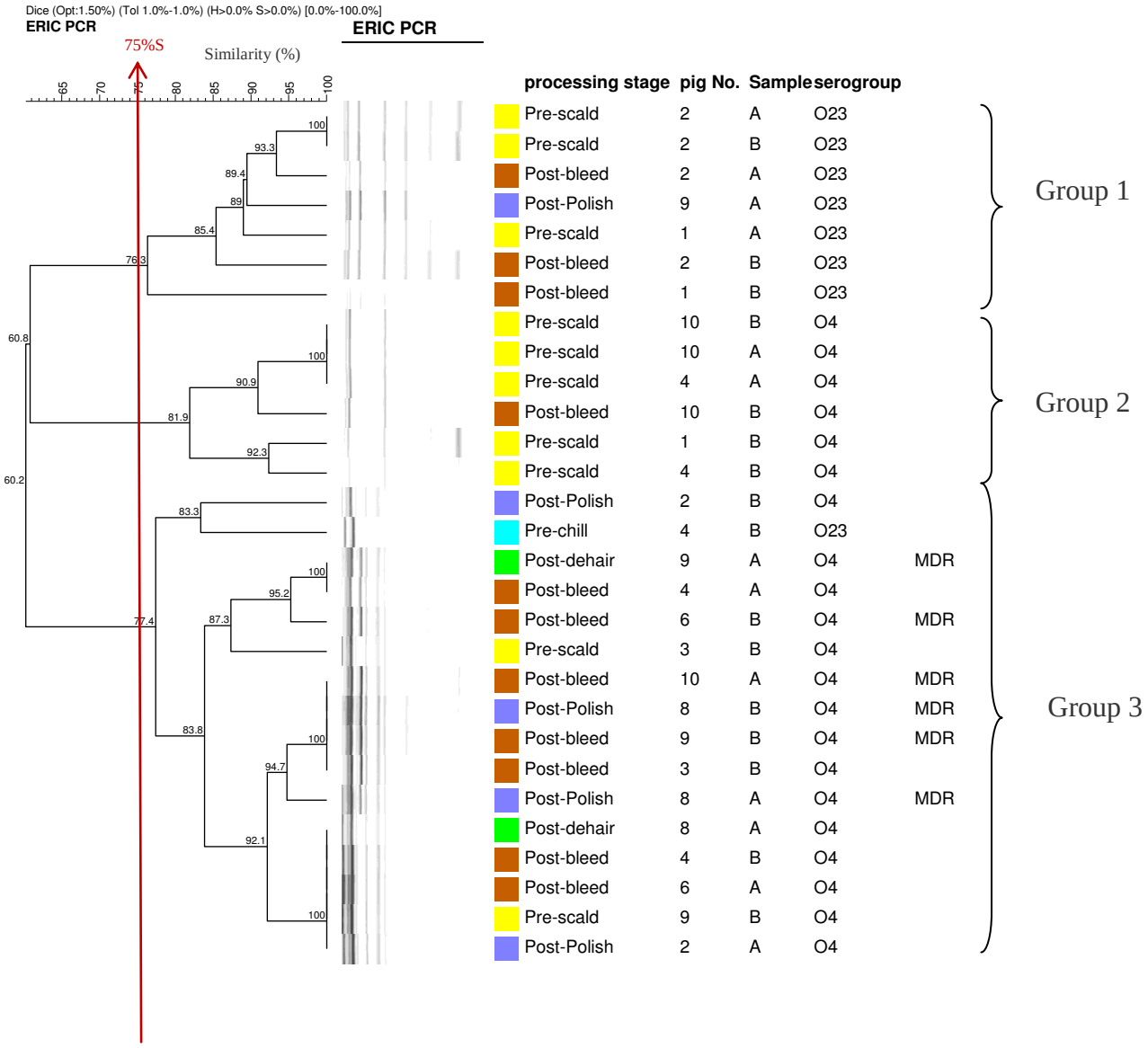



Figure 4.4. Dendrogram obtained from cluster analysis (Dice coefficient; UPGMA) of ERIC-PCR types of 29 Salmonella spp. isolates from pig carcasses during slaughter processing. Groups are defined at 75% similarity level.
(Brown: post-bleeding; yellow: pre-scalding; green: post-dehairing; purple: post-polishing; blue: pre-chilling)

resistance to all six antibiotics tested (Figure 4.5). Cluster analysis of the fingerprints divided the strains into three groups at 75% similarity as shown in Figure 4.5. The serogroups were highly associated with the genogroups, since the O4 serotypes were clustered in Groups 2 and 3, and the O23 serotypes were clustered in Group 1, except one isolate. All of the MDR isolates belonged to serogroup O4 and clustered in Genogroup 3 (within a subgroup with 84% similarity). The ERIC-profile of the MDR isolate, Sal-post-dehair 9A, was identical (100%S) with Sal-post-bleed 4A, but the latter did not present antimicrobial resistance. It may indicate that the antimicrobial resistance genes were carried on plasmids rather than on the chromosome, and subsequently lost.

The same trend of the genetic changes along the processing was seen in both organisms. Like the distribution pattern of E. coli strains, Salmonella strains recovered at the post-bleeding stage were clustered in a different genogroup from pre-scalding samples (pairwise $\Phi_{PT}$=0.633;Table 4.2). Furthermore, the majority of post-dehairing and post-polishing samples tended to cluster in the predominant genogroup.

## 4.4 DISCUSSION

The objective of this study was to investigate the genetic diversity amongst the E. coli and Salmonella isolates using DNA fingerprinting with a view to identifying process stages which result in flora changes. Molecular genotyping by ERIC-PCR is a fast and cost-effective method for investigating the genetic diversity of bacterial strains, although the repeatability of this technique has

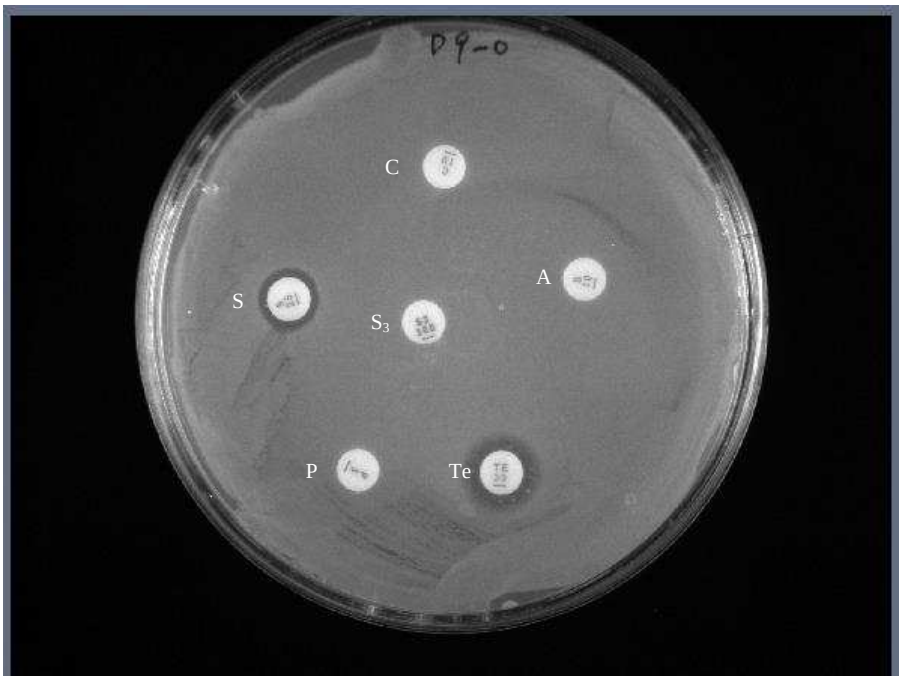



Figure 4.5. An example of antibiotic susceptibility examination of a multi-drug resistant Salmonella isolate (Sal-post-dehair 9A). Six antibiotic discs, including ampicillin (A), chloramphenicol (C), penicillin (P), streptomycin (S), sulphonamide compound ($S_3$), and tetracycline (Te), were tested.

Table 4.1. Detection of multi-drug resistant Salmonella isolates.

| **Isolate code** | **Multi-drug resistance profile*** | **Somatic O group** | **ERIC-PCR genogroup** |
|---|---|---|---|
| Sal-post-dehair 9A | $ACPSS_3Te$ | O4 | Group3 |
| Sal-post-bleed 10A | $APSS_3Te$ | O4 | Group3 |
| Sal-post-polish 8A | $APSS_3Te$ | O4 | Group3 |
| Sal-post-polish 8B | $APSS_3Te$ | O4 | Group3 |
| Sal-post-bleed 9B | $PS_3Te$ | O4 | Group3 |
| Sal-post-bleed 6B | $PS_3$ | O4 | Group3 |

***** Disc code: A, ampicillin; C, chloramphenicol; P, penicillin; S, streptomycin; $S_3$, sulphonamide compound; Te, tetracycline.

Table 4.2. Distribution of the major genotype groups of E. coli and Salmonella isolates amongst processing stages.

| | | Pork processing stages | | | |
|---|---|---|---|---|---|
| | | Post-bleed | Pre-scald | Post-dehair | Post-polish |
| E. coli | major genogroup | IV | III | III | III |
| | number and percentage of isolates in group at this stage | 25 (57%) | 38 (76%) | 23 (66%) | 3 (43%) |
| Salmonella | major genogroup | 3 | 2 | 3 | 3 |
| | number and percentage of isolates in group at this stage | 7 (64%) | 5 (50%) | 2 (100%) | 4 (80%) |

been questioned (Weijtens et al., 1999; Meacham et al., 2003). However, with the normalisation facilities of the analysis software, ERIC-PCR gave stable and reproducible results in the present study. It appears that ERIC-PCR is suitably robust to obtain the information on diversity of E. coli isolates needed for this study.

The ERIC pattern data in the present study suggested a high diversity of E. coli types amongst the various slaughter stages. The results corroborate previous findings which reported that highly diverse populations of E. coli can be found during pork slaughter (Warriner et al., 2002; Wu et al., 2009). Namvar and Warriner (2006) investigated the E. coli transmission in a Canadian pig slaughterline by ERIC-PCR genotyping. Forty-one genotypes were defined (n=655), with 30 ERIC types found after bleeding, 8 after scalding, and 9 after singeing/polishing. Compared with the results of the present study, a similar pattern was seen in that the greatest diversity of genogroups was recovered from post-bleeding but this then decreased subsequently. This reduction following the slaughter process also indicates the selective effects of the processing stages, particularly after the scalding and singeing processes.

It appears that most of the isolates recovered at the post-bleeding stage are relatively transient types due to their absence in later operations. This selection between bleeding and pre-singeing stages was due either to the exposure of predominant genotypes on the skin after washing between bleeding and pre-singeing stages, or to the removal of weakly attaching genotypes by the wash. Namvar and Warriner (2005) have demonstrated that most E. coli

genotypes recovered from pork carcasses by swabbing exhibited strong attachment to pork skin, but some could be removed by rinsing.

Most isolates from pre-scalding and post-dehairing clustered in Group III the largest group in the dendrogram, indicating the majority of the E. coli isolated from post-dehairing was genetically related to those isolated from pre-scalding. This may suggest that the approximate 2 $\log_{10}$ cfu $cm^{-2}$ decrease of E. coli numbers between these two points just reduced the existing population numerically. However, it was noted that isolates of Group IV, VI and VII were not present at post-dehairing and later sampling points. This may indicate that the scalding operation did reduce the number of E. coli by a selection of some genotypes, but those that were left to later stages then became the predominant genotypes on the carcasses. Namvar and Warriner (2006) studied the genotypic changes of E. coli isolates on pork carcasses during slaughter processing, and showed that only two genotypes were recovered at the scalding stage. However, the genotypes found in later stages (scraping, evisceration, and cooling) were matched to those recovered in earlier operations, concluding that although scalding reduced the levels of enteric organisms, the survivals can be present on carcasses again in later processes.

Like scalding, singeing is also an effective decontamination process but contamination can happen during the polish process. Four possibilities for the source of contamination after scalding or singeing were considered: (1) those E. coli isolates found could be thermo-resistant strains and could survive through the hot-water scalding; (2) those E. coli isolates found were hidden in a

non-fully scalded/singed part, then re-distributed onto the carcass surface by the later dehairing/polishing process; (3) the E. coli isolates were redeposited from the equipment of the slaughter line; (4) faecal leakage occurs during dehairing and polishing and is redistributed over the carcass and other carcasses via the slaughter line equipment. The persistence and redistribution of bacterial contaminants was observed with arcobacters (Houf and Van Driessche, 2007) and campylobacters (Ellerbroek et al., 2010) on animal carcasses. The precise heat resistance of E. coli recovered after the scalding process in this study remains unknown and needs to be further examined.

Group VI and Group VIII were independent groups which were only found associated with one processing stage in this trial (only recovered on post-bleeding and post-polishing carcasses, respectively). The fact that Group VI disappeared after the pre-scalding wash stage would support the idea that much of this is superficial contamination readily removed. Furthermore, these distinct groups suggest that various sources of E. coli contaminants were associated with these stages. The surface of a bled carcass still had hair-on and was covered with visible dirt, blood, and faeces. Wu et al. (2009) noted that E. coli isolates were found to be of higher genetic diversity from stunned carcasses than from faeces. It suggested that the surfaces of carcass at the early stage of slaughter were exposed not only to E. coli originating from the pig's own faeces, but also to cross-contamination between pigs or from the environment. The fact that Group VI disappeared after the wash stage would support the idea that much of this is superficial contamination readily removed. The wash stage is not seen in most UK slaughter lines, so the removal of the

superficial contamination in other slaugherhouses is during scalding. Thus, this may suggest that high levels of microorganisms would be introduced into the scalding water or equipment.

In addition, the independent group found post-polishing suggested other sources of contamination which may not have originated directly from the same carcass. This may again indicate cross-contamination is occurring via the machinery, which is supported by the E. coli counts which increase at this stage. In a Belgian study of the prevalence of arcobacters in a pork slaughterline, Houf and Van Driessche (2007) concluded that slaughter equipment can act as a vector due to the detection of the same genotypes on different carcasses slaughtered on the same day. The new E. coli strains can be introduced to carcasses by contact with the polisher blades (Warriner et al., 2002). In the present study, however, no samples were taken from the polisher or the slaughter environment so no positive correlation with this source was possible.

Although most of the incidence of Salmonella on the slaughterline can originate from the animals themselves (Vieira-Pinto et al., 2005; 2006), the redistribution of contaminants could not explain the change of Salmonella serotypes along the processing in the current study, which indicated cross-contamination between carcasses also needs to be considered. Salmonella isolates with high similarity and identical strains were recovered not only from the same carcass, but also from different carcass at the same process stage. This is similar to the distribution of the E. coli genogroups. The genotyping

results suggested that horizontal transmission occurred within the same processing stage and the genotypes could be shared between carcasses during the process. These results are in agreement with a Belgian study (Botteldoorn et al., 2004), which indicated that only 25% of the Salmonella positive pork carcasses were contaminated with the same serotype or genotype found in the corresponding carcass faeces or lymph nodes.

It is known that antimicrobial treatment of animals may contribute to the development of resistant organisms in the treated animals (Chopra and Roberts, 2001). In previous studies, antimicrobial resistant Salmonella strains have been found from pigs at slaughter (Korsak et al., 2003; Agustin et al., 2005; Schmidt et al., 2012). In the present study, the MDR Salmonella isolates were most frequently resistant to penicillin, sulphonamides, and tetracycline. The report of the sales of antimicrobials in veterinary medicine in the UK indicated that these three antimicrobials were also the most frequently used antimicrobials in food animals (cattle, pigs, sheep, and poultry) in 2005-2010 (DEFRA, 2011a). This may explain the MDR profiles found in the present study. Although lack of the herd information meant it was not possible to trace the use of antibiotics on these pigs in the farm, this MDR-Salmonella carriage by healthy pigs is of major concern due to the potential of horizontal transfer of resistance genes (Bonardi et al., 2003).

The major genogroup of E .coli isolates post-bleeding were different from the pre-scalding genogroup. The same trend was found with Salmonella between

the post-bleeding and the pre-scalding genogroups. This change suggests that there were the same factors causing the changes of the major population genotypes of E. coli and Salmonella during the dressing process.

# CHAPTER 5 EFFECTS OF SINGEING AND POLISHING PROCESSES ON CARCASSES DURING PORK SLAUGHTER

## 5.1 INTRODUCTION

Many studies, as well as those described in the previous chapters, have demonstrated the substantial reduction of bacterial contamination caused by singeing, and the following re-contamination/ cross-contamination of the carcasses during the polishing process (Berends et al., 1997; Bryant et al., 2003; Pearce et al., 2004). However, the question arises of where the organisms which contaminate the carcasses during polishing originate. During this study, work in the FSA MO1040 study showed from the singe thermal imaging that potential "hot" and "cold" spots, and that singing was not uniform (Tinker et al., 2007). Thus, in the present study the effects of singeing and polishing on different carcass sites were studied to determine differences in survival or viability of bacteria through these processing stages.

The objectives were firstly to observe the effects of the singeing process on different sites of pig carcasses, and to identify the transfer of bacterial contaminants across this operation. The sites on the carcasses to be sampled were chosen based on the temperature variation between “hot spots” (well-singed areas; such as the belly area) and “cold spots” (areas of a carcass with lower temperature changes; such as trotters and anus area) as identified by thermal imaging (Figure 5.1). The sampling was conducted in Slaughterhouse B in England, chosen because it was possible to sample between the singeing and polishing stages. E. coli was used as the representative bacterium for

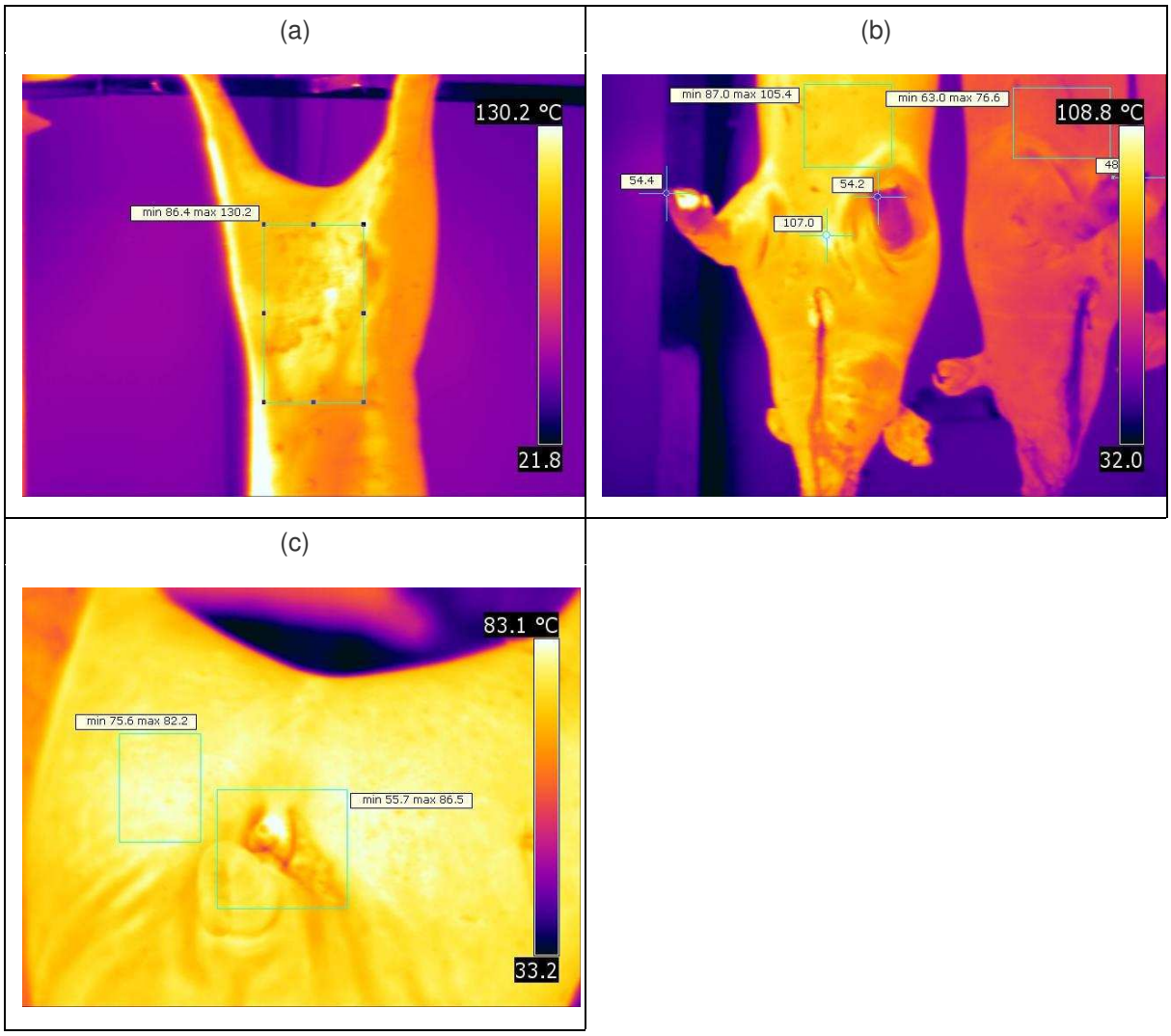



Figure 5.1. Thermal images of the carcasses post-singe (from work carried out as part of FSA project MO1040; kindly provided by Tinker, D., 2007), taken with thermal imaging camera: model Flir ThermaCAM PM695 PAL.

(a) belly area: with a typical singeing temperature range 86 – 103 °C ("hot spot")
(b) trotter area: temperature down to 54°C ("cold spot")
(c) anus area: temperature down to 55°C ("cold spot")

studying the contamination events during the process as it had been demonstrated to be an effective monitor by the previous studies. Both enumeration and genotyping of E. coli were undertaken as this had been shown to be effective in understanding cross contamination in the previous study, Chapter 4.

Two visits were made to this slaughterhouse. Typical isolates recovered in Visit I were confirmed as E. coli by indole testing. However, subsequent characterisation of some of these isolates showed that indole testing alone may not exclude other indole-positive Gram negative bacteria. Therefore in Visit II both the indole test and the oxidase test were used to confirm the isolates as E. coli. Details of the strain characterisation which led to this alteration in procedure are discussed in Chapter 6.

In the second part of this study, the time of carcasses entering the slaughterline was investigated during Visit II. Time of processing would be important if the equipment was contaminated before processing started or if contamination built up on equipment during the period of activity. To understand the impact of time, the carcasses at the beginning of processing and at the mid-point were swabbed post-bleeding and pre-chilling. For more detailed tracing of the sources of contaminants or the routes of transmission, equipment samples (from the dehairer and the polisher) and faecal content samples (taken from gut samples after evisceration) were also collected in the later visit.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Slaughterhouse

Slaughterhouse B (Figure 5.2) is a commercial slaughterhouse in England. The throughput of Slaughterhouse B was 320 carcasses per hour (approximate 3000 carcasses per day). The main features comprised a vertical steam scalding module, at 52-66˚C for 5 min; the singer type was sarcophagus-style with a single base flame, and a singeing residence time of approximate 8 sec. More detailed information of the processes is listed in Appendix 5.1. This slaughterhouse was first visited in October 2007 and the second visit was in October 2009.

### 5.2.2 Carcass sampling

#### 5.2.2.1 Online sampling points

As shown in Figure 5.2, the first process sampling point was pre-singeing, when the carcasses had been scalded and dehaired. The second sampling point was post-singeing, immediately after the carcasses had left the singer and before they were rinsed by cold water. The third sampling point was post-polishing, when the carcasses had passed through the polishing operation. In Visit II, swab samples were collected with the same methods at the same sampling points as in the former visit. Ten carcasses were swabbed at each designated sampling point on Visit I and 15 carcasses were sampled on Visit II.

Before the slaughter activity started and after the mid-day break, swab samples were also taken from the internal surfaces of the slaughter equipment including the dehairing machine and the polisher.

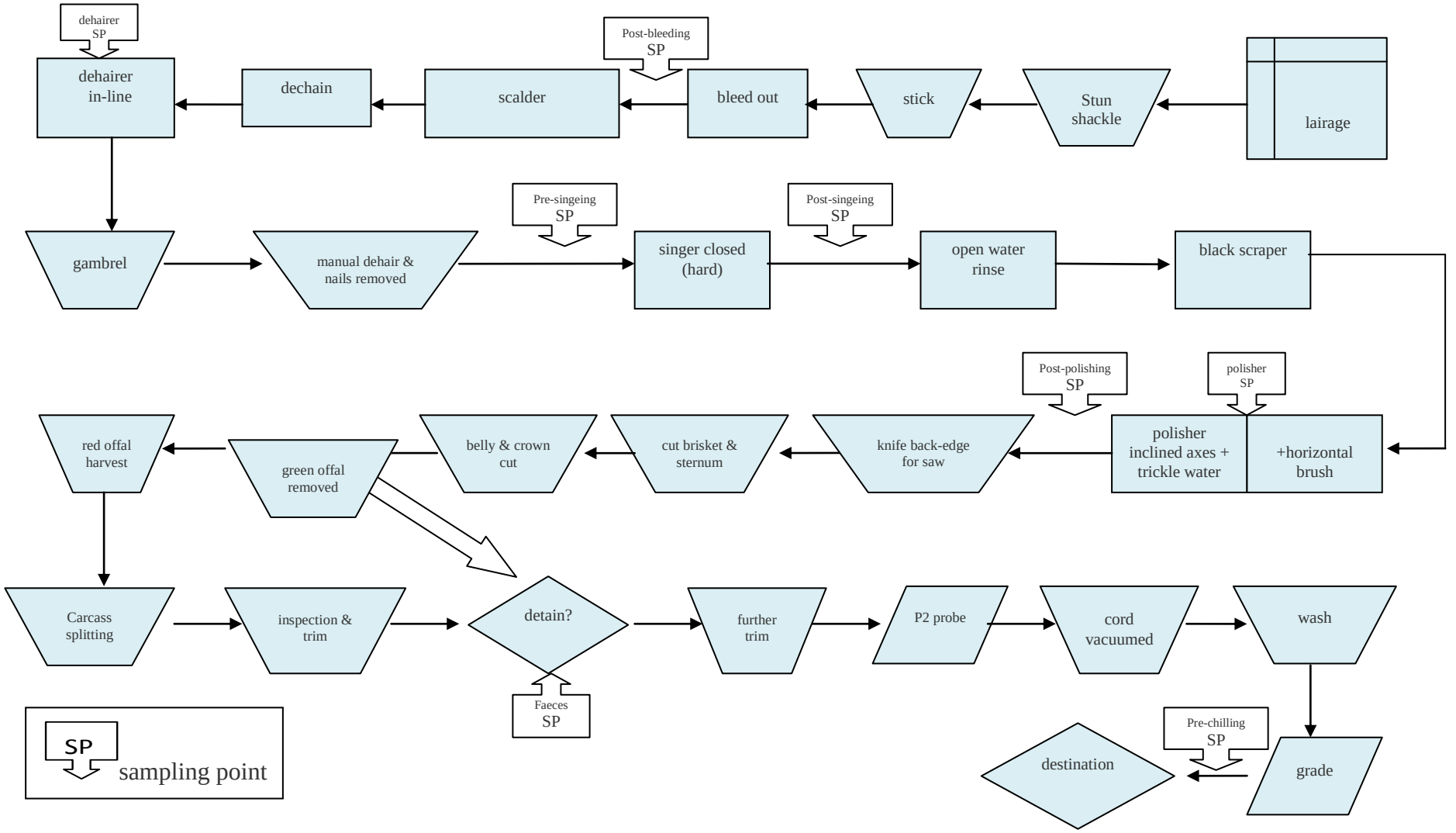



Figure 5.2. Slaughterline for Slaughterhouse B (provided by Tinker D, 2008).
(SP: the designated sampling point; rectangles: automatic operations; trapezium: manual operations; parallelograms: inspections; rhombus: detaining area)

#### 5.2.2.2 Part swabbing

Designated areas (trotter, belly, and anus; Figure 5.3) on the carcass were sampled using a pre-moistened Polywipe sponge (50 $cm^2$; MWE, UK) which was agitated within a distance of twice the sponge's width on a designated site of carcass. The sampled area was approximate 100 $cm^2$.

#### 5.2.2.3 Full length swabbing

The FSA recommended method for sampling the full length of carcasses was used as described in Section 2.1. Two sampling points, post-bleeding and pre-chilling,were been sampled on Visit II.

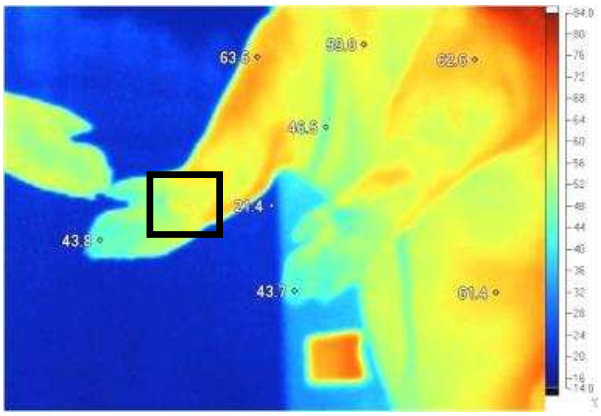
#### 5.2.2.4 Rectal faeces sampling

In Visit II, the whole sets of the green offal of four selected carcasses (including the first two carcasses processed at the beginning of processing activity and two random carcasses after the mid-day break) were removed from the slaughterline to a detainment area. The rectal faeces were collected by squeezing out the faeces from the rectum into a 50 ml Falcon™ polypropylene conical tube (BD Biosciences, US).

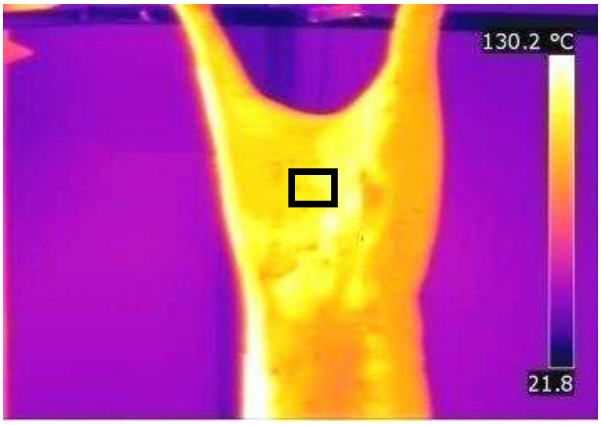
### 5.2.3 Equipment surface sampling

Equipment surface samples were taken from the dehairer and the polisher by sponge swabbing as described in Section 2.2.

(a)




(b)




(c)

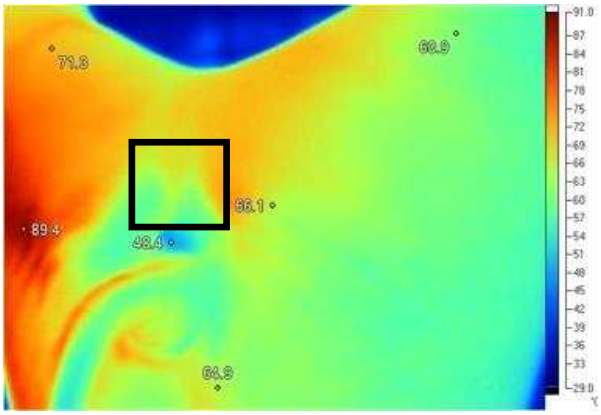



Figure 5.3. Sampled areas on pork carcasses: (a) trotter; (b) belly; and (c) anus. The area swabbed (marked in the boxes) was approximate 100 $cm^2$.

### 5.2.4 Bacterial sample preparation

For sponge swab samples, the preparation was as described in Section 2.3. For faecal samples, 90 ml of buffered peptone water (Oxoid, UK) was added to 10 g of faecal material and mixed using a Vortex-Genie-2T vortexer (Scientific Industries Inc, US) for 1 min. The suspension was then serially diluted with MRD to the appropriate dilution.

### 5.2.5 Enumeration of microorganisms

The enumeration of E. coli was conducted as described in Section 2.4.2. The LOD of different samples used in this study were given as follows:

- whole carcass swab: LOD = - 2.00 $\log_{10}$ cfu $cm^{-2}$
- specific area swab: LOD = - 0.82 $\log_{10}$ cfu $cm^{-2}$
- faecal and caecal contents sample: LOD = 0.70 $\log_{10}$ cfu $g^{-1}$
- the LOD of equipment swabs was not able to be defined due to the undefined size of each area swabbed.

### 5.2.6 Confirmation of E. coli

The indole test was performed as described in Section 2.5.1. The oxidase test was carried out using Oxidase identification strips (Oxoid, UK) as described in Section 2.5.2.

### 5.2.7 Genotyping of E. coli isolates

ERIC-PCR genotyping and data processing was conducted using the method

described in Section 2.6.

## 5.3 RESULTS

### 5.3.1 Effects of singeing

#### 5.3.1.1 Total viable counts and E. coli counts

In the first trial (Figure 5.4a), the mean ECC of the anus swabs were highest at the pre-singeing stage (2.21±0.78 $\log_{10}$ cfu $cm^{-2}$) but significantly ($P<0.01$) reduced to just above the limit of detection post-singeing. On the anus area, there was a significant increase of E. coli ($P<0.01$) after polishing. The increase in numbers from the anus samples could be due to redistribution only on these polisher blades, or local faecal leakage. On the trotter and belly areas, no E. coli were detected post-singeing and post-polishing indicating that the singeing process effectively removed the organism from these two carcass sites and there was no deposition from the polisher during this trial.

In Visit II (Figure 5.4b), the initial counts at the three designated sites at pre-singeing were approximate 1 $\log_{10}$ cfu $cm^{-2}$ higher than the ECCs at the same sites in the former trial. However, in both trials the range of the mean ECCs at pre-singeing amongst these sites was similar. After singeing, E. coli was recovered at the anus site and the trotter but not on the belly. The mean ECCs on the anus post-singeing were higher ($P=0.01$) than those of the trotter although a similar level of reduction in counts (2.5 $\log_{10}$ cfu $cm^{-2}$ for the anus samples and 2.4 $\log_{10}$ cfu $cm^{-2}$ for the trotter samples) was seen for both sites

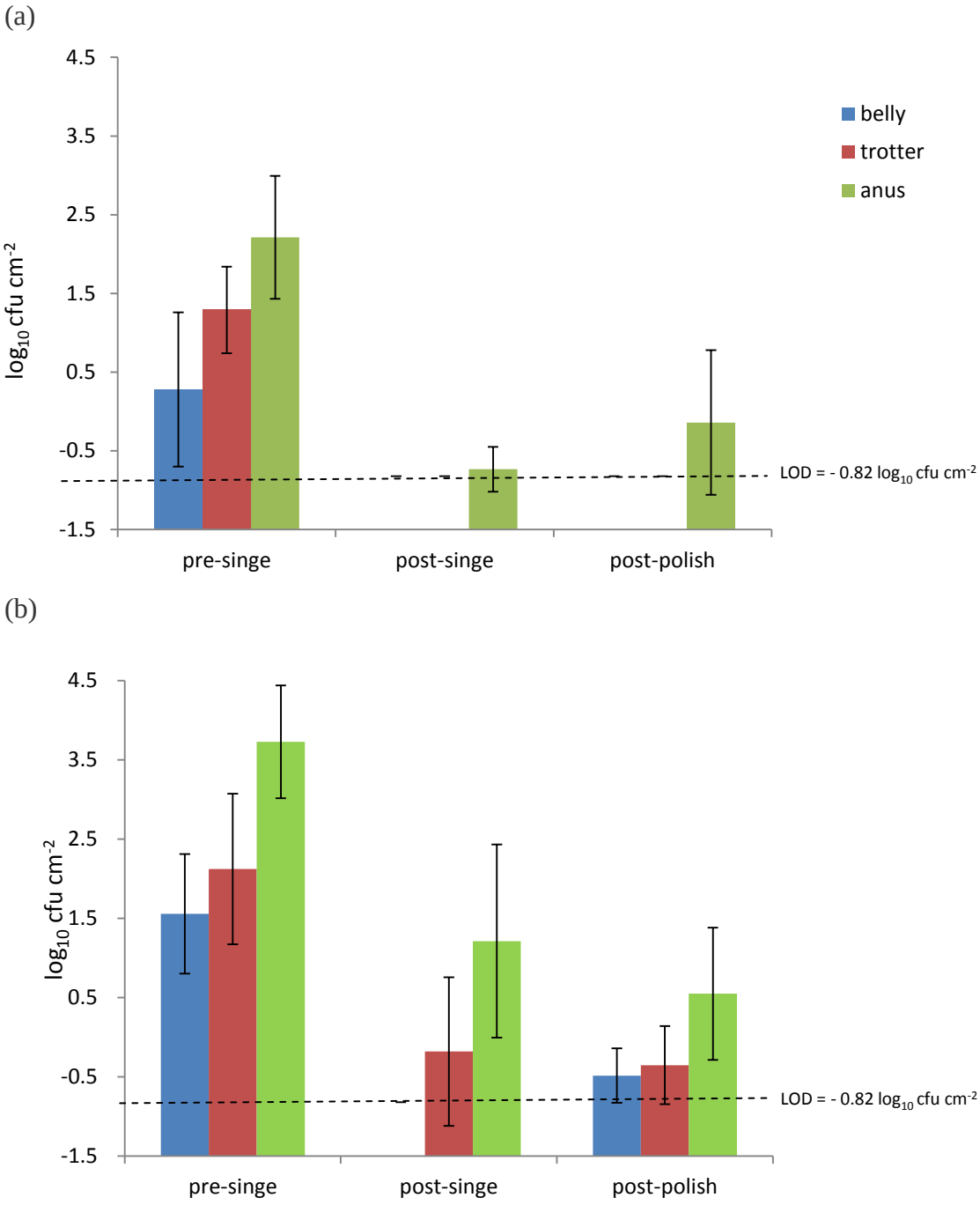



Figure. 5.4. The mean E. coli counts on the three carcass sites (belly, trotter, and anus) through the singeing and polishing processes. (a) Visit I (n=10); (b) Visit II (n=15).

($p<0.01$). E. coli was recovered post-polishing at all three sites. The counts on trotter and anus post polishing were not statistically higher ($P>0.05$) than on these sites post singeing, where the counts on the belly were increased to -0.48 $\log_{10}$ cfu $cm^{-2}$ post polishing.

In regards to the number of carcasses positive for E. coli after singeing, in the former trial (n=10), in the anus area 10% of the carcasses were E. coli positive, which increased to 40% post-polishing. In the later trial (n=15), 87% of anus samples were E. coli positive post-singeing. This increased to 93% post-polishing. Sixty-seven percent of trotter samples were positive for E. coli post-singeing and post-polishing (n=15). For the belly part, E. coli were not detected post-singeing but were recovered on 73% of post-polishing samples. The high number of E. coli positive carcasses detected after singeing at the anus and trotter sites suggested that the singeing operation is less effective at these cold spots.

#### 5.3.1.2 ERIC-PCR genotyping of the isolates

Although the numbers of E. coli recovered in Visit I were limited, ERIC-PCR was performed on isolates from six stages (trotter pre-singe, belly pre-singe, anus pre-singe, anus post-singe and anus post-polish) and the dendrogram derived by cluster analysis of these isolates is shown in Figure 5.5. Thirty of the 33 isolates were clustered into 4 groups at 55% similarity ($\Phi_{PT}=0.562$; $P<0.01$). Group 1 is composed of 4 identical isolates from the anus post-polish from a single pig carcass (Pig 8) and this group showed only 5.87% similarity with the other groups. Group 2 is composed of isolates from the trotter

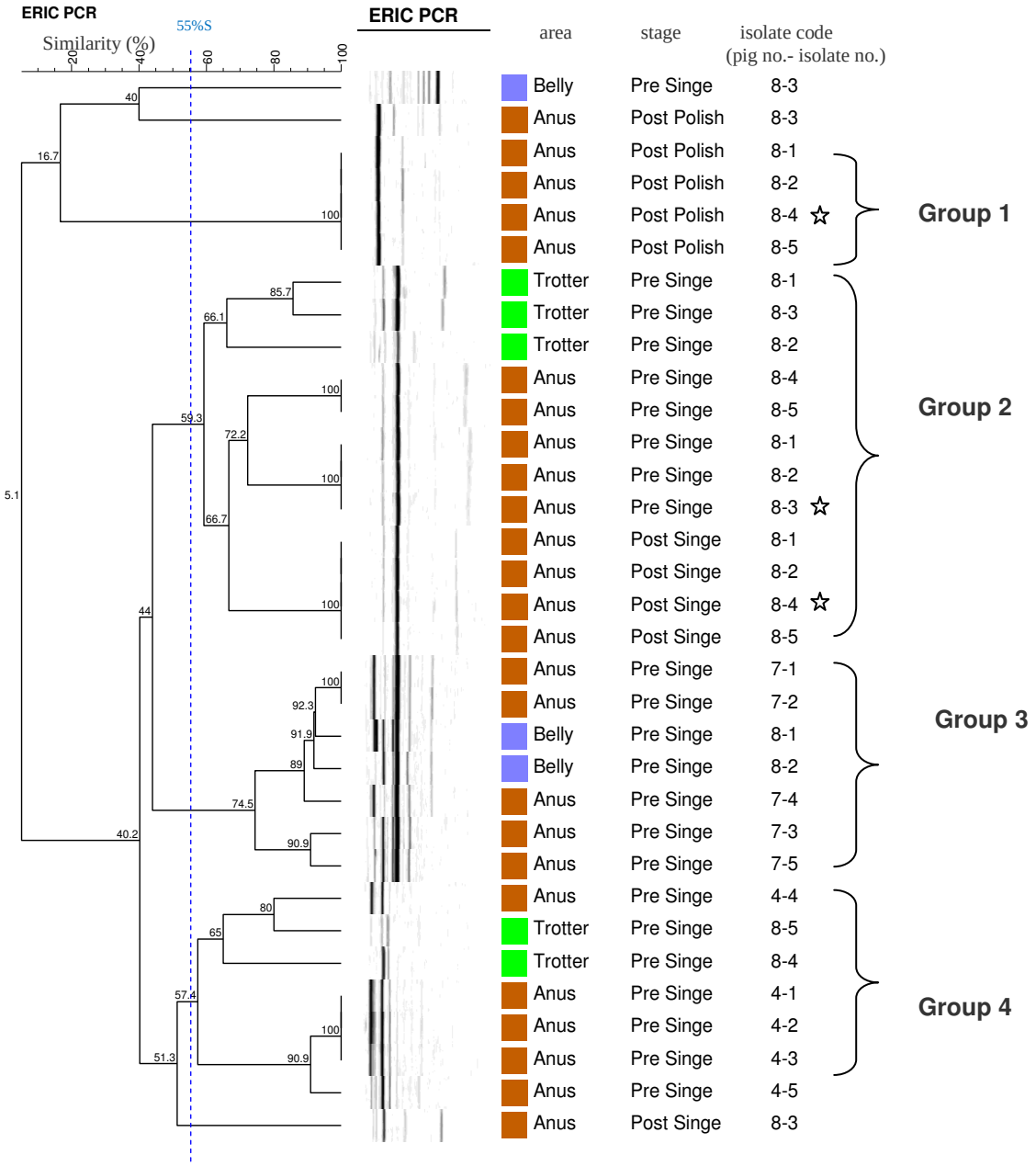



Figure 5.5. Dendrogram obtained from cluster analysis (Dice coefficient; UPGMA) of ERIC-PCR types of E. coli isolates (n=33) from pig carcass samples in Visit I. Groups were defined at 55% similarity level.

(blue: belly isolates; green: trotter isolates; brown: anus isolates; asterisks: isolates selected for further characterisation)

pre-singe, the anus pre-singe and the anus post-singe again all from Pig 8. This group shows a high degree of similarity of flora before and after singeing on this carcass demonstrating survival of strains through the singe process. However, it should be noted that identical strains were not shown pre- and post-singeing or from different sites on the carcass. The genotypes grouped in Groups 3 and 4 were all from the pre-singe samples which were sampled from the trotter, belly, and anus area on different pig carcasses. It suggested that these genotypes were well-distributed on different areas of the carcasses. However, mostly they did not appear to survive the singe process. Only one isolate related to Group 4 (isolate 8-3) was found after singeing and this isolate was from the anus area (one of the "cold-spots").

A total of 437 E. coli isolates collected during Visit II were genotyped. The full illustrated dendrogram is shown in Appendix 5.1. Similarity values amongst the isolates from different sampling sites varied from 21.6% to 100%. Thirty-one groups and six isolates were distinguished at a 55% similarity threshold ($\Phi_{PT}$=0.515; P<0.01).

The genotypes found at each sampling site are listed in Table 5.1. Sixteen genotypes were recovered from the belly pre-singeing and the trotter pre-singeing. Moreover, the compositions of the genotypes are similar between these two sampling points, where 12 genotypes are in common. Seventeen genotypes were recovered from the anus post-singeing, which was the greatest number of genotypes amongst the carcass processing stages and increased from the pre-singeing stage. Only 6 genotypes matched those found pre-singeing.

Table 5.1. A summary table of the cluster analysis for ERIC-PCR profiles of 437 E. coli isolates sampled during Visit II. ERIC-types were determined at 55% similarity threshold ($\Phi_{PT}$=0.515; P<0.01).

| ERIC-types | pre-singe | post-singe | post polish |
|---|---|---|---|
| Belly | n=69 ; 16 ERIC-types:<br>1, 2, 3, 4, 6, 7, 11, 12, 13, 14, 15, 16, 18, 19, 20, 28 | Not detected. | n=25 ; 10 ERIC-types:<br>1, 3, 4, 6, 7, 8, 10, 17, 19, 27 |
| trotter | n=75 ; 16 ERIC-types:<br>1, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 15, 19, 20, 28, 30 | n=32 ; 8 ERIC-types:<br>3, 4, 5, 6, 11, 12, 13, 28 | n=36 ; 8 ERIC-types:<br>3, 4, 8, 9, 16, 20, 21, 29 |
| anus | n=72 ; 10 ERIC-types:<br>1, 3, 4, 7, 8, 11, 13, 25, 26, 27 | n=60 ; 17 ERIC-types:<br>1, 3, 4, 6, 7, 15, 17, 18, 19, 22, 23, 24, 25, 26, 29, 30, 31 | n=67 ; 10 ERIC-types:<br>1, 3, 4, 6, 12, 13, 14, 19, 26, 28 |

*Unique types (only present in one sampling site) were highlighted.

Four types were unique (only found at one sampling point) indicating that many of the new strains present on the anus area appeared after the singeing process. Seven out of eight genotypes found on the trotter post-singeing were the same as the genotypes found pre-singeing suggesting the possibility of strains surviving the singeing process. However, it is clear that not all genotypes survived this process. No E. coli were found on the belly post-singeing but 10 ERIC-types were found post-polishing. Nine out of these 10 genotypes were found on pre-singeing sites with 7 genotypes found on post-singe sites. Hence all may have come from original carcasses and survived singeing process to get onto carcass in the polisher.

### 5.3.2 Effects of time entering the slaughterline

#### 5.3.2.1 E. coli counts of early and later slaughtered carcasses

Carcasses (n=4) at the beginning of the production period (early slaughtered carcasses) and carcasses (n=4) before the break (later slaughtered carcasses) were swabbed at post-bleeding and pre-chilling stages (Figure 5.6). Both the early and later slaughtered carcasses showed similar initial counts and more than 3.8 $\log_{10}$ cfu $cm^{-2}$ decrease in the number of E. coli over the slaughter process. The effects of the time entering the slaughterline were not significant ($P>0.05$).

#### 5.3.2.2 E. coli counts of slaughterline equipment

To examine the contamination on machines on the processing line, the dehairer and the polisher were sponge swabbed (Table 5.2). It was not possible to

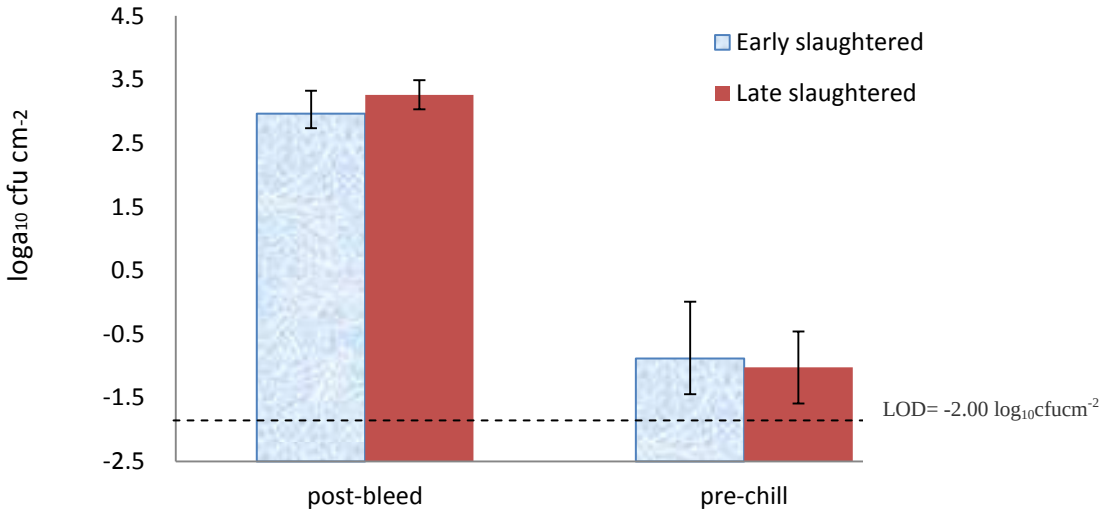



Figure 5.6. The mean ECC of early slaughtered carcasses (n = 4) and later slaughtered carcasses (n = 4) at the post-bleeding and the pre-chilling stages.

collect samples when the machines were in operation. Thus, the samples were taken before start of process and during a break of operation. The ECCs are presented as log cfu per sponge due to the difficulty of the estimation of the area swabbed. Prior to the start of slaughter activity, E. coli was detected at all three sampling points (entrance, middle part, and exit) for the dehairer, and in the first rank and middle rank of brushes for the polisher. The numbers of E. coli on these machines prior to start were high and were nearly at the same level as at the mid-day break. According to the European Commission Decision 2001/471/EC, it is unacceptable if more than 1 cfu $cm^{-2}$ of Enterobacteriaceae is found on contact surfaces, including scalding and polishing instruments (EU Commission, 2001). E. coli is only one species in the family of Enterobacteriaceae so numbers of Enterobacteriaceae should be higher than the E. coli counts. Although the sizes of swabbing area could not be measured and thus precise counts were determined, the numbers would not be acceptable as these machines had been cleaned overnight.

#### 5.3.2.3 Comparison of DNA fingerprints of E. coli isolated from rectal faeces with those from carcasses

After enumeration, five E. coli isolates were isolated from the swabs of carcasses post-bleeding and pre-chilling and from the rectal faeces of two early slaughtered and two later slaughtered pigs, and their genotypes were compared. This comparison aimed to determine how much of the flora of the final carcass (at pre-chilling) is from the original pig (at post-bleeding) or from faecal carriage which could be introduced onto the carcass at any stage (e.g. evisceration or faecal leakage at polishing). The genotypic relationships

Table 5.2. The E. coli contamination on (a) the dehairer and (b) the polisher of the slaughterline before and in the break of activity.

(a)

| | dehairer | | |
|---|---|---|---|
| | entrance | middle | exit |
| **pre-start** | 2.45 | 4.17 | 4.35 |
| **SD** | 0.03 | 0.06 | 0.04 |
| **break** | 3.67 | 4.71 | 4.19 |
| **SD** | 0.03 | 0.01 | 0.03 |

(unit: $\log_{10}$ cfu sponge$^{-1}$)

(b)

| | polisher | | | |
|---|---|---|---|---|
| | entrance scrapers | first brush | mid brush | last brush |
| **pre-start** | ND[1] | 2.02 | 1.48 | --[2] |
| **SD** | | 0.20 | 0.01 | |
| **break** | 1.81 | 1.88 | 2.10 | 2.64 |
| **SD** | 0.31 | 0.17 | 0.21 | 0.25 |

(unit: $\log_{10}$ cfu sponge$^{-1}$)

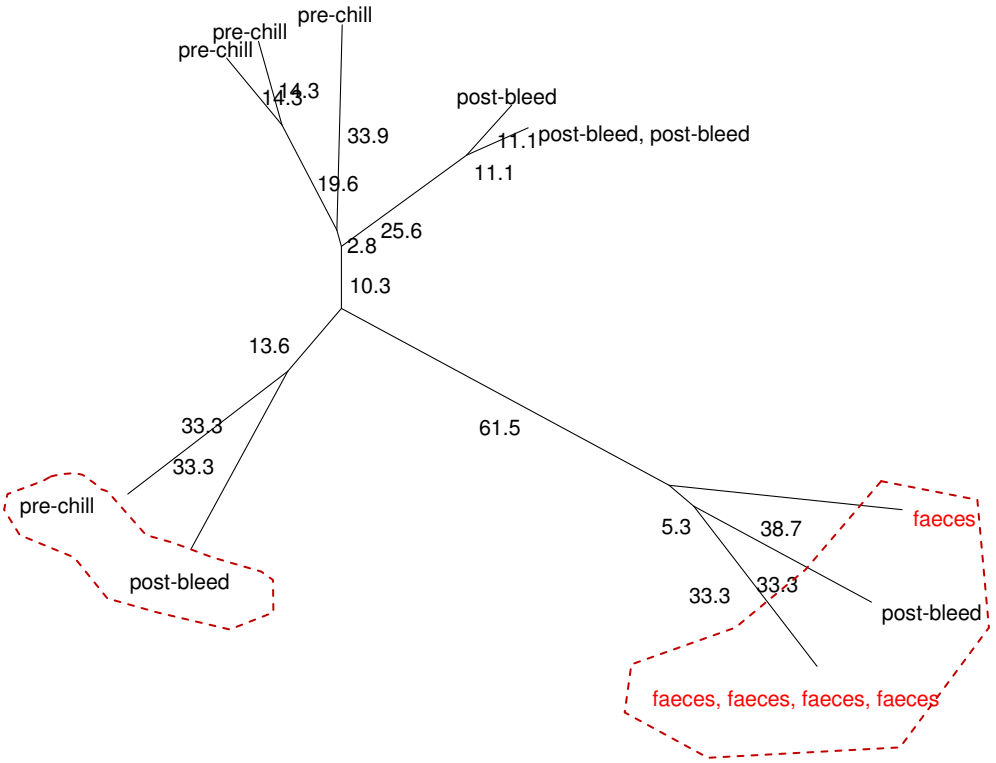
1. ND: not detected.
2. Sample not collected.

amongst the isolates of the individual pigs (Figure 5.7) are shown by rendered tree plots. The rendered tree is useful to reduce complexity and to compare the genotypic distance between isolates. A branch composed of only one kind of sample (e.g. post-bleed: 5.7b, c; or pre-chill: 5.7d) suggested that it was a unique genotype at that stage and examples were found on all four plots. In the cases of early slaughtered pig 1 and late slaughtered pig 2 (Figure 5.7a and 5.7d), the link of post-bleed and pre-chill isolates indicated that these genotypes were carried though the whole slaughter process. In the plots of early slaughtered pig 2, later slaughtered pig 1 and pig 2 (Figure 5.7b, c and d), the faecal isolates were distributed on the branches and were grouped with most of the carcass isolates suggesting that these branches are the overlapped genotypes amongst faecal, post-bleed, and pre-chill isolates. The frequent presence in these branches of isolates associated with the final pig population also indicated that faecal contents might be a major source of the final surface contamination. It was also noticed that for late slaughtered pig 2 (Figure 5.7d), a branch containing a faecal isolate and a pre-chill isolate but no post-bleed isolate might indicate these strains were transmitted onto the final carcass during polishing or the evisceration process.

#### 5.3.2.4 Diversity of the dehairer and polisher E. coli isolates

The isolates derived from the dehairer (n=16) and the polisher (n=18) were subjected to ERIC-PCR typing and were compared with the carcass isolates. There were 14 genotypes from the dehairer and the polisher isolates. Extracted sections of the dendrogram (Figure 5.8) show several examples of identical genotypes (100% similarity of the ERIC-PCR type) found between the

**a. Early slaughtered pig 1**




**b. Early slaughter pig 2**

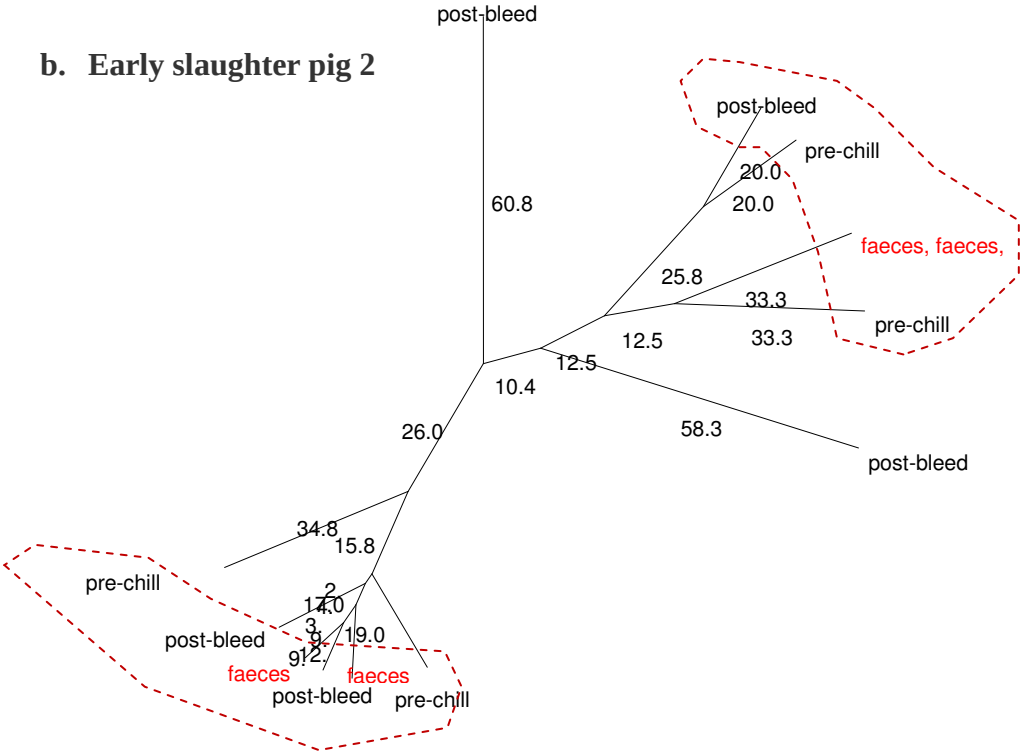
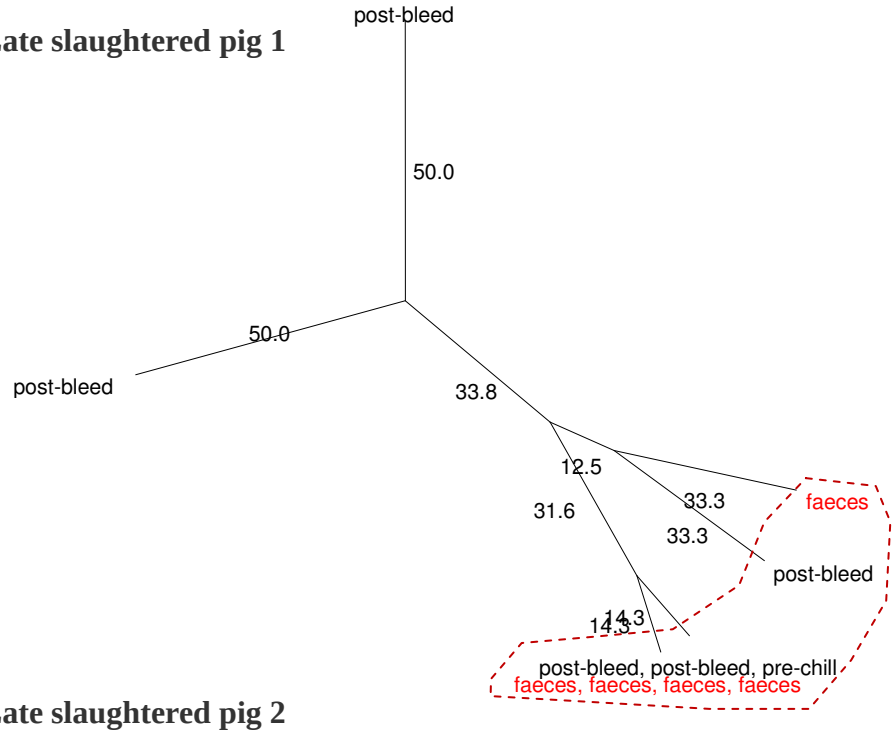



Figure 5.7. Rendered tree plot obtained from cluster analysis of ERIC-PCR typing of the E. coli isolates from individual pigs: (a) and (b) are early slaughtered pigs. Dotted circles were clusters composed of isolates from different origins. Numbers on the branches represent genetic distance (%). Clusters were defined with a genetic distance below 55%.

**c. Late slaughtered pig 1**




**d. Late slaughtered pig 2**

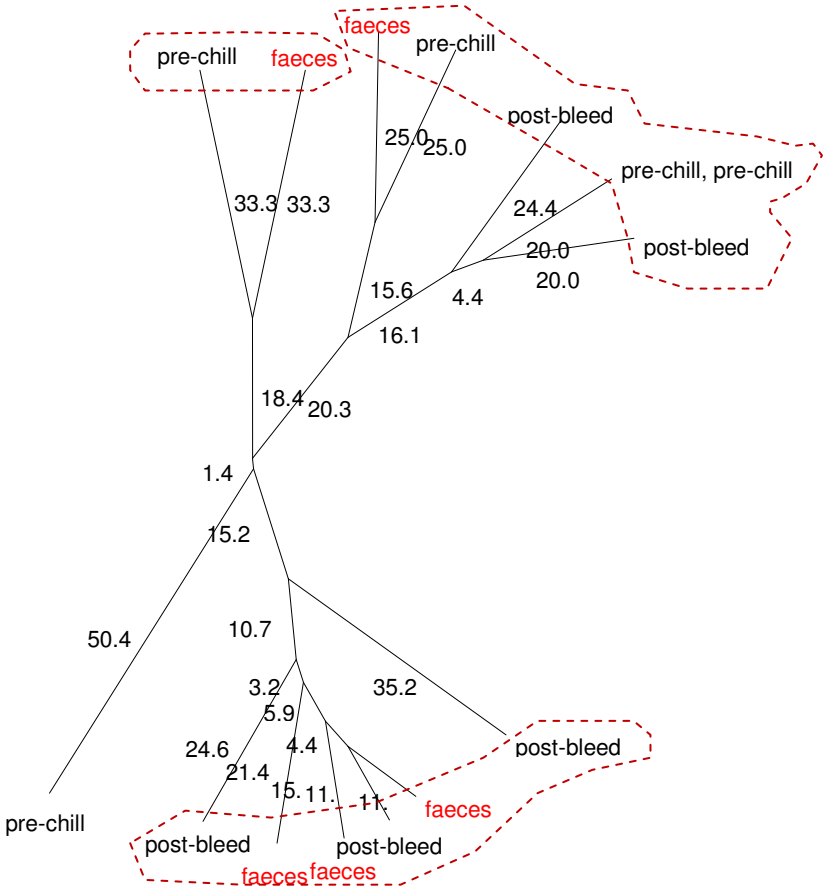



Figure 5.7. Continued. Rendered tree plot obtained from cluster analysis of ERIC-PCR typing of the E. coli isolates from individual pigs. (c) and (d) are later slaughtered pigs.

equipment (the dehairer or the polisher) and from the carcasses at pre-singeing, post-singeing, and post-polishing stages. Those isolates demonstrated that the equipment was involved in the transmission of the bacteria during processing. For example, an identical genotype found from the dehairer at break was also found on pigs sampled before and after the break (panel F, Figure 5.8). An identical genotype found from the dehairer pre-start (panel D) and on the belly of pigs 4, 6, 14 pre-singeing may indicate the equipment strain contaminated the carcasses. On the other hand, an identical genotype found from the polisher in the break (panel B) and on the trotter of pig 7 pre-singeing and the anus of pigs 1, 2, 3, 4, 5, 7 post-polishing may indicate the carcasses contaminated the equipment because these pigs passed through the polishing process before the break.

## 5.4 DISCUSSION

The primary design of a singeing system is for meat quality, not for hygiene reasons. Thus, the singeing process is intended to leave an attractive clean appearance and to develop a rind. It may not be enough to act as an effective microbial control on every part of the carcass. When singeing is carried out incorrectly and/ or when very large amounts of bacteria are located in the deeper layers of the skin, a reduction of only 5 -30% may be achieved (Berends, et al., 1997).

In many studies singeing was seen as a uniform treatment on carcasses and the temperature variation across the whole carcass had not been considered. It has been reported that during singeing temperatures differed at different sites on a

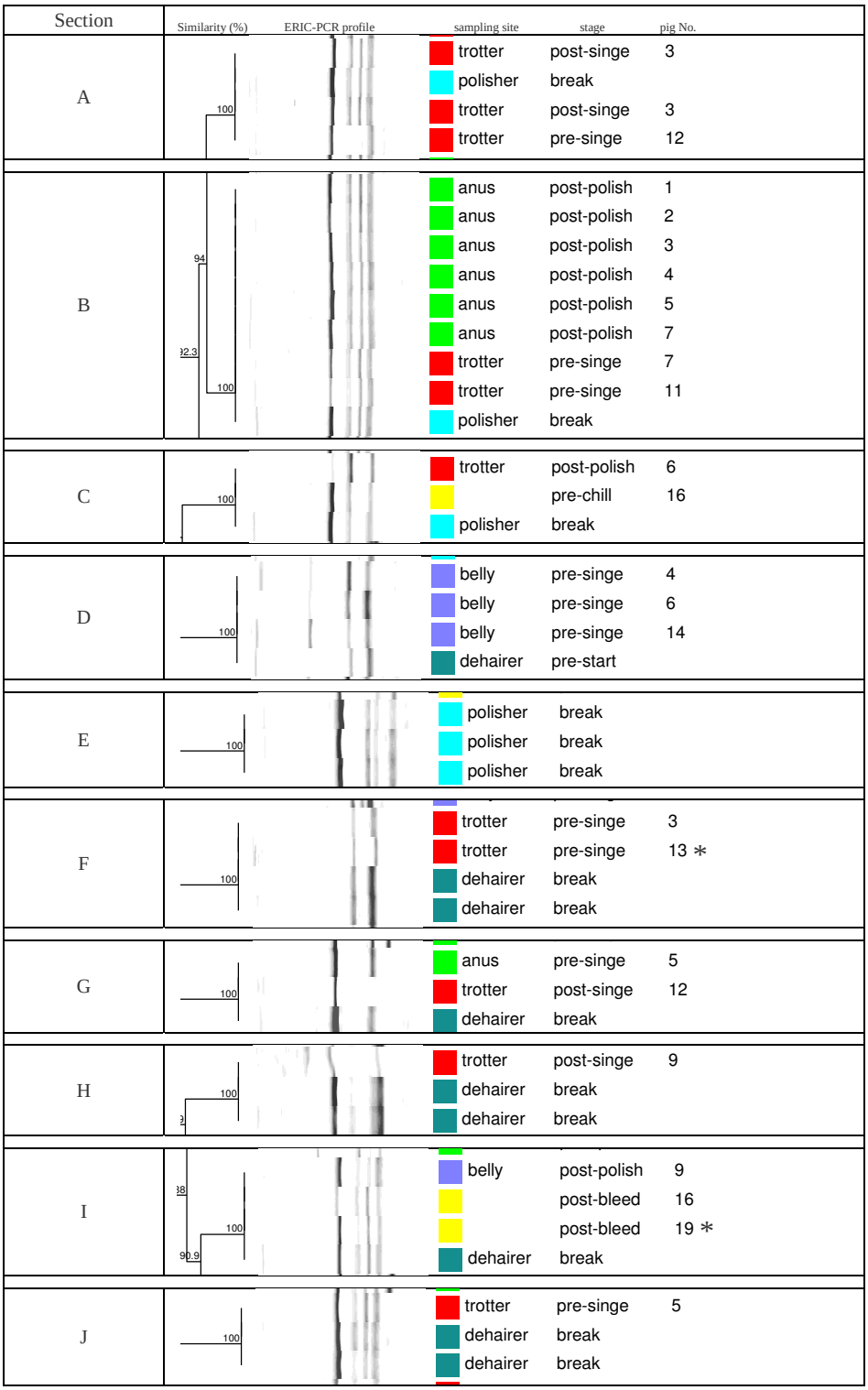



Figure 5.8. Demonstration of identical isolates from the equipment of the slaughterline and the carcasses. Isolates marked with an asterisk were sampled from the carcasses processed after mid-day break.

carcass, as much evident by thermal images taken post-singeing (Richards et al., 2009). In the present study the efficacy of the singeing process was conducted by examined the presence of E. coli at different sites on the carcass. The hypothesis was that the "cold spots" would allow more bacteria to survive the singeing process than the "hot spots" and the bacteria could be redistributed subsequently via polishing. In the present study, the ECCs of the anus were higher than those of the trotters or the belly at the pre-singeing stage (where the carcasses had already been scalded and dehaired). This is different from the observation of Schaeferseidler et al. (1984), in which the bacterial contamination levels were uniform over the length of the scalded carcasses. However, count variation between sites on the carcass have been reported (Palumbo et al., 1999; Pearce and Bolton, 2005; Zweifel et al., 2008). The difference of initial counts between carcass sites may affect the level of reduction during the singeing process.

Singeing can decrease the aerobic and the E. coli counts 2.0 - 2.5 log cfu $cm^{-2}$ (Gill and Bryant, 1993; Delhalle et al., 2008) and remove almost all Enterobacteriaceae (Morgan et al., 1987). The current study has shown that bacterial counts at each site were reduced significantly ($p<0.01$) by the singeing process, with more than a 99.5% reduction of ECCs. However, the "hot spot" of the belly area was the only site where no E. coli was recovered post-singeing in both trials. This clearly indicates that the level of heat applied to the belly area results in the surface being properly decontaminated. On the other hand, in both trials, E. coli was recovered at post-singeing on the anus area. This might be associated with the orientation of the flames which could

not reach this part directly. This frequent recovery of E. coli could be a result of the combination of higher initial numbers, lower temperature exposure, and new strains from faecal leakage. Although the singeing process demonstrates its potential for de-contamination, bacteria might survive in deeper skin folds or in the hair follicles (Berends et al., 1997).

On the belly part, E. coli was not recovered after the polishing process in one visit but was found in the other. Despite being visibly clean, E. coli had reappeared on the belly of polished carcasses whereas the counts showed no significant increase ($P>0.05$) on trotter or anus. The reappearance of E. coli on belly indicates contaminations across the carcasses during polishing. Despite having been well-singed, bacterial recontamination of the belly area was expected due to recontamination associated with polishing (Yu and Palumbo, 2000; Bolton et al., 2002; Spescha et al., 2006). It has been demonstrated that the coliform counts after polishing on the belly were approx. 1.5 log cfu $cm^{-2}$ higher than after singeing (Pearce et al., 2004), and the percentage of Enterobacteriaceae positive carcasses increased from 12% to 19% (Spescha et al., 2006).

In Visit II, E. coli was recovered on the trotter and the anus areas post-singeing, and the mean ECCs of these areas remained the same post-polishing. This provides evidence that the ‘cold spots’ may allow survival of the organism through singeing and thus suggests a source from which contamination may spread in the polishing process. This result supports the hypothesis that singeing can significantly reduce the bacterial contamination on the carcass

surface, but it is not sufficient in eliminating the contamination (Borch et al., 1996).

The DNA fingerprinting gave further details about the flora changes during the singeing and polishing processes. In Visit I, E. coli genotypes from the trotter pre-singe were divided between Group 2 and Group 4, where genotypes from the anus pre-singe were also present. It is possible that the faecal E. coli have contaminated the pig trotters while they were walking around in faeces produced by the pigs as suggested by Rostagno et al. (2005). Only a few E. coli were obtained after the singeing and polishing process and all of them were from the anus area of the same carcass. The clustering of the anus isolates obtained pre-singeing and post-singeing indicated that E. coli strains in this group were surviving the singeing process. However, isolates from the anus post-polishing had a low similarity with other groups indicating that these isolates have probably come from a different population, and had not originated from the anus area of this pig. This new population could originate from cross contamination with other carcasses or the equipment (Namvar and Warriner, 2006).

The greater number of isolates recovered in Visit II provided a better understanding of bacterial transmission. Although the bacterial populations originally on pig carcasses were significantly reduced by singeing, it is apparent that some bacteria did survive the process as an identical ERIC-PCR type was isolated both before and after singeing from the trotter (found on pig No.10). Furthermore, several genotypes found post-polishing possessed high

similarity to the genotypes of pre-singeing isolates (from 86.6% to 100% similarity), which indicated those genotypes may survive the singeing process and reappear in the subsequent process. Those genotypes were found on the trotter pre-singeing and post-polishing on pigs No.9, and 13, and also on the anus pre-singeing and post-polishing on pigs No.1, 13, and 14. Although the survival of bacteria under the singeing process has been mentioned in literature (Borch et al., 1996; Berends et al., 1997; Alban and Stark, 2005), this kind of genetic evidence on the provenance of isolates has rarely been reported.

In this study, the influence of time into the slaughter day was also investigated. No significant difference in E. coli contamination was found between the first pigs slaughtered and those slaughtered 2 hours later, either at the beginning of the slaughter process (sampled at post-bleeding) or at the end of the process (sampled at pre-chilling). In a study conducted by Namvar and Warriner (2006) with similar scope, higher numbers of E. coli were found from the carcasses post-bleeding in the initial hours of processing than the later carcasses, possibly due to the overnight stay in the holding area of those early entering pigs. Studies also indicated that 2 hours or overnight lairage increased the Salmonella carriage by slaughter pigs (Swanenburg et al., 2001; Milnes et al., 2009). However, contradictory findings from another study indicated that up to 18 hours lairage in clean holding pens did not increase Salmonella shedding (Hurd et al., 2001). The lack of significant differences in numbers of E. coli between early and later pigs entering the plant may suggest that contamination prior to the slaughter activity was not a major problem.

The relatively high numbers of E. coli recovered from the in-line dehairer and from the polisher prior to the slaughter activity suggest that the equipment was not sanitised effectively. Hutchison et al. (2007) studied the bacterial contamination of contact surfaces in UK slaughterhouses and found a mean total aerobic counts of 4.4 log cfu $cm^{-2}$ on the polisher before the slaughter process, and concluded that the polisher is one of the most difficult surfaces to clean and sanitise. The dehairer, as well as the central part of the polisher, is a semi-closed system which it may be difficult to clean thoroughly with daily washes. This factor enhances the possibility that the increase of bacterial contamination after these processing stages is due to cross-contamination from the equipment (Gill et al., 1995; Yu et al., 1999) However, the ECCs of these areas at the mid-day break had only changed slightly (an increase of less than 0.6 $log_{10}$ cfu $sponge^{-1}$). This indicates that the E. coli are not accumulating to any great extent during the processing activity although they can attach and persist on these machines. Regarding the genotyping of the equipment isolates, an identical genotype was found in the isolates on six out of the first seven carcasses and on the polisher. This indicates that the E. coli could be transferred between the carcasses when they enter the polishing process within the same day. The presence of identical genotypes from the dehairer and carcasses pre-singeing, and from the polisher and carcasses post-polishing clearly demonstrates that cross-contamination is happening in the dehairer or polisher machinery, sometimes due to poor cleaning and sometimes due to cross-contamination events during processing.

Vieira-Pinto et al. (2006) have reported that with the detection of the same

genotypes, about 80% of Salmonella positive pig carcasses were associated with faecal-related contamination and 69% was self-contamination by their own faeces. This self-contamination is in agreement with the findings of the present study. On three out of the four pigs sampled, the faecal genotypes were closely related to many of the carcass genotypes whether at the beginning or the end of slaughter operations. It indicates the possibility of the E. coli shedding during the slaughter activity and contaminating the external surface. However, in the case of early slaughtered pig 1, the faecal isolates were closely related to only one post-bleeding isolate and most of the isolates were grouped in another branch. Furthermore, isolates with low similarities to the faecal isolates were also found on each examined pig. Although the contamination from faecal flora plays an important role in microbiological dissemination, it should be considered that cross-contamination from other pigs' faeces (not just this pig's own flora) could happen during the slaughter operations.

As a direct result of this present study it can be seen that the singeing process did not uniformly decontaminate the carcasses. Although singeing is still the most effective means of decontaminating slaughtered carcasses, the surface temperature variations can lead to survival of bacteria at particular sites. The survivors are then redistributed back onto the decontaminated sites of the carcass itself, or spread to other carcasses during polishing. In addition to the cross-contamination during the polishing operation observed with the prevalence of E. coli, genotyping demonstrated that identical types were found on the singed carcasses and the polisher suggesting survival and then spread of contaminants via the machinery. The genotyping results also reveal the

presence of E. coli on concurrently and subsequently polished carcasses. The demonstration that the equipment is not adequately cleaned and thus spreads contaminants to the subsequent day's carcasses is also a case for concern.

# CHAPTER 6 ISOLATION AND CHARACTERISATION OF E. COLI FROM PIG CARCASSES, CAECAL CONTENTS AND EQUIPMENT

## 6.1 INTRODUCTION

In the present study, the relationship of E. coli isolates from the slaughtered carcasses, the gut contents, and the polishing machine was investigated to provide a better understanding of the routes of transmission. As discussed in earlier chapters, the polishing operation has been considered as a control point (CP) as part of a HACCP plan of the swine slaughterline (Borch et al., 1996) and has also been identified as the key step for defining the final microflora on carcasses (Gill and Bryant, 1992; Berends et al., 1997; Hald et al., 2003). However, it is unclear what the key sources of the bacteria reintroduced after singeing are. Faecal leakage, carcass to carcass cross-contamination, or existing equipment contamination have all been proposed as the key factor (Dockerty et al., 1970; Gill et al., 1995; Yu et al., 1999; Aslam et al., 2004). In the previous chapter, it was demonstrated that the equipment was associated with carcass contamination. Therefore, in this study the contribution made by the gut contents of the pig was examined in detail. This is because at least two of the processes prior to the final carcass could be involved in redistributing faecal contents, including the polishing and evisceration processes. In this study the populations of E. coli taken from a carcass pre-chilling were compared with the gut flora from the same carcass.

One of the criticisms of studying changes in bacterial populations at different stages of processing is that if the population as a whole is very diverse, then at each stage if only a limited number of colonies per carcass are examined, this may represent only a subset of the whole population. Hence, changes may be seen which are not overall population changes but the changes due to random sample selection. Thus, in this study the potential impact of the sampling size was investigated as it was anticipated that gut contents would show a very diverse population. On this occasion, as normally only low numbers of E. coli are recovered from the pre-chilled carcasses, all of the E. coli colonies on a plate at an appropriate dilution were collected to constitute the 'whole population' sample. Fifty colonies were isolated from the caecal contents of each pig as representing the gut flora.

## 6.2 MATERIALS AND METHODS

### 6.2.1 In-plant sampling

The in-plant sampling was conducted at Slaughterhouse C (Figure 6.1) in January 2010. It was a small scale abattoir in the East Midlands of England. The sampling of pre-chilling carcasses was carried out as described in Section 2.1, and the middle rank of brushes of the polisher was sampled as described in Section 2.2. The caecal contents (10 g) of each sampled carcass were collected by piercing a hole in the caecum using a No.10 guarded disposable sterile scalpel (Swann-Morton, UK) and collecting contents into a 50 ml Falcon™ polypropylene conical tube (BD Biosciences, US).

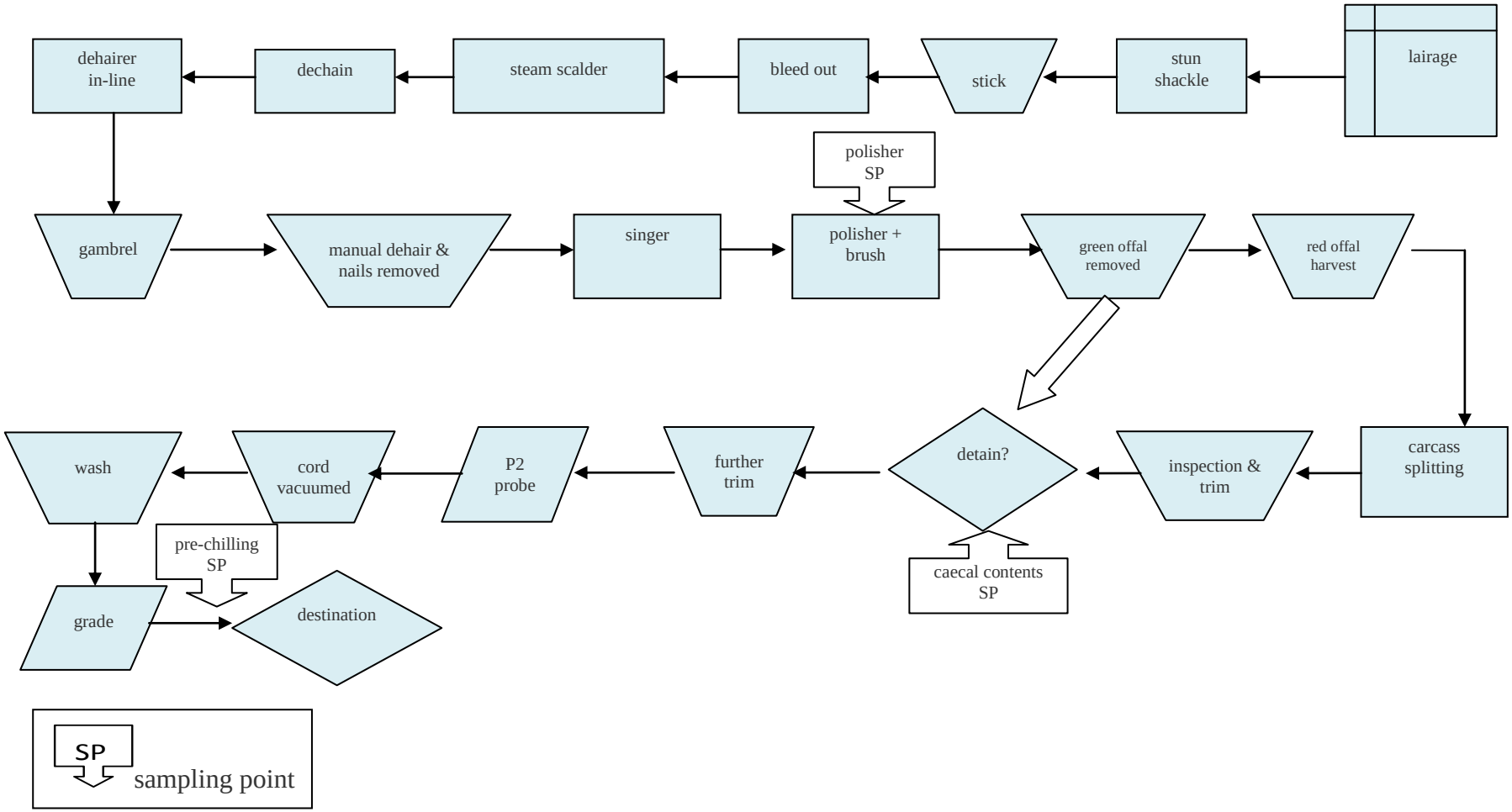



Figure6.1 Slaughter line for Slaughterhouse C (Reproduced by kind permission of Dave Tinker & Associates 2010)
SP: the designated sampling point; rectangles: automatic operations; trapezium: manual operations; parallelograms: inspections; rhombus: detaining area

### 6.2.2 Enumeration of E. coli

The bacterial dilutions were prepared as described in Section 2.3. The detection and enumeration of E. coli was carried out on TBX agar as described in Section 2.4.2.

### 6.2.3 ERIC-PCR of E. coli isolates

Isolation and confirmation of E. coli was carried out as described in Section 2.5. ERIC-PCR genotyping was conducted as described in Section 2.6.

## 6.3 RESULTS

### 6.3.1 Recovery of E. coli

A total of eight pig carcasses were randomly chosen for this study. The first four pigs (Pig 1 to Pig 4) were randomly sampled on the line without fixed intervals between them whereas the last four pigs (Pig 5 to Pig 8) were sampled consecutively. All of the eight pigs originated from the same farm. E. coli was recovered from the carcass swab and the caecal contents of each selected pig. The ECCs of the caecal contents ranged from 4.53 to 6.80 $\log_{10}$ cfu $g^{-1}$ (Figure 6.2a). The ECCs of the pre-chilling carcasses ranged from -1.55 $\log_{10}$ cfu $cm^{-2}$ to -0.15 $\log_{10}$ cfu $cm^{-2}$ (Figure 6.2b). For the four consecutively processed pigs (Pig 5 to Pig 8) the ECCs varied from -1.40 to -0.15 $\log_{10}$ cfu $cm^{-2}$ indicating that the extent of contamination of each pig was independent from adjacent carcasses. A low number of E. coli was recovered from the polisher (0.9 $\log_{10}$ cfu $sponge^{-1}$, Figure 6.2c) during the operation break, and the post-activity

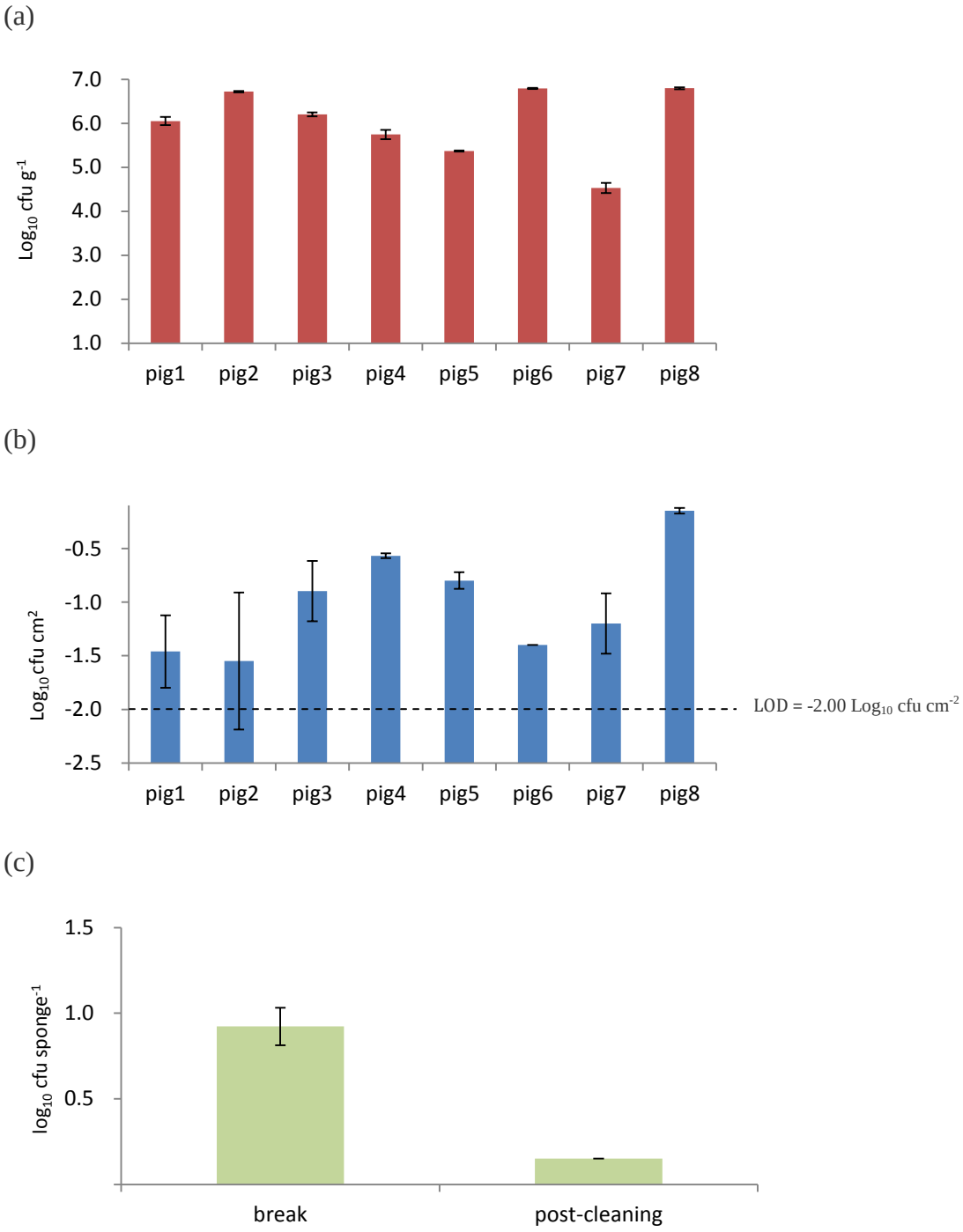



Figure 6.2. E. coli counts of (a) caecal contents and (b) pre-chilling carcasses of eight individual pigs, and (c) the polisher during break and post-cleaning. Pigs 1-4 were randomly sampled and pigs 5-8 were consecutive pigs in the line.

cleaning effectively reduced the ECC to 0.1 $\log_{10}$ cfu sponge$^{-1}$.

### 6.3.2 DNA fingerprinting of the E. coli isolates

The diversity of ERIC-PCR profiles of 538 E. coli isolates recovered from carcasses, caecal contents, and the polisher is shown in the dendrogram, Appendix 6.1. Forty-two ERIC-types were distinguished amongst the isolates with a threshold of 70% similarity ($\Phi_{PT}$= 0.684; P<0.01). At the bottom of the dendrogram, a very distinct grouping (7.8% - 33.6% similarity) was constituted by 6 caecal isolates and 1 carcass isolate showed highly diverse E. coli strains present during pork production.

Based on the ERIC-PCR genotyping data, the distribution of all genotypes is shown in Figure 6.3. It was noted that 18 genotypes were shared between the carcass isolates and the caecal isolates. All of the genotypes isolated from the polisher equipment (n=6) were also detected from pre-chilled carcasses and caecal contents. These genotypes shared amongst the polisher, caecal contents, and surface of carcasses demonstrated that the polisher was involved in the spread of contaminants during processing.

The variety of genotypes recovered from each carcass (Figure 6.4) shows the distribution of the carcass only, the caecal only, and the overlapped genotypes for individual pigs. Only Pig 2 did not possess a carcass-only genotype, whereas the other pigs harboured 2 to 7 genotypes found only on the carcass, indicating that self-contamination from its caecal matter may not be a major source of surface contamination. Most of the pigs harboured equal or more caecal-only genotypes than

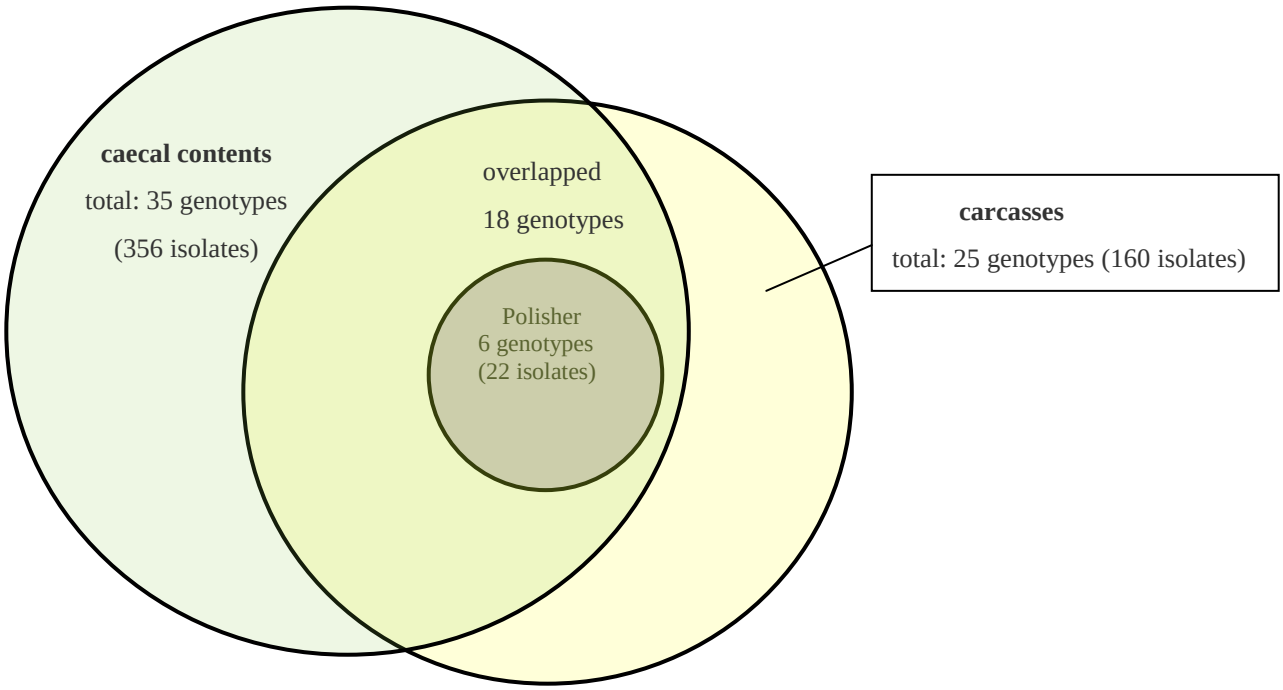
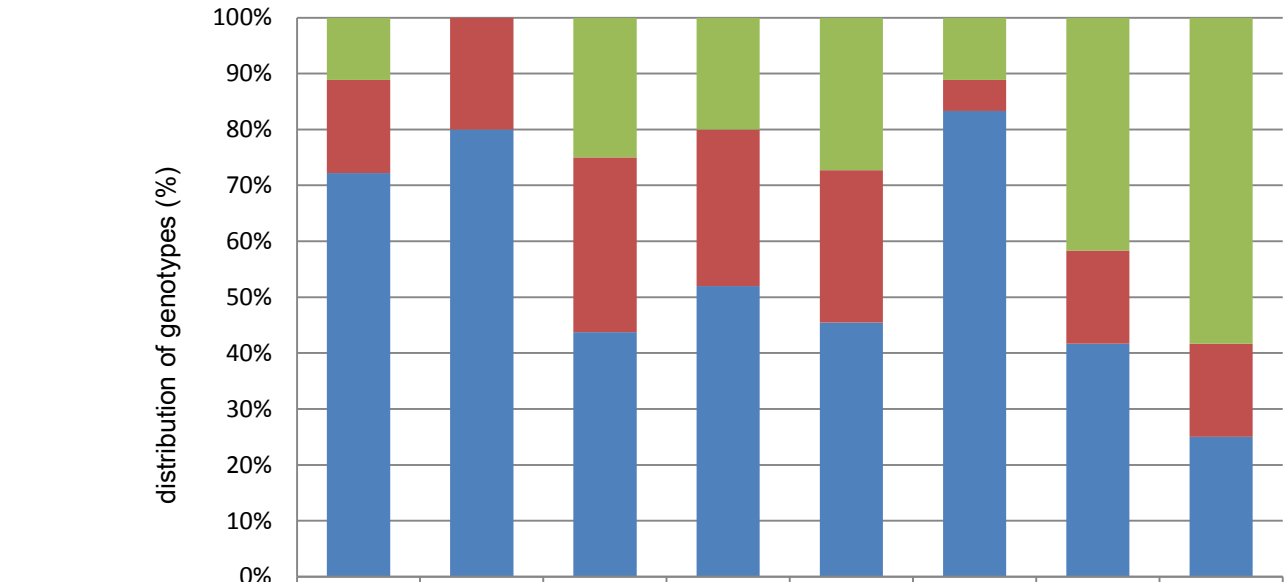



Figure 6.3. The distribution of ERIC-genotypes amongst carcasses, caecal contents and polisher.



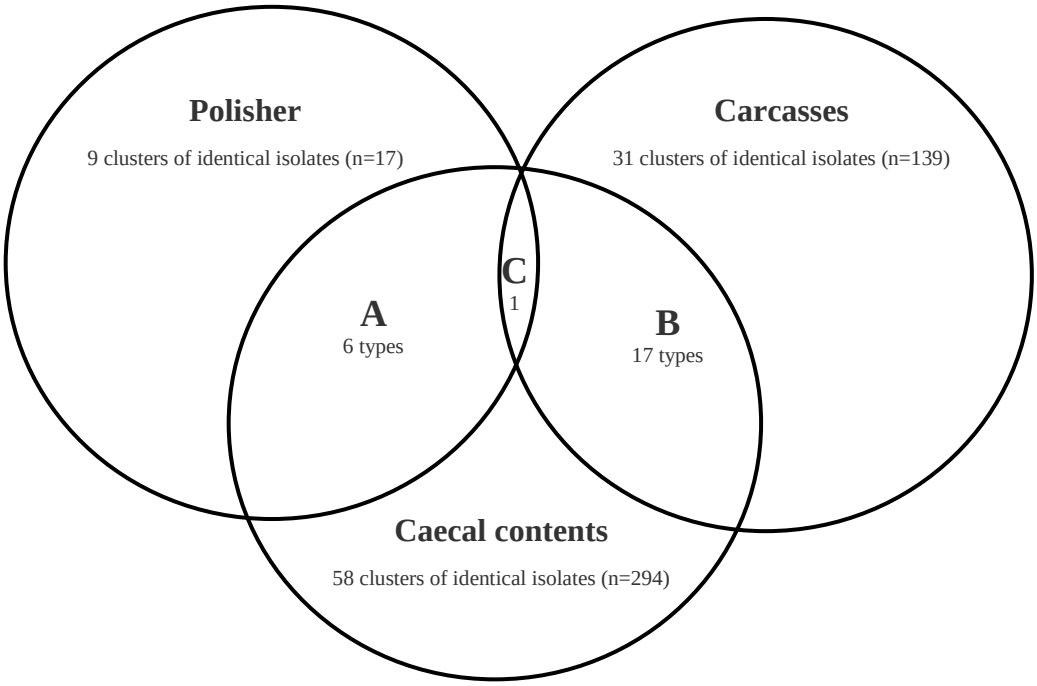

| | pig1 (n=55) | pig2 (n=53) | pig3 (n=64) | pig4 (n=66) | pig5 (n=54) | pig6 (n=53) | pig7 (n=48) | pig8 (n=123) |
|---|---|---|---|---|---|---|---|---|
| carcass only genotypes | 2 | 0 | 4 | 5 | 6 | 2 | 5 | 7 |
| overlapping genotypes | 3 | 2 | 5 | 7 | 6 | 1 | 2 | 2 |
| caecum only genotypes | 13 | 8 | 7 | 13 | 10 | 15 | 5 | 3 |

Figure 6.4. Relatedness of genotypes from different sites on individual pigs. Overlapping genotypes were ones found on both the carcass and in the caecum. Carcass only and caecum only genotypes were those found only from the surface of the carcass and from caecum, respectively.

the carcass-only types except Pig 8. This pig showed relatively high counts of E. coli on the carcass surface and 70% of the E. coli genotypes (n=10) were not related to the pig's caecal flora indicating that the major source of surface contamination was most probably been from other pigs.

A total of seventy-four sub-clusters of identical types (with 100% similarity) were found on the dendrogram. An analysis of the clusters of identical strains between sampling sites (Figure 6.5) showed that 61.3% of the carcass isolates (n=160) were identical with caecal isolates again indicating that surface contamination originated from gut matter whether of itself or other pigs. The results also revealed very close relationships of genotypes isolated from caecal contents and those from the polisher, where 6 identical ERIC-types were shared between caecal contents and polisher isolates.

Figure 6.6 is an example plot extracted from the full dendrogram and shows that identical strains were recovered at different sampling sites. It demonstrated that the same strains can be found from carcasses and caecal contents, and even from the polisher. In Figure 6.6, the cluster of strain 1 is composed of isolates from the polisher and caecal isolates from Pigs 3, 5, and 8 and indicated the transmission from caecal flora to the equipment. Since the polisher was sampled after these pigs were processed, it might support faecal leakage as a route of contamination of the polisher as this strain was not picked up from these carcasses. In addition, the cluster of strain 2 demonstrated that the contaminants in the caecum of Pig 3 were spread to the polisher and then were transmitted to the carcass surfaces of Pig 3 and the later processed Pig 5.




| | | |
|---|---|---|
| A | same genotypes shared by caecal contents and polisher | 6 genotypes (36 caecal and 12 polisher isolates) |
| B | same genotypes shared by caecal contents and carcasses | 17 genotypes (124 caecal and 92 carcass isolates) |
| C | same genotype shared by carcasses, caecal contents, and polisher | 1 genotype (1 carcass, 3 polisher, and 2 caecal isolates) |

Figure 6.5. Number of clusters of identical ERIC-type recovered amongst carcasses, caecal contents and polisher. The identical types shared between sampling sites were shown in the area A, B, and C.

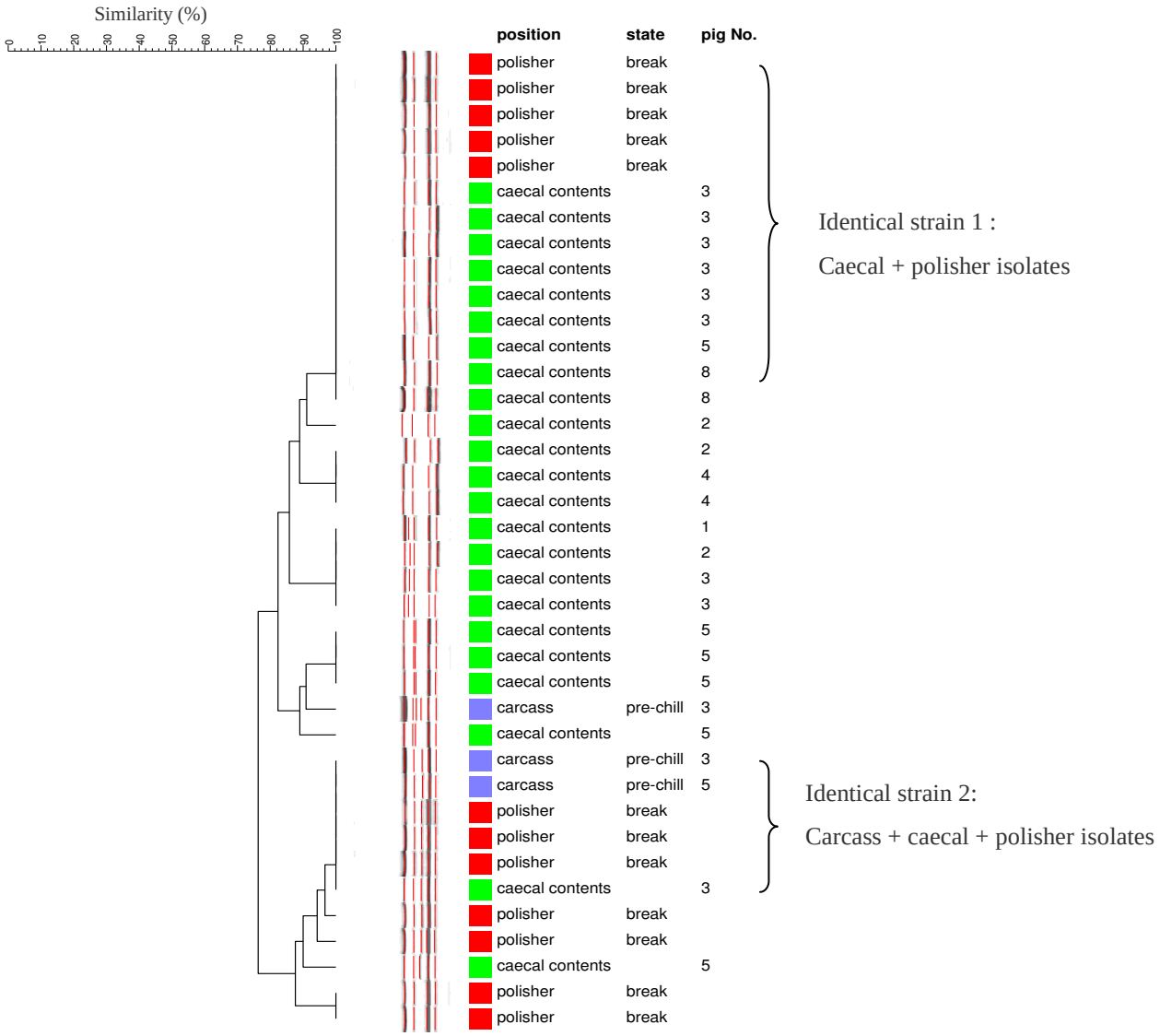



Figure 6.6. Representative examples of the identical strains found between different sampling sites. This dendrogram was extracted from Appendix 6.1.

## 6.4 DISCUSSION

As with other investigations about the genetic diversity of E. coli in the guts, a high diversity of E. coli genotypes was observed in the caeca not only within the sampled pigs as a whole, but even within individual caecal samples. However, one pig showed only 8 caecal-genotypes suggesting that there were still individual differences between pigs, and pigs from the same herd developed individual flora populations, although some of these were shared with other pigs.

In microbiological testing, typing of selected colonies often underestimates the diversity (Weijtens et al., 1999). In a study conducted by Lautenbach et al. (2008), the impact of sampling sizes on the determination of diversity of E. coli strains was investigated. Their results demonstrated that the ability to characterise E. coli strain diversity is directly related to the number of colonies sampled and the underlying prevalence of the strain. Thus, sampling of five randomly selected colonies could identify 24% - 98% of the whole population depending on the prevalence of these selected strains. Practically, examining five colonies can find at most five genotypes, so a flora diversity greater than five genotypes would not be able to be accurately presented. The results in the present study showed that the number of genotypes in the 'whole population' of the individual carcasses ranged from 2 to 13. Five out of the eight sampled pigs had more than 5 carcass genotypes. It indicates that taking five representative colonies as in the studies in previous chapters may underestimate the diversity of the entire population. This shows the limitations of the methodology used

in this work. Increasing the number of isolates selected could make the findings more robust and may possibly obtain more detailed information, but, however, in practice it should be considered that the expansion of sample size also increases the time and the cost of analysis and may not significantly change the conclusions drawn.

The present results suggest that the majority of E. coli strains found on the carcass originated from the gut flora, which agrees with a study of Campylobacter conducted by Malakauskas et al. (2006). However, as the 'overlapped genotypes' for individual pigs were not the majority of the final carcass flora, it can be concluded that the final carcasses may suffer more cross-contamination than self-contamination.

Because chilling is the last step of slaughter (Dockerty et al., 1970), the pre-chilling carcass is the final carcass although some reduction in counts can take place during the chilling process (Carr et al., 1998; Lenahan et al., 2009; Tomovic et al., 2011). The detection of E. coli on the final carcasses suggested that few strains can survive the whole process and therefore be present in the final products. Although most genotypes in the final carcass flora could be found in caecal contents, there were still 17% genotypes which were only found from the pre-chilling carcasses, suggesting the existence of other sources of cross-contamination. The sources of these genotypes could be the carcasses in previous herds, or the dominant strains in the slaughter environment, such as residual strains in the polisher as seen in the previous chapter. It still needs more efforts to identify the actual sources of these genotypes.

Polishers immediate cross contamination whether with carcass to carcass transmission during polishing (Borch et al., 1996) or from a contaminated polishing equipment (Yu et al., 1999; Yu and Palumbo, 2000). To this study the caecal contents as a source of contamination of the polisher was observed. Furthermore, most of the polisher isolates were identical to the caecal isolates, rather than with the carcass isolates, providing support for the theory that shedding by faecal leakage is an important source of E. coli within the polisher.

In general, the final carcass flora was primarily related to caecal contents. The present study provides evidence that enteric bacteria spread through the polishing equipment of the pork slaughter line are related to those from the caeca and therefore supports faecal leakage as a key source of contamination. The difficulty of cleaning means that bacteria may become established on polishing machines, constituting a source of cross contamination (Borch et al., 1996). These findings conclude that monitoring and controlling the slaughter equipment as an intermediate of transmission is very important to comply with HACCP requirements and to ensure the safety of the final products.

# CHAPTER 7 HEAT TOLERANCE AND PHENOTYPIC CHARACTERISTICS OF E. COLI STRAINS FROM PIG CARCASSES

## 7.1 INTRODUCTION

The reduction effects of scalding and singeing operations on bacterial counts, as well as on genotypes, have been observed and discussed in the previous chapters. As differences in E. coli genotypes have been seen across these operations involving heat, the impact of heat on different genotypes of the E. coli isolates were examined with particular interest.

In this study, heat tolerance of several E. coli isolates was determined using a 55°C water bath incubation as a simulation of scalding conditions. Commercial scalding conditions normally range from 58°C to 65 °C, depending on the type and duration of treatment. The temperature of 55°C is that estimated to be achieved on the surface of carcasses, and is used frequently when studying heat intervention during meat production or processing (Juneja and Miller, 1997; Bolton et al., 2003; Juneja, 2004; Sallami et al., 2006; Osaili et al., 2007)

In the first part of the study, three representative E. coli isolates recovered from Slaughterhouse A and three representative isolates from Slaughterhouse B were selected for study. The isolates were chosen from the same or different genogroups based on the genotyping dendrograms in Figures 4.2 and 5.5 (marked with asterisks). The codes of the Slaughterhouse A isolates were:

post-bleed73 (genogroup VI), pre-scald103 (genogroup III), and post-dehair105 (genogroup III). The codes of the Slaughterhouse B isolates were: pre-singe83 (genogroup 2), post-singe84 (genogroup 2), post-polish84 (genogroup 1). The heat tolerance of these isolates were determined and compared. The isolates represented groups which were present before and after a heat processing stage. The hypothesis was that strains found after a heat process could have higher heat resistance than those found before, and that strains from the same genogroup would show similar heat tolerances.

It has been known that the expression of the global regulator gene rpoS, encoding the RpoS sigma factor, leads to an enhancement of stress responses when E. coli cells enter stationary phase (Aldsworth et al., 1999; Dodd and Aldsworth, 2002). The plasmid pSB367, containing spvRA (from Salmonella Dublin) fused with luxCDABE from Photorhabdus luminescens and conferring resistance to 50 µg/ml ampicillin, was constructed for bioluminescence-based detection of RpoS expression (Swift and Stewart, 1994) and used in this study. Expression of the spv operon requires functional RpoS and uses expression of bioluminescence as a reporter. E. coli transformed with the pSB367 plasmid emits light when induction levels of RpoS are sufficient.

Because the growth phase has an impact on heat resistance, experiments were carried out on both exponential phase and stationary phase cultures, with the anticipation that the importance of RpoS would be examined if differences in heat resistance were evident.

## 7.2 MATERIAL AND METHODS

### 7.2.1 Thermal resistance

The thermal resistance of E. coli isolates was determined by the method described by Namvar and Warriner (2005). The isolate was cultured in nutrient broth (NB; 7146a, Acumedia, Ml, US) at 37℃ overnight and was adjusted to the initial concentration ($10^8$ cfu $ml^{-1}$) by measuring absorbance and adjusting to A= 0.8 at 600 nm. A thin-walled glass universal containing 9.9 ml of NB was pre-warmed in a water bath maintained at 55 ±1℃ and 0.1 ml of the E. coli suspension was added. Samples of 0.1 ml were periodically withdrawn (from time zero, then at 0.5, 1, 3, 5, and 10 min) and immediately pipetted into pre-cooled NB on ice. Each cooled sample was serially diluted in NB and dilutions were plated onto nutrient agar ( 7145a, Acumedia, Ml, US) and incubated at 37℃ for 24h.

### 7.2.2 Calculation of D-values

The calculation of D-values was carried out using the linear regression method described by Ahmed et al. (1995). Colonies were enumerated and the data plotted as logarithmic counts ($\log_{10}$ cfu $ml^{-1}$) against time (sec). D-values were calculated from the straight portion of the survival curves by linear regression. For stationary cultures, at least four values in the straight portion with a correlation coefficient ($r^2$)>0.90 were used for the linear regression analysis. For exponential cultures, at least three values (or all the values if only two counts available) in the linear portion ($r^2$>0.90) were used.

### 7.2.3 RpoS induction

The induction of the rpoS gene was determined using the method described by Swift and Stewart (1994). The RpoS-dependant reporter pSB367 plasmid was used for the detection and quantification of the rpoS expression.

#### 7.2.3.1 Preparation of pSB367 plasmid

The pSB367 plasmid was extracted from the E. coli pSB367 strain provided by Mr. Philip Richards from the University of Nottingham laboratory culture collection. The E. coli pSB367 was an ampicillin resistance clone which was capable of growth in ampicillin supplemented media. To prepare nutrient broth supplemented with ampicillin (NB-amp50), 1 ml of 50 mg $ml^{-1}$ filter sterilised ampicillin solution was added to 1000 ml sterile NB. The E. coli pSB367 was cultured in NB-amp50 at 37˚C overnight. Plasmid DNA was prepared using the commercial Zyppy™ Plasmid Miniprep Kit (Cambridge Bioscience, UK) following the manufacturer's instructions. Briefly, 100 µl of alkaline lysis buffer was added into 600 µl overnight culture. When the bacterial lysis was completed (the turbid lysate became clear), 350 µl neutralization buffer was added and the cellular debris floated to the surface. The debris and the lysate were separated by centrifugation (at 13,000 g for 5 min). The cleared lysate was moved to the filter column set (Zymo-spin™ II) and the filter column set was then centrifuged at 13,000 g for 2 min. The plasmid DNA was retained in the column and washed twice with wash buffer (containing 70% ethanol). After wash steps the plasmid DNA was eluted with 10 µl sterile double distilled water and stored at -20 ˚C until required.

7.2.3.2 Preparation of electro-competent E. coli cells

Cultures of E. coli strains were grown at 37 ˚C in LB broth with shaking for a period of 18 h. The cultures were then inoculated 1:50 to fresh LB broth and incubated to $A_{600nm}$ ~ 0.5. The cultures were chilled on ice for 15 min and then centrifuged at 4,000 g (JA-10, Beckman) at 4˚C for 15 min. The supernatant was discarded and the pellet was resuspended in 200 ml ice-cold sterile distilled water and centrifuged at 4,000 g at 4˚C for 15 min. Resuspension of the pellet and centrifugation was repeated once in 100 ml ice-cold sterile distilled water and then once in 20 ml ice-cold 10% sterile glycerol solution (v/v in distilled water). The final pellet was resuspended in 1 ml ice-cold 10% glycerol; 40µl aliquots of electro-competent cells were rapidly frozen using liquid nitrogen and then stored at -70˚C until use.

7.2.3.3 Electroporation of plasmid DNA into competent E. coli cells

The plasmid DNA was dialysed before electroporation. DNA (5 µl) was dropped on a floating Type-VS Millipore membrane (Millipore, US) in a petri plate filled with sterile double distilled water for 10 min. The dialysed pSB367 plasmid DNA (2 µl) was then mixed with 40 µl prepared competent cells to make a suspension in an ice-cold electroporation cuvette (Bio-Rad, US). To electroporate cells, the mixture was pulsed at 25 µF, 2.5 kV, and 200 Ω using the Gene-Pulser™ electroporator (Bio-Rad, US). Pulsed cells were added into 1 ml LB broth and recovered at 37℃for 1h. After recovery, 200 µl of the transformed culture was spread on nutrient agar supplemented with 50 µg $ml^{-1}$ ampicillin (NA-amp50) and incubated at 37℃ for 20 - 48h. The successfully

transformed cells grew on NA-amp50.

#### 7.2.3.4 Bioluminescence assays

The transformed E. coli strains were inoculated in pre-warmed NB-amp50 and were incubated at 37℃ with shaking (200 rpm). Isolates were subcultured three times by means of 1:100 dilutions into NB-amp50 when the $A_{600nm}$ reached to 0.15. The RpoS activity during cell growth was monitored by bioluminescence and absorbance at 600nm respectively using a Tekan microplate reader (Tekan, Switzerland). Readings of Relative Light Unit (RLU) and O.D. were taken by GeNios Pro software every 30 min within 18h duration. The method of calculation of induction time for rpoS as was described by Aldsworth et al. (1998). The induction time of RpoS-mediated gene expression was derived by the intersection between lines drawn through the stationary and exponential portions of the growth curves and bioluminescence curves plotted against incubation time.

### 7.2.4 Sequencing of rpoS

#### 7.2.4.1 PCR amplification of rpoS regions

Six regions of the rpoS gene were amplified using the method developed by Jordan et al. (1999). Reaction mixtures of 50 µl contained 1µl DNA, 16 pmol of primers (MWG Eurofins, Germany), 25mM dNTPs (Promega, US), 1 mM $MgCl_2$ (Promega, US), 4.6µl of 10x PCR buffer, 1 U of Taq DNA polymerase (Thermal Scientific, UK) and DNase free water to make the total volume 50 µl. The primers used were listed in Appendix 7.1 and were used individually in

each PCR reaction.

#### 7.2.4.2 Sequencing of rpoS amplicons

PCR products that gave a single band on an agarose gel were purified with Zymoclean™ Gel DNA Recovery Kit (Cambridge Bioscience, UK). The agarose gel with the PCR products was cut and dissolved into 3 volumes of agarose gel dissolving (ADB) buffer and then incubated at 55℃ for 5-10 min until completely dissolved. The dissolved agarose solution was further purified with the Zymo-spin column filtration system. The purified DNA was eluted with 10 µl sterile double distilled water and stored at -20 ℃ until required. The DNA sequencing services were provided by MWG sequencing laboratory, a commercial sequencing centre in Germany. DNA sequence similarity searches utilised the BLAST search available on the National Institute of Health website (http://www.ncbi.nlm.nih.gov/blast).

### 7.2.5 V3 region of 16S rDNA PCR

PCR was performed using a protocol described by Ercolini et al. (2003). The V3 variable region of the 16S rDNA was amplified using the primer set of V3F (5'-CCTACGGGAGGCAGCAG-3') and V3R (5'-ATTACCGCGGCTGCTGG-3'). The PCR mixture (30 µl) contained 0.2 µM of each primer, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, 2.5 µl of 10X PCR buffer, and 2.5 U of Taq polymerase. The reaction was started by a denaturation at 94°C for 5 min. A touchdown PCR was then performed with the initial annealing temperature 66°C, and then decreased 1°C every cycle for 10 cycles; finally, 20 cycles were performed at 56°C. The extension for each

cycle was carried out at 72°C for 3 min, while the final extension was at 72°C for 10 min. Aliquots (2 µl) of PCR products were checked on 2% TAE agarose gels.

### 7.2.6 API 20E biochemical test

The commercial kit API 20E® (BioMerieux, France) was performed as given by the manufacturer's manual. The results were analysed using APIWEB™ software online from the API website.

## 7.3 RESULTS

### 7.3.1 Measurement of heat tolerance

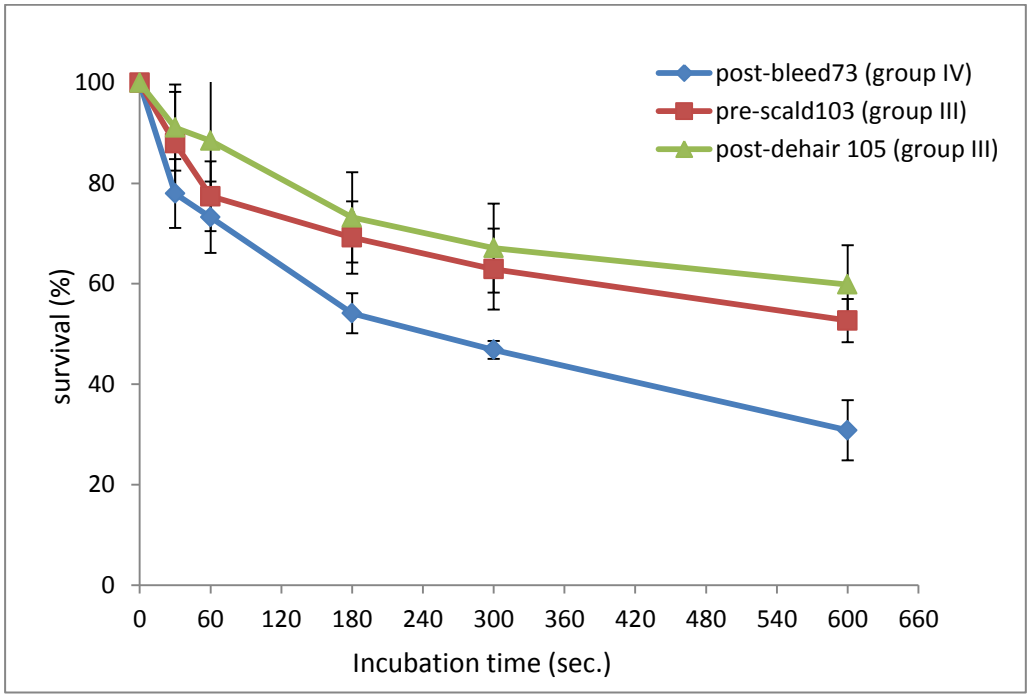
The percentage survival curves for the Slaughterhouse A strains are shown in Figure 7.1 and the mean $D_{55°C}$ values (time to reduce bacterial populations by 1 log or 90% at 55°C) are presented in Table 7.1. The data used in D value calculation are shown in Appendix 7.2. It should be noted that the $D_{55°C}$ values of isolates post-bleed73 and pre-scald103 in two batches of testing of exponential phase culture were calculated using only the counts at time 0s and 30s because E. coli was not detected after 60s (Appendix 7.2). As the extrapolation of the linear curve plotted using the counts of time 0s and 30s would give a count below the limit of detection at 60s, this calculation is a reasonable approximation.

The isolates from genogroup III in stationary phase showed greater tolerance to

heat and retained 46% to 60% viability after 10 min thermal treatments ($D_{55°C}$ ranged from 3.97 min to 5.23 min). This was in comparison to post-bleed73, the genogroup IV strain which showed a mean $D_{55°C}$ value of 2.37min, and the percentage survival after 3 min heat treatment was significantly lower ($p<0.05$) than pre-scald103 and post-dehair105 (genogroup II strains). Of the mid-exponential phase cells, none of the strains tested were detected after 10 min (Figure 7.1b).

In the other set of heat tolerance experiments with slaughterhouse B strains, one of the post-polishing strains was chosen due to a very low level of similarity comparing with strains at other stages. The post-polishing strains were of particular interest with regard to the heat tolerance in order to establish if they had survived the singe process. One of the post-polishing isolates from group 1 and two isolates from group 2, one pre-singeing and one post-singeing, were chosen for the determination of their heat resistance. With regard to the percentage survival of the stationary phase cells after 10 min heat treatment (Figure 7.2a), the strain pre-singe83 showed 44% survival and the post-singe84 showed 43% survival. However, the strain post-polish84 was not detected after 5 min heat treatment. Figure 7.2b shows that pre-singe83 and post-singe84 were less heat tolerant at mid-exponential phase when compared with their stationary phase cultures, and were not detected after 10 min incubation. However, for the post-polish84 isolate, the thermal reduction curves were similar in cultures of both growth stages. This isolate was heat sensitive and could not withstand 5 min heat treatment.

(a)




(b)

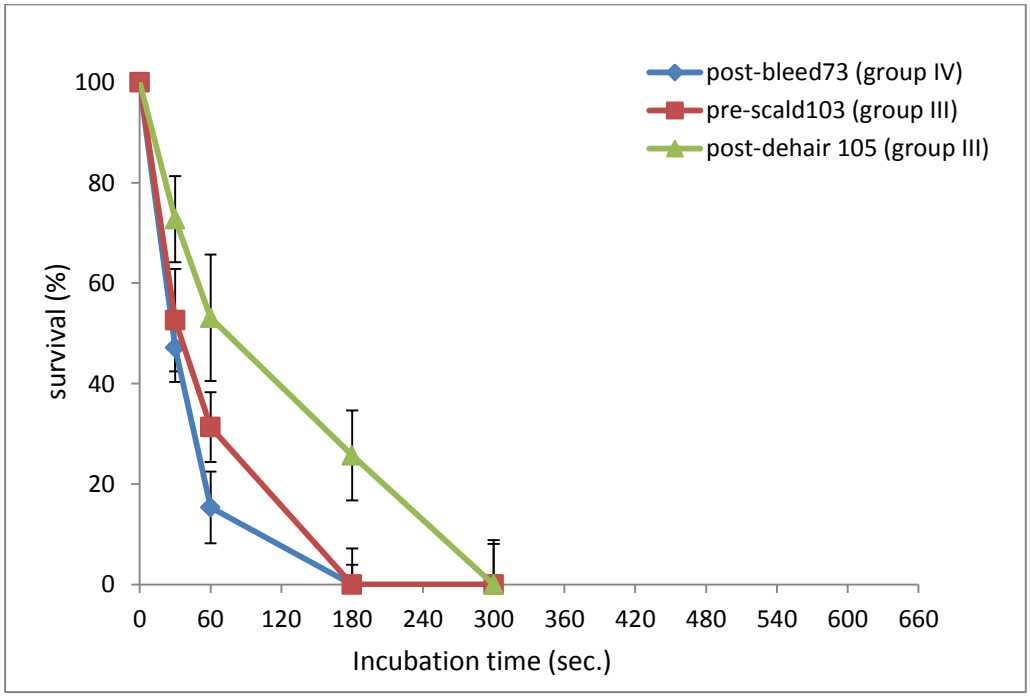



Figure.7.1. Percentage survival curve of three E. coli strains (from Slaughterhouse A) at 55˚C in (a) stationary phase and (b) exponential phase. Sampling was commenced from time zero.

Table 7.1. Heat resistance of E. coli isolates from Slaughterhouse A: expressed as D-values† minutes at 55°C

| | | Growth phase | |
|---|---|---|---|
| | | Stationary | Exponential |
| Source of isolates | Genomic group | $D_{55°C}$ | $D_{55°C}$ |
| post-bleed73 | IV | 2.37 ± 0.31 [a] | 0.17 ± 0.07 [a] |
| pre-scald103 | III | 5.23 ± 1.50 [b] | 0.20 ± 0.10 [a] |
| post-dehair 105 | III | 3.97 ± 2.65 [ab] | 0.70 ± 0.05 [b] |

† D-values shown are the means of three replicate experiments, each performed in triplicate and expressed as mean ±standard deviation in minutes. Means within same column with the same letter are not significantly different (P > 0.05).

Table 7.2. Heat resistance of E. coli isolates from Slaughterhouse B: expressed as percentage survival and D-values† minutes at 55°C

| | | Growth phase | | | |
|---|---|---|---|---|---|
| | | Stationary | | Exponential | |
| Source of isolates | Genomic group | $D_{55°C}$ | % survival at 10min | $D_{55°C}$ | % survival at 10min |
| Pre-singe83 | 2 | 3.06 ± 0.20 | 44.4% | 0.45 ± 0.25 | 0% |
| Post-singe84 | 2 | 2.58 ± 0.71 | 42.8% | 0.81 ± 0.14 | 0% |
| Post-polish84 | 1 | 0.50 ± 0.23 | 0% * | 0.22 ± 0.03 | 0% |

†D-values shown are the means of two replicate experiments, each performed in triplicate and expressed as mean ±standard deviation in minutes.
* significant difference (p< 0.05) from the other two isolates tested.

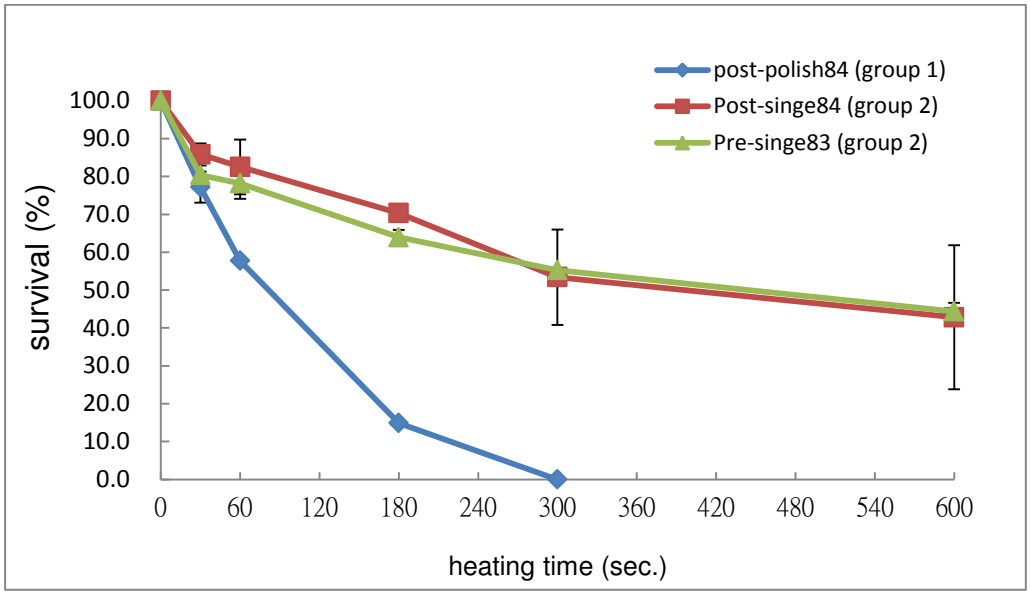
### 7.3.2 Expression of RpoS

The post-polish84 isolate showed a relatively low heat tolerance and no significant increase of protection to heating was found in the stationary stage cultures, whereas strains pre-singe83 and post-singe84 possessed greater heat tolerance in stationary phase than in mid-exponential phase. Thus, the expression of rpoS of these isolates was examined.

Two aspects of RpoS expression were studied, which were RpoS induction time and the level of bioluminescence per unit optical density during the growth cycle. The RpoS induction time was calculated based on the growth curve and bioluminescence curve. An example of how the induction time is measured is shown in Figure 7.3a. The induction time of each culture was estimated and shown in Figure 7.3b. The pre-singeing isolate and post-singeing isolate entered stationary phase at 6.6 h and at 6.8 h of incubation respectively, and showed induction of RpoS just before growth entered stationary phase. However, no RpoS-induction was observed in the heat-sensitive strain. The heat-sensitive strain also showed relatively late stationary phase entry, at 7.4 h of incubation. The bioluminescence per unit optical density (RLU/O.D.), Figure 7.4, shows the level of RpoS expression related to cell density. The ratios of RLU/O.D. of the two RpoS induced strains were over $1.9 \times 10^5$.

However, although the RpoS induction was not seen in the heat-sensitive strain, bioluminescence was still detected but at a relatively low level, below $3 \times 10^4$.

(a)




(b)

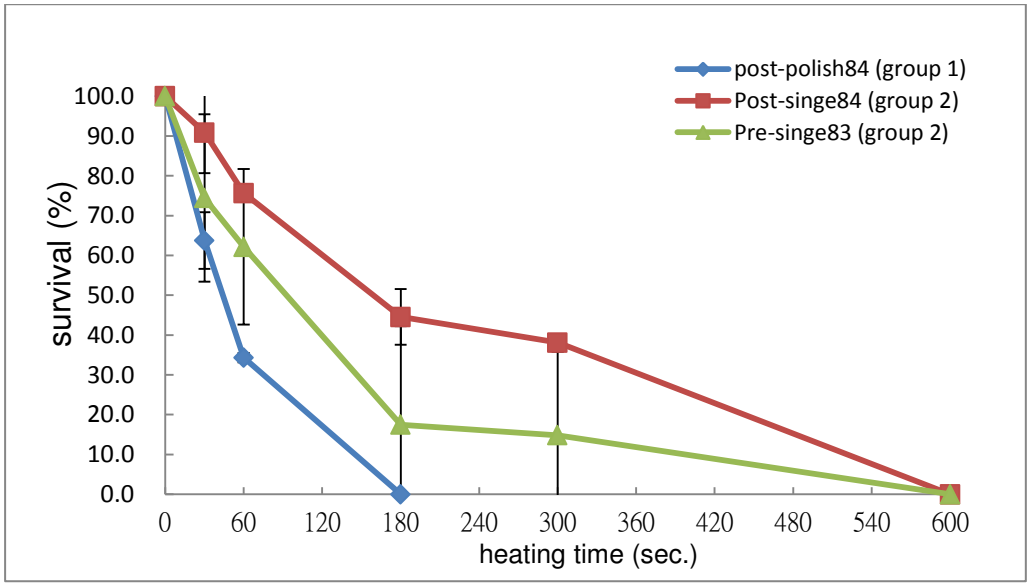



Figure.7.2. Percentage survival curve of three E. coli isolates (from Slaughterhouse B) at 55˚C at (a) stationary phase and (b) exponential phase. Sampling was commenced from time zero (approximate initial conc. $1\times10^{7}$ cfu $ml^{-1}$).

The RLU/O.D. was increased slightly with time during incubation but it was not related to the growth phase. Thus, in two of the isolates typical RpoS induction in stationary phase was evident which related to the higher heat resistance seen in stationary phase. However, strain post-polish84 did not show RpoS induction in stationary phase culture. This would explain the lack of heat tolerance of the stationary phase culture observed.

### 7.3.3 Further characterisation of RpoS

#### 7.3.3.1.1 rpoS sequence alignments

Because of the low similarity to other genotypes, the low tolerance to heat, and the absence of rpoS induction whilst still producing low level of bioluminescence from pSB367, further characterisation of the heat-sensitive isolate post-polish84 was performed. The rpoS gene of one of the strains showing RpoS induction (pre-singe83) and of the non-induced isolate (post-polish84) was sequenced and the sequences compared by sequence alignment. In Figure 7.5, the six PCR amplicons from the two tested isolates are aligned. In section A, B, C, D, and E, the sequences from the two isolates were 97-99% aligned except for a few single base polymorphisms and gaps. However, the amplicons of section F differed between these two isolates.

#### 7.3.3.2 Sequencing and BLAST

The amplicon of rpoS section F of the non-induced isolate was compared on the public nucleotide database NCBI-nBLAST. The sequence of strain

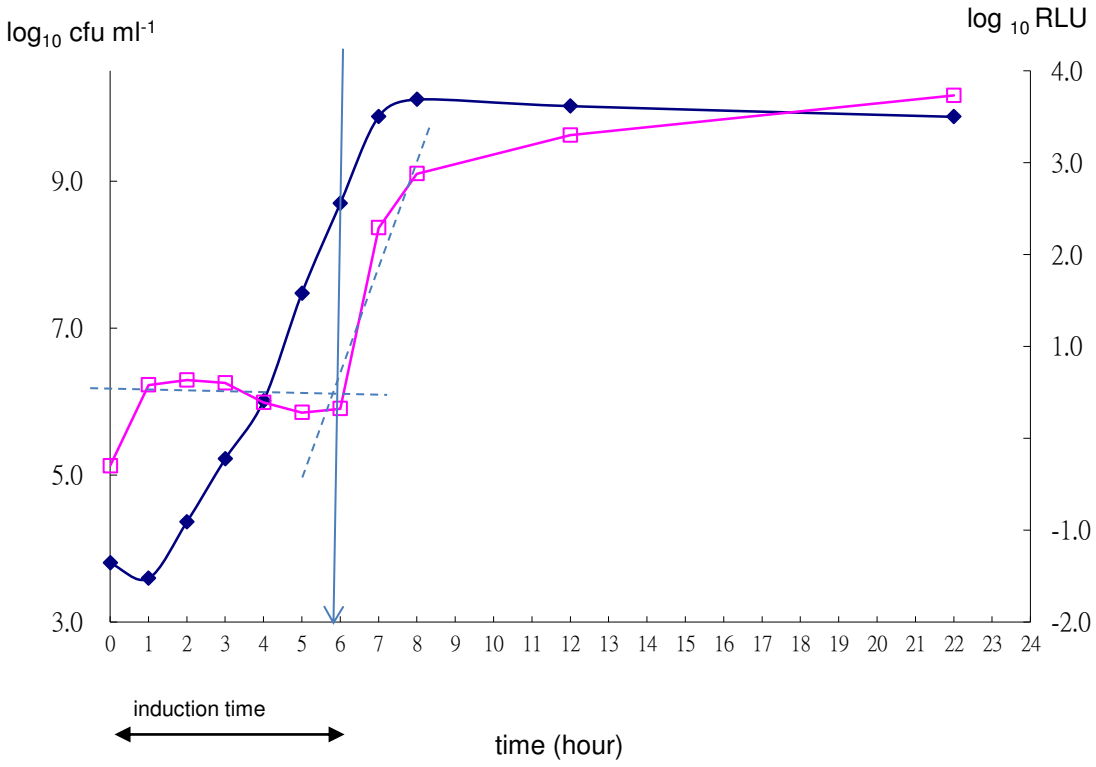



Figure 7.3a. An example of the induction time for rpoS-mediated gene expression in a culture of the E. coli strain (pre-singe83) transformed with pSB367. The induction time was defined by the intersection between dotted lines drawn through the horizontal and exponential portions of the bioluminescence curve (Aldsworth et al., 1998). (closed squares: growth curve; open squares: bioluminescence)

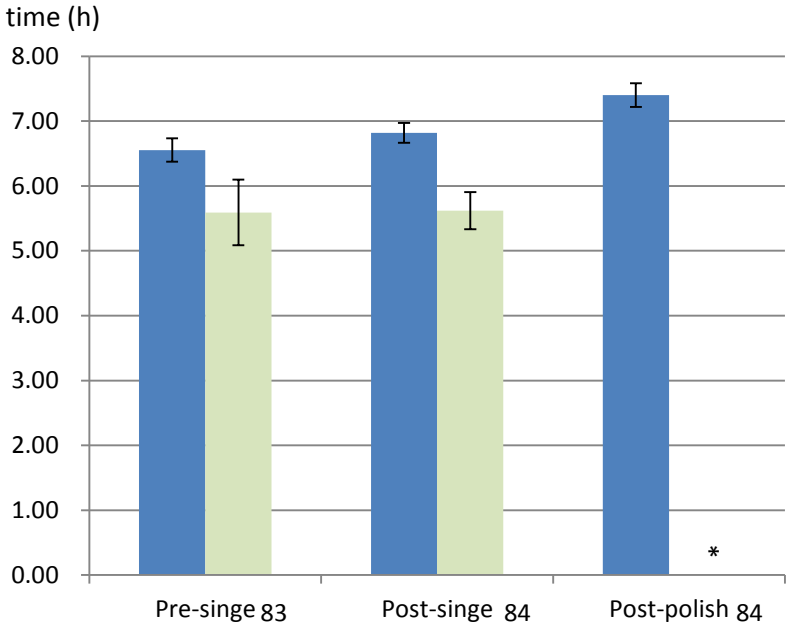



Figure 7.3b. Induction times and stationary phase entry times for representative E. coli isolates obtained from different slaughter steps. Tested strains were transformed with bioluminescent reporter plasmid pSB367. Light bars: induction time of RpoS; Dark bars: time of entry into stationary phase.

* No obvious induction of RpoS was observed in the post-polish strain.

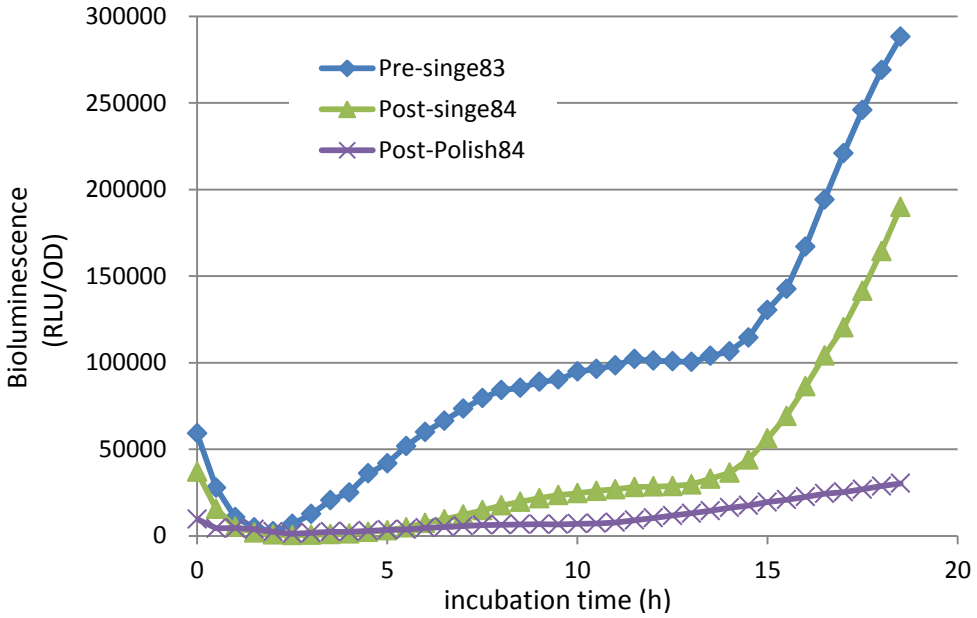



Figure 7.4. Bioluminescence of representative E. coli isolates during growth. The values of bioluminescence were displayed as Relative Light Unit per Optical density at 600 nm.

pre-singe83 belonged to E. coli rpoS gene but the sequence of strain post-polish84 did not. The highest match was a fragment belonging to the Trk gene (e value $3e^{-124}$; max identity 94%), a transporter encoded in the genome of Aeromonas veronii B565 (CP002607.1). Phenotypic characterisation was therefore carried out and the biochemical test API20E also identified this strain as Aeromonas hydrophila/ caviae/ sobria (98.8% ID; T=0.92). This identification was unexpected so the species identity of strain post-polish84 was checked. The V3 region of 16S rDNA of the isolate was amplified with PCR, sequenced and identified by NCBI- nBLAST. The BLAST result indicated this isolate was identified as Aeromonas (max identity 100%).

## 7.4 DISCUSSION

### 7.4.1 Characterisation of thermal tolerance

This study was an attempt to understand whether the changes in genotypic characteristics of the E. coli population seen through the slaughterline were a result of response to the thermal challenges of the slaughter processes resulting in selection of a sub population.

Amongst the Slaughterhouse A isolates (n=135), two representatives, a post-bleeding isolate and a pre-scalding isolate, were obtained from the stages before heat treatment during processing. The post-bleeding isolate belonged to genogroup IV, which was composed mainly of the post-bleeding isolates but which were not seen to be present in subsequent stages of operations. Thus, it

Amplicon A


Amplicon B


Amplicon C


Amplicon D


Amplicon E


Amplicon F

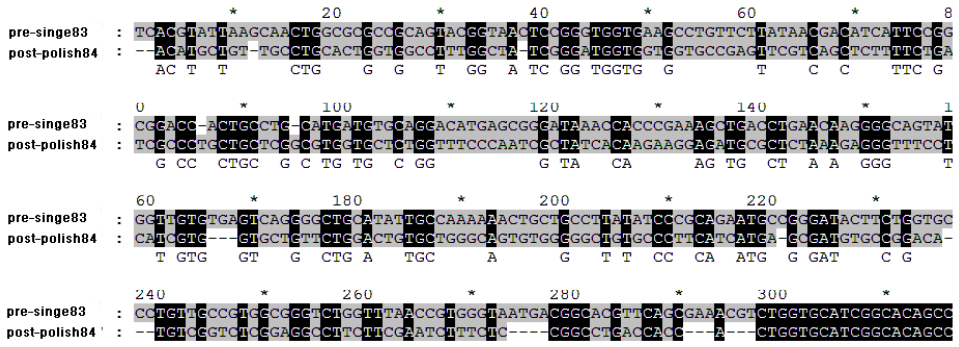



Figure 7.5. The sequence alignment of rpoS primer set A - F. Sequences were of the heat tolerant strain pre-singe83 and heat sensitive strain post-polish84. Primers are given in Appendix 7.1.

was assumed that the isolates from genogroup IV may be less heat tolerant due to their absence in later processes. This was supported by the results that the loss of viability of the post-bleeding isolate was the most rapid of those tested.

The thermal reduction curves may consist of stages, such as activation, shoulder, reduction, and tailing (Nguyen et al., 2010). In the thermal reduction graphs, the heat inactivation started almost immediately. No obvious activation and shoulder stages were seen on the curves, but tailing was observed after 5 min heat treatment for stationary phase curves of strains pre-scald103, post-dehair105, pre-singe83 and post-singe84. First-order kinetics of thermal inactivation assumes that bacteria in the population have the same probability of dying but in nature bacterial populations are heterogeneous (Singh et al., 2010). Tailing indicates changes in resistance after a certain period of heat treatment. McCann et al. (2006) concluded that the tailing effects may be associated with the production of heat shock proteins in the subpopulation, which protect cells of E. coli from denaturisation at high temperatures.

Despite the non-linearity of the inactivation curves, D values can be derived from the linear portions and used for comparison of heat resistance (Juneja, 2004; Eblen et al., 2005; Nguyen et al., 2010). The $D_{55°C}$ values of the stationary phase of E. coli representatives obtained in this study ranged from 2.41 to 3.97 min (excluding the post-polishing isolate). In a review of heat resistance of E. coli (Sorqvist, 2003), the author reported that $D_{55°C}$ values for E. coli strains varied from 0.9 to 22.3 min. However, these values are affected by

many environmental factors and direct comparisons cannot be made unless these factors are considered, such as detection methods, media used, pH and $a_w$ (Sorqvist, 2003; Conesa et al., 2009). Dlusskaya et al. (2011) reported that E. coli strains found in a slaughter plant were more resistant to heat than reference lab strains. An extremely heat-resistant E. coli isolate exhibited a $D_{60\ ^{\circ}C}$ value up to 71 min whereas the $D_{60\ ^{\circ}C}$ of the reference strains ranged from 0.1 to 0.5 min. This could indicate that some wild-type E. coli strains on the slaughtered animals have greater heat tolerance.

The levels of percentage survival in stationary phase were significantly greater than those in exponential phase, suggesting that the stationary phase afforded the cells extra protection against heat damage. Cells in exponential phase are normally more sensitive than those in stationary phase (Rees et al., 1995; Cheville et al., 1996; Komitopoulou et al., 2004). The greater resistance in stationary phase can result from the expression of RpoS, which has been reported a major regulator of stationary phase gene expression and increasing resistance to many stress conditions (Dodd, 2005). On the point of food safety, RpoS expression plays an important role for the resistance and virulence of food borne pathogens during food processing or storage (Cheville et al., 1996; Aldsworth et al., 1998; Jorgensen et al., 2000).

Of particular interest in this study was the role of rpoS expression in heat stress responses. An association between heat tolerance and RpoS expression was observed in the tested isolates. Survival after 10 min heat treatment was observed in the two strains with RpoS induction. The isolate which did not

show an RpoS induction rapidly lost viabilities. These findings provide explanations of the variation in heat resistance among strains, although the heat sensitive isolate was latterly identified not an E. coli strain, but as an Aeromonas strain.

### 7.4.2 Identification of the atypical isolate

The sequence matches for both section F of rpoS and the V3 region of 16S rDNA indicated that this isolate belonged to Aeromonas. The failure to screen this strain out by the isolation and confirmation methods used was unexpected. Each presumptive E. coli isolate picked from the selective medium (TBX agar) was subjected to a secondary confirmation by the indole test and only indole positive isolates were subcultured and genotyped. However, Aeromonas was also an indole positive bacterium. Due to the ability of the organism to grow on TBX agar, it was possible that this Aeromonas isolate was accidentally picked from the TBX culture plates and subsequently identified as E. coli from the indole test.

Aeromonas is a common bacterium found on pork and in pork dressing plants (Singh, 1997; Yu and Palumbo, 2000; Fontes et al., 2011). Fontes et al. (2011) reported the presence of Aeromonas on 76% of pork diaphragm samples from a total of 154 samples. Gill and Jones (1995) sampled 48 skinned pork loins at two pig slaughterhouses, and Aeromonas hydrophila or Aeromonas caviae was isolated from all of the samples. High numbers of Aeromonas were also found on equipment, such as the dehairer and shackling table (Gill and Jones, 1995;

Yu and Palumbo, 2000).

With regards to the ERIC-PCR typing of Aeromonas species, Fontes et al. (2011) reported that the ERIC-PCR method worked on Aeromonas species but was unreliable as Aeromonas isolates with identical DNA sequence may give different ERIC-PCR band patterns. This explains why an ERIC profile was obtained from this isolate but the isolate had a very low similarity (5.1%S) with other isolates found.

The unexpected isolation of Aeromonas from TBX plates showed the importance of using proper measures for species confirmation. For more reliable identification of E. coli, the oxidase test was applied. Aeromonas species are oxidase positive and can easily be differentiated from E. coli by performing the oxidase test. Thus, the use of three characteristics to confirm E. coli isolation (TBX selective culturing, indole test, and oxidase test) was adopted for collecting E. coli isolates from the second visit to Slaughterhouse B in 2009 (Chapter 4) and with the visit to Slaughterhouse C in 2010 (Chapter 5).

Because of the utilisation of an Aeromonas isolate, the comparison of heat tolerance amongst the pre-singeing, post-singeing, and post-polishing isolates was compromised. While this was unfortunate and reduced the power of the study, it does not invalidate the major parts of the experiments. The pre-singeing isolate and post-singeing isolate from the same genogroup showed similar thermal resistance. This confirmed the findings with the Slaughterhouse

A isolates, and demonstrated that isolates of the same genotype have similar characteristics of heat responses. Like E. coli, it has been reported that the level of rpoS expression affected the resistance to stresses of Aeromonas strains (Zhao et al., 2007). The Aeromonas isolate found here was not able to induce rpoS at stationary phase, which explained its heat sensitivity in stationary phase. In addition, the recovery of a non-thermotolerant bacterium indirectly indicates that the bacteria recovered on the anus area after polishing may not be able to survive the heat treatments such as scalding and singeing. Those bacteria could therefore be from a site shaded from heat exposure, be from the carcass internally and being deposited by faecal leakage, or from the environment.

# CHAPTER 8 DETECTION OF VIRULENCE FACTOR-CARRYING E. COLI FROM SLAUGHTERED PIGS

## 8.1 INTRODUCTION

Pigs may be a vehicle for transmission of pathogenic E. coli strains to slaughterlines because strains carrying pathogenic determinants have been isolated from carcasses, pork cuts and the environments of slaughterhouses (Bouvet et al., 2002b; Loukiadis et al., 2006; Veilleux and Dubreuil, 2006; Xia et al., 2010). As previously discussed, a great diversity of E. coli genotypes was found amongst the isolates recovered in the pig slaughterhouses. Several genotypes were observed surviving the processing and were represented on the final products. If any virulence factor was carried by these "survival" genotypes, these pathovars might result in human illness when the meat enters the food chain. The carriage of pathogenicity genes by the E. coli isolates was therefore investigated.

In this study the carriage of virulence factor genes of E. coli isolates was investigated using PCR, as primers for the detection of a range of genes have been published. The presence of the toxins and the fimbrial genes associated with enterotoxigenic E. coli (ETEC) were examined by detection of the plasmid borne heat stable toxin gene (STI), the heat labile toxin gene (LT) and the gene for the F4 fimbriae. Strains were also examined for the presence of the intimin gene (eae) associated with enteropathogenic E. coli (EPEC) and enterohaemorrhagic E. coli (EHEC), for the verotoxin gene stx associated with Verotoxin producing E. coli (VTEC) including EHECs, and for the astA gene

which codes for a plasmid borne toxin (EAST1) associated with some enteroaggregative E. coli (EAggEC). As the toxin genes of ETEC and EAggEC are encoded on plasmids, the plasmid profiles of strains carrying ETEC or EAggEC genes were investigated.

Following initial investigations, selected isolates were subjected to IdentiBac microarray analysis, which was carried out by the Animal Health and Veterinary Laboratories Agency, Weybridge. IdentiBac is a system of DNA microarrays for the simultaneous detection of multiple genes in bacteria and viruses including subtyping, antimicrobial resistance genes, toxin- and virulence genes. In this study two DNA chips were used, including virulence factor genes and the antimicrobial resistance (AMR) genes.

## 8.2 MATERIALS AND METHODS

### 8.2.1 Presence of pathogenicity genes

The pathogenic determinant genes were examined by PCR. The sequences of the primer sets used for the virulence gene determination are listed in Appendix 8.1.

#### 8.2.1.1 Detection of ETEC virulence genes

The genes of the heat-labile enterotoxin (LT), the heat-stable enterotoxin 1 (ST1) and the F4 fimbriae were examined with a multiplex PCR method developed by Dr. Helen Davies, University of Nottingham. The PCR mixture (25 µl) contained 2.5 µl reaction buffer (10X; Thermo Scientific, UK), 2.5 µl

$MgCl_2$ (25 mM), 3 µl dNTPs mixture (10mM), 1 µl of each primer (10 pmol; MWG Eurofins, Germany), 1 u Taq DNA polymerase, 1 µl of DNA template, and sterile distilled water. The PCR reaction was carried out with one cycle for 5 min at 94°C, and then 30 cycles comprising 1 min at 94°C, 1min at 54°C, and 1min at 72°C. The final cycle was at 72°C for 7 min. The PCR products (5 µl) and the DNA marker (100 bp DNA ladder; Promega, US) were resolved on a 2% TAE agarose gel by electrophoresis (80V for 1.5h).

#### 8.2.1.2 Detection of EPEC virulence gene

For EPEC detection, a 482 bp amplicon of the eae gene was amplified (Stacyphipps et al., 1995). The PCR assay was performed in 25 µl reaction mixture containing 2.5 µl reaction buffer (10X; Thermo Scientific, UK), 200 µM dNTPs mixture, 2 pmol of the primer set (MWG Eurofins, Germany), 1.5 mM $MgCl_2$, 1 u Taq DNA polymerase, 1 µl of DNA template, and sterile distilled water. The PCR reaction was carried out with one cycle for 5 min at 94°C, and then 35 cycles comprising 1.5 min at 94°C, 1.5 min at 64°C, and 1.5 min at 72°C. The PCR products (5 µl) and the DNA marker (100 bp DNA ladder) were resolved on a 1.5% TAE agarose gel by electrophoresis (80V for 1.5 h).

#### 8.2.1.3 Detection of VTEC virulence gene

The isolates were firstly examined with a primer set (LIN3 and LIN5; MWG Eurofins, Germany) which was used for the detection of all members and variants of the verocytotoxin family, i.e. stx1c, stx1d, stx2c, stx2d, and stx2e

(Lin et al., 1993). The PCR reaction mixture (25 µl) containing 2.5 µl reaction buffer (10X), 1mM dNTPs mixture, 6 pmol of the forward and the reverse primer, 15 mM $MgCl_2$, 1u Taq DNA polymerase, 1µl of DNA template, and sterile distilled water. The PCR reaction was carried out with one cycle for 5 min at 94°C, and then 40 cycles comprising 1 min at 94°C, 1.5 min at 43°C, and 1.5 min at 72°C. The PCR products (5 µl) and the DNA marker (100 bp DNA ladder) were resolved on a 1.5% agarose gel by electrophoresis (80V for 1 h).

If isolates were stx-positive, a multiplex PCR method was used to distinguish between variants stx1 and stx2 (Cebula et al., 1995). The reaction mixture (25 µl) containing 2.5 µl reaction buffer (10X), 10 mM dNTPs mixture, 50 pmol of each primer (MWG Eurofins, Germany), 1.5 mM $MgCl_2$, 1 u Taq DNA polymerase, 1 µl of DNA template, and sterile distilled water. The PCR reaction was carried out with one cycle for 5 min at 94°C, and then 35 cycles comprising 1.5 min at 94°C, 1.5 min at 60°C, and 1.5 min at 72°C. The PCR product (5 µl) was analysed with 1.5% agarose gel electrophoresis. The stx2-positive isolates were further examined for the stx2e variant (Pohl et al., 1992). DNA samples (1 µl) were amplified in a 25 µl reaction mixture constituting 2.5 µl reaction buffer (10X), 1 mM dNTPs mixture, 10 pmol of the forward and the reverse primer, 15 mM $MgCl_2$, 1.25 u Taq DNA polymerase and sterile distilled water. The PCR was carried out with denaturation for 5 min at 94 °C, and then 30 cycles comprising 2 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The PCR products (5 µl) and the DNA marker (1kb DNA ladder; Promega, US) were resolved on a 1.5% TAE agarose gel by

electrophoresis (80V for 1 h).

8.2.1.4 Detection of EAggEC virulence gene

The presence of the plasmid borne gene, astA, was detected by a PCR assay which was performed in 25 µl reaction mixture containing 2.5 µl reaction buffer (10X), 200 µM dNTPs mixture, 4 pmol of the primer set (astAF and astAR; MWG Eurofins, Germany), 2 mM $MgCl_2$, 1 u Taq DNA polymerase, 1 µl of DNA template, and sterile distilled water (Jenkins et al., 2006). The PCR reaction was carried out with one cycle for 5 min at 94°C, and then 30 cycles comprising 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a final extension for 10 min at 72°C. The PCR products (5 µl) and the DNA marker (100 bp DNA ladder) were resolved on a 2% TAE agarose gel by electrophoresis (80V for 1.5 h).

IdentiBac DNA micro-array analysis

Crude DNA (200 ng/µl) was sent to the Animal Health and Veterinary Laboratories Agency for the IdentiBac analysis. Extracted DNA was labelled with biotin (L) and amplified in a linear multiplex PCR in an array. The biotin labelled single-stranded PCR product is then hybridised to the corresponding probes. The bound PCR product is detected using a horse-radish peroxidase – streptavidin conjugate, which converts the substrate (seramun green) into a coloured precipitate. In the data generated by IdentiBac, array signals greater than 0.5 are considered positive. Signals between 0.4 and 0.5 are ambiguous and may need further confirmation by other methods. The array image was captured and visualised with ArrayMateTM and the intensity of each probe

was recorded. The virulence genes and AMR genes examined are listed in Appendix 8.2.and Appendix 8.3, respectively.

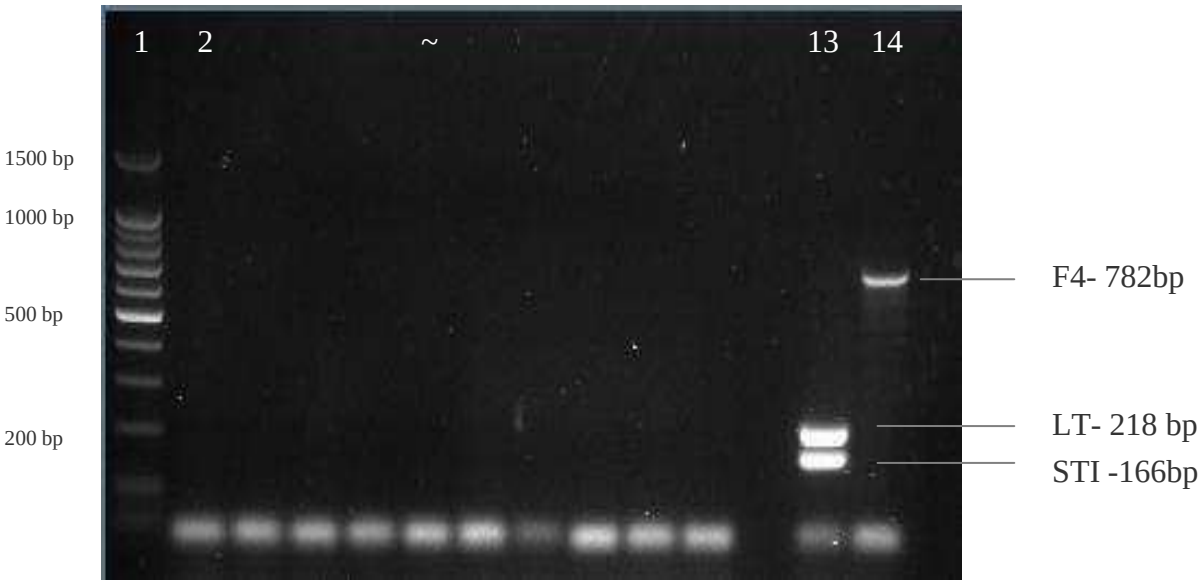
Detection of virulence plasmids

The E. coli strain for the virulence plasmid detection was inoculated in 10 ml LB broth and incubated at 37°C for 18 – 24 h. The plasmid extraction was performed as previously described in Section 7.2.3.1. The plasmid profiles were resolved by electrophoresis using 0.7% TAE agarose gel (45V for 8 h).

## 8.3 RESULTS

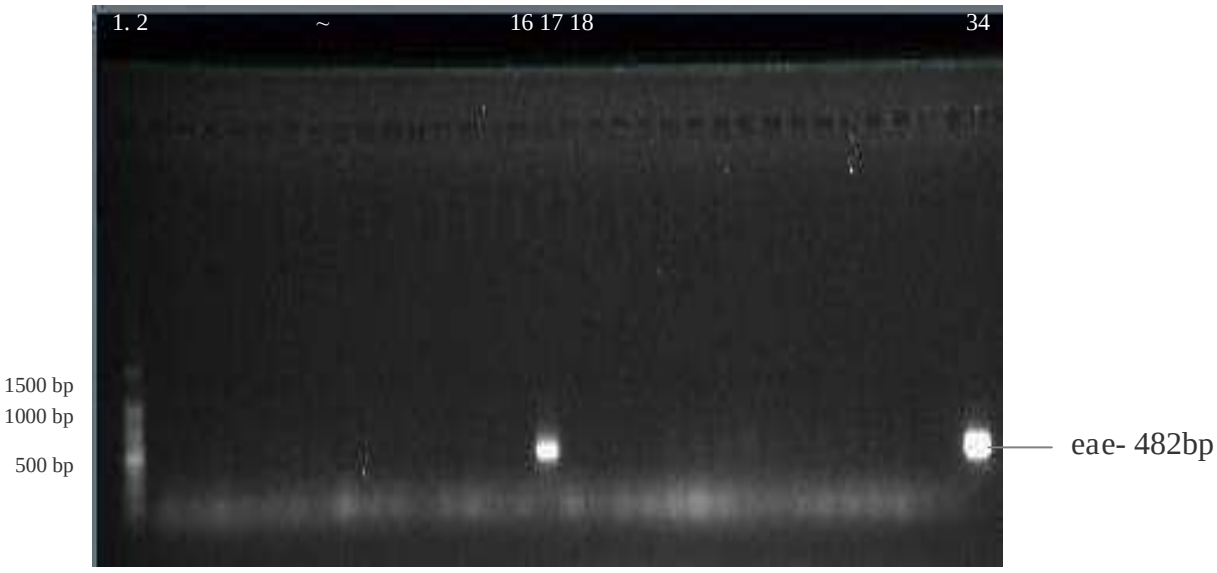
### 8.3.1 Detection of virulence determinants

A total of 1065 E. coli isolates sampled from slaughterhouses B and C, either on the carcasses at different processing stages or in gut contents of slaughtered pigs, were examined for the virulence genes associated with ETEC, EPEC/EHEC, VTEC, or EAggEC strains. Examples of the PCR detection of the virulence factors are shown in Figures 8.1 –8.4. Where the specific primer pair for the virulence factor genes yielded an amplicon of the expected size, strains were considered positive. The presence of the eae gene associated with EPEC/EHEC strains is shown in Figure 8.2. The stx genes were examined, first using a multiplex method which detected stx1 and stx2, as shown as Figure 8.3a. Further identification of the stx-positive isolates used a specific primer set for stx2e, and all stx-positive isolates were confirmed as carrying the stx2e variant gene (Figure 8.3b). The PCR detection of the gene of enterotoxin EAST1 (astA) is shown in Figure 8.4.




Lane 1: 100 bp DNA marker; lane 2 to Lane 11: pig E. coli isolates (negative for the target genes); lane 12: blank; lane 13: LT and STI positive control strain (H10407); lane 14: F4 positive control strain (Y04158).

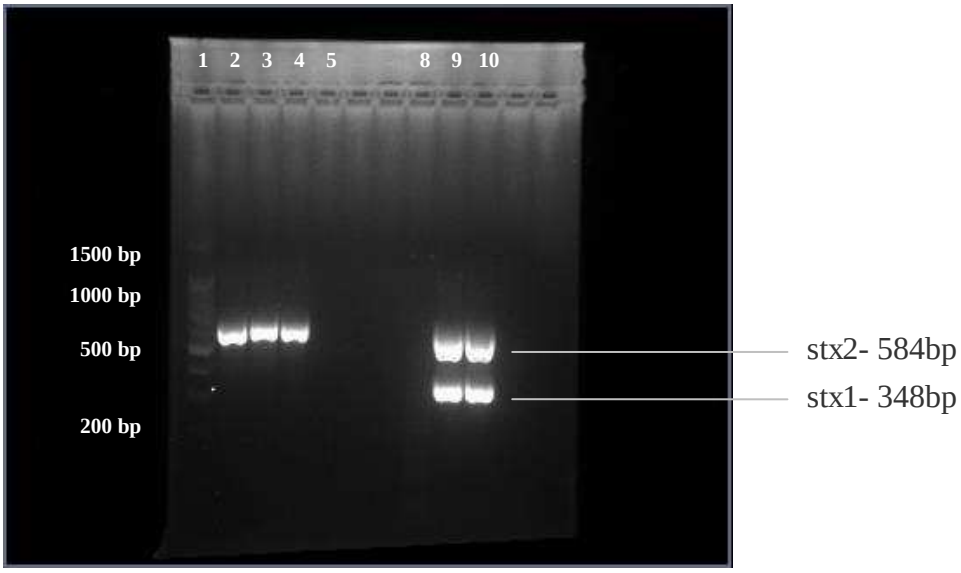
Figure 8.1. PCR amplification of F4, LT, and STI genes (2% TAE agarose gel, 80V for 1.5h).




Lane 1: 100 bp DNA marker; lane 2 to 16 and 18 to 33: pig E. coli isolates (eae-negative); lane 17: pig E. coli isolate (eae-positive); lane 34: eae positive control strain (O157, EDL933).

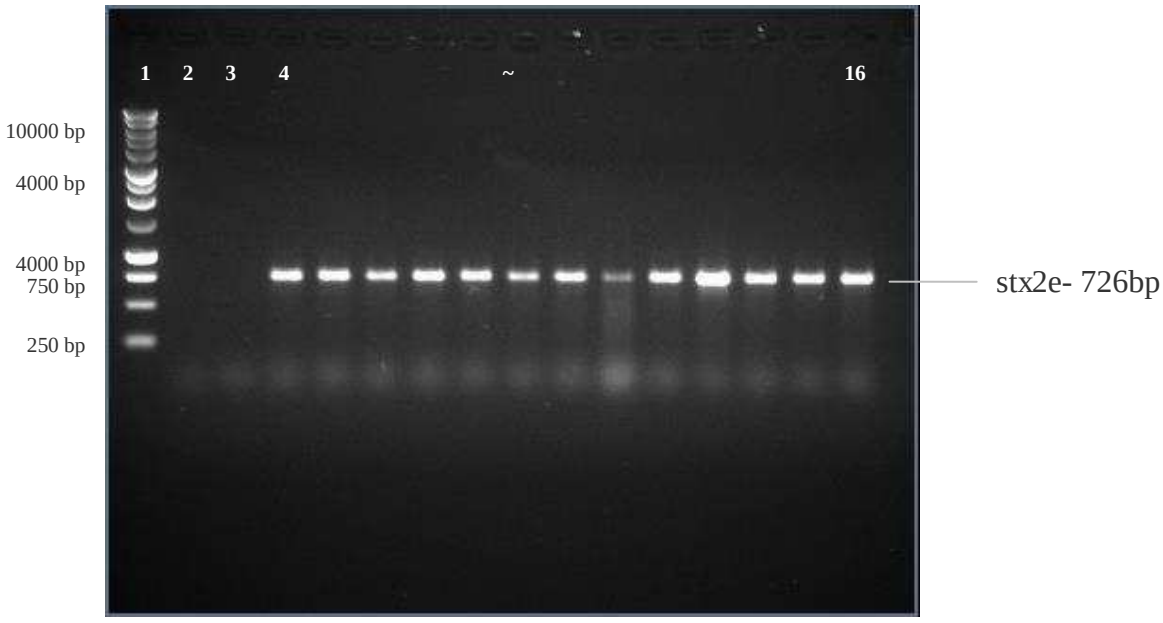
Figure 8.2. PCR amplification of eae gene (1.5% TAE agarose gel, 80V for 1.5h).

(a)



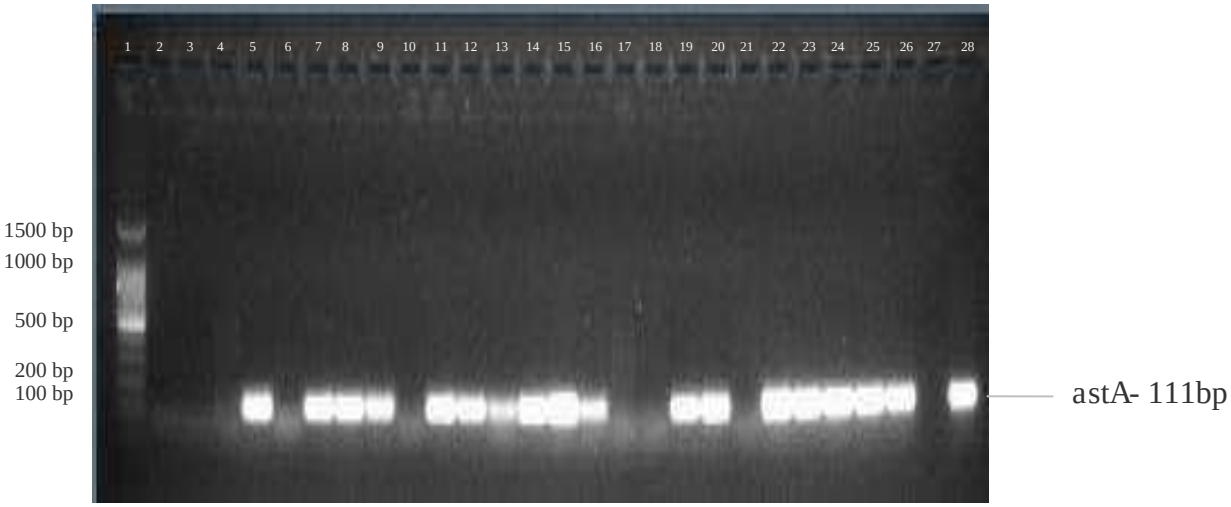

Lane 1: 100bp DNA marker; lane 2 to 4: pig E. coli isolates (stx2-positive); lane 5 to 8: pig E. coli isolates (stx-negative); lane 9 and 10 : stx1- and stx2-positive control strain (O157 EDL933)

(b)




Lane 1: 1Kbp DNA marker; lane 2 and 3,: negative control strain O157 EDL933 (stx2e-negative); lane 4 to 16:: stx-positive pig E. coli isolates (stx2e-positive)

Figure 8.3. (a) amplification of stx genes using multiplex PCR;.(b).amplification of stx2e gene (both of 1.5% TAE agarose gel, 80V for 1h).




Lane 1: 100 bp DNA marker; lane 2,3,4,6,10,17,18,21: pig E. coli isolates (astA-negative); lane 5,7-9, 11-16, 19,20,22-26: pig E. coli isolates (astA-positive); lane 28: astA positive control strain (H10407).

Figure 8.4. PCR amplification of astA gene (2% TAE agarose gel, 80V for 1.5h).

The numbers of strains in which virulence genes were detected are shown in Table 8.1. The virulence factor genes associated with ETEC, EPEC, or VTEC strains were infrequently detected whereas the astA gene associated with EAggEC was detected in 22.5% of isolates. On the basis of these characterisations, sixteen isolates carrying STI enterotoxin gene were identified as ETEC associated strains. However, none of the isolates carried LT or F4 genes. Isolates carrying the stx2 verotoxin gene and the eae gene were detected, but no isolate was harbouring both the genes, suggesting that a true EHEC strain was not present in this E. coli collection. More than one quarter of tested isolates (25.7%) carried at least one virulence gene, and 71.9% of pigs tested carried pathogenic associated E. coli strains, indicating a high prevalence of potentially pathogenic E. coli strains amongst the UK pigs.

Amongst the isolates carrying a virulence factor (n= 274), 5.7% carried two or more virulence genes (Table 8.2). The most complex combination of virulence genes, in which the isolate possessed STI, stx2e, astA, was sampled from the caecal contents of pig 5 at Slaughterhouse C. These combinations of genes indicate a great diversity of pathogenic isolates.

To confirm the correct amplicons had been found, some of the chosen amplicons were sequenced and aligned with the NCBI-nBLAST databases (Table 8.3). Each PCR amplicon acquiring a high alignment score to the target virulence gene confirmed that the correct amplicon was present. The sequencing of the eae genes also provided further identification that these

Table 8.1. Virulence profiles of E. coli isolates from slaughtered pigs.

| Pathogenicity association | Virulence factor | Positive isolates (n= 1065) | Positive pigs (n= 57) |
|---|---|---|---|
| **ETEC** | F4 | **0** | **10** (17.5%) |
| | LT | **0** | |
| | STI | **16** | |
| **VTEC/EHEC** | stx1 | **0** | **7** (12.3%) |
| | stx2 (stx2e) | **13** | |
| **EPEC/EHEC** | eae | **19** | **8** (14.0%) |
| **EAggEC** | astA | **240** | **41** ( 71.9%) |

Table 8.2. E. coli isolates carrying two or more virulence genes.

| Combination of pathogenicity | Virulence factor | Number of isolates |
|---|---|---|
| ETEC/VTEC | STI, stx2e | 2 * |
| ETEC/EAggEC | STI, astA | 1 |
| VTEC/EAggEC | stx2e, astA | 8 |
| EPEC/EAggEC | eae, astA | 2 |
| ETEC/VTEC/EAggEC | STI, stx2e, astA | 1 * |

* Selected isolate(s) in this group was further characterised by DNA array.

Table 8.3. NCBI-BLAST results confirming the sequences of PCR amplicons of the virulence determinants.

| Pathogenicity association | Virulence gene | Most significant alignment | e value* | Max identity | Number of isolates sequenced |
|---|---|---|---|---|---|
| EPEC/ EHEC | eae | EPEC2 subgroup; eae gene for intimin | 0.0 | 100% | 3 |
| VTEC/ EHEC | stx2e | E. coli stx2eA and stx2eB genes for shiga toxin 2e subunit | 0.0 | 99% | 3 |
| ETEC | ST1 | E. coli heat-stable enterotoxin I gene, complete cds | $1e^{-48}$ | 99% | 2 |
| EAggEC | astA | Heat stable enterotoxin 1 (astA) gene, complete cds | $6e^{-23}$ | 100% | 2 |

* The expected score for aligning a random pair of amino acid is required to be negative.

eae-carrying isolates tested belonged to EPEC-2 subgroup.

### 8.3.2 Genotype analysis of the virulence determinant-carrying strains

The ERIC-PCR profiles amongst the virulence gene carrying strains were re-analysed to examine genotype relatedness. In Figure 8.5, forty-five virulence factor gene carrying isolates were clustered into four distinct groups ($\Phi_{PT}$=0.419; P<0.01). The isolates clustered in Group 1 were mainly stx2e/astA combination strains. The eae carrying isolates were mainly clustered in Group 3. However, Groups 2 and 4 were two small groups which constituted a mixture of STI, eae, and stx2e isolates. It was noticed that all of the isolates in Group 1 were from Slaughterhouse C, whereas all of the isolates in Group 3 and Group 4 were from Slaughterhouse B. A mixture of both slaughterhouse strains was only seen in Group 2 (n=6) with only two isolates from Slaughterhouse B. This indicates that the isolates, even those categorised in the same pathogenic type, may be genetically distinct if recovered from different origins. It was observed that four of the stx2e/astA isolates and as STI/stx2e/astA isolate from Pig 5 sub-clustered in an identical genotype within Group 1. In Group 1, there was a non-identical STI-carried isolates also from the same pig. As the STI is carried on a plasmid, it may suggest that the latter strain has arisen by gaining the additional virulence factor on a plasmid.

### 8.3.3 Plasmid profiling of ETEC and EAggEC strains

The ST1 gene of ETEC as well as the astA gene of EAggEC are encoded on virulence plasmids. STI-encoding plasmids could range in size from 1 to over

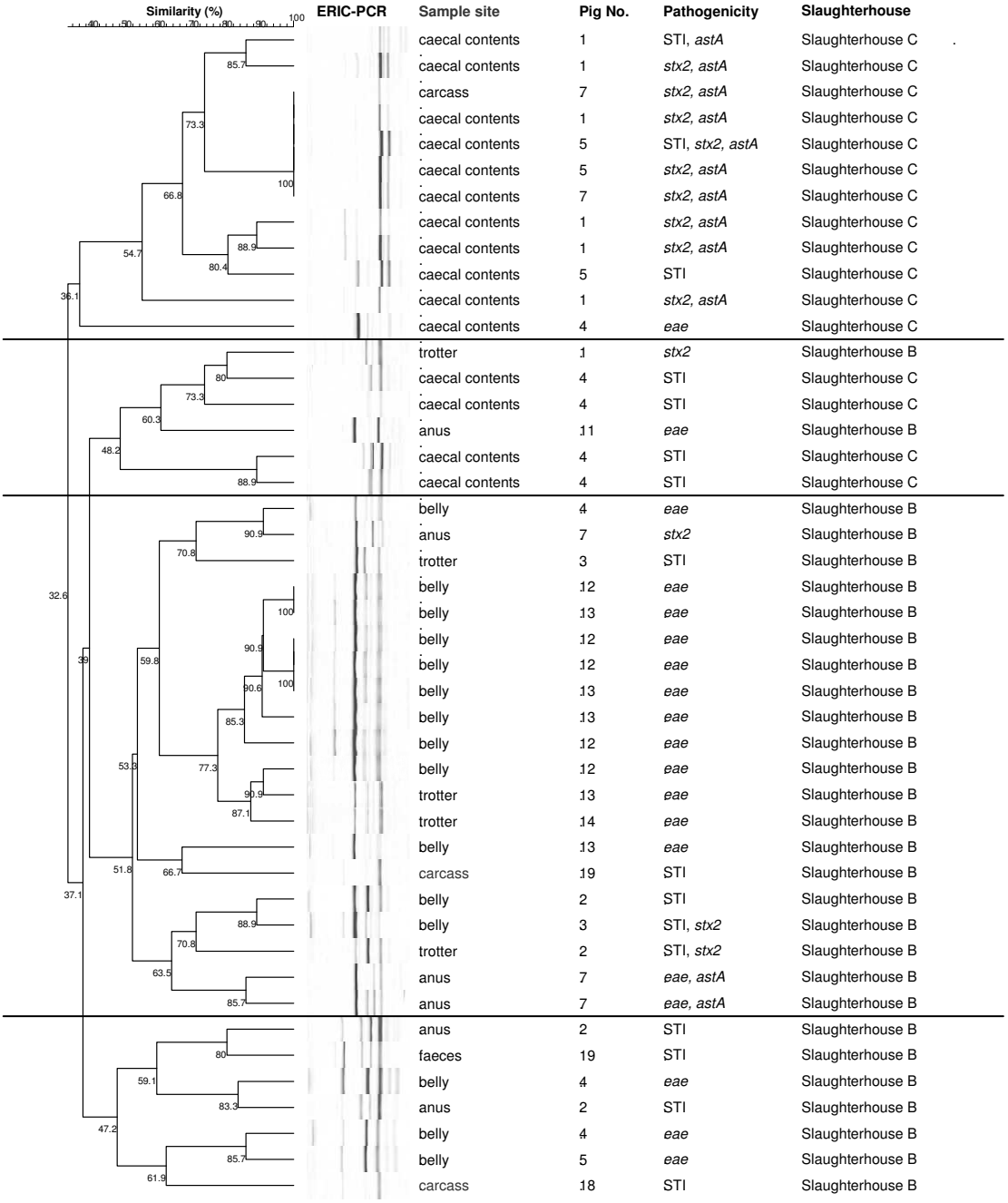


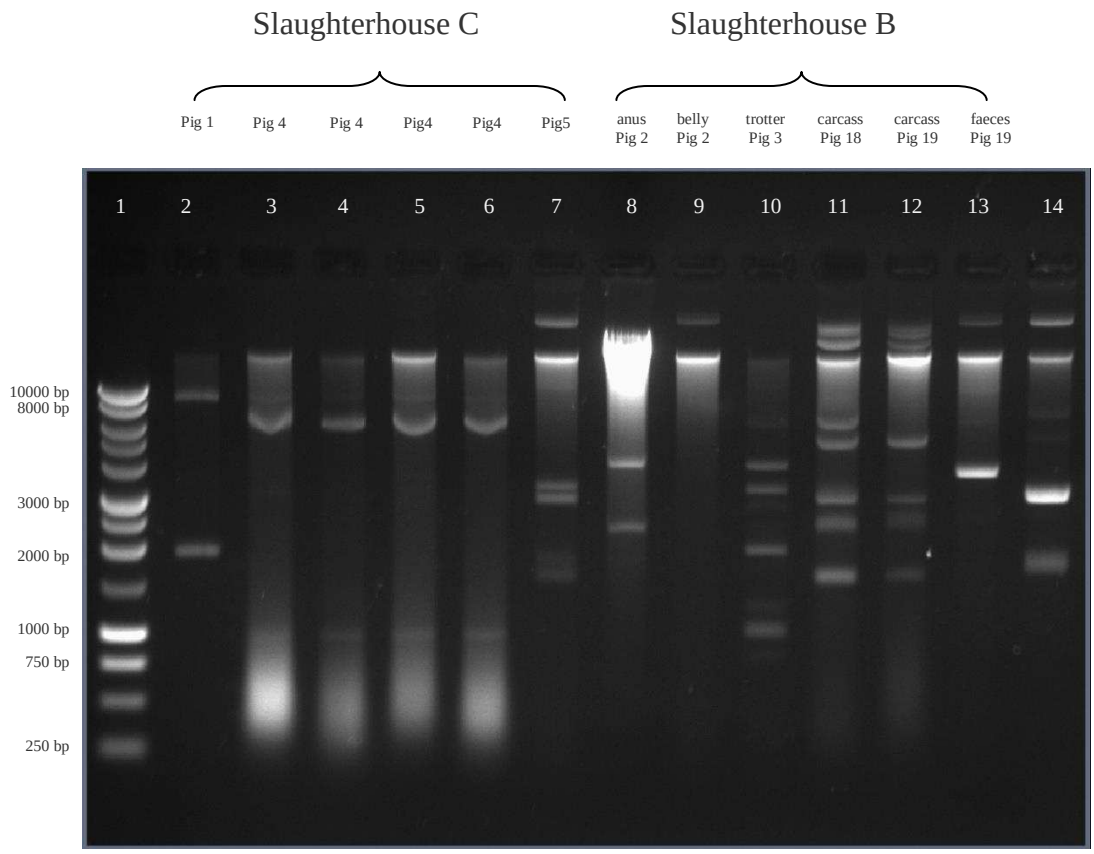

Figure 8.5. Cluster analysis (Dice coefficient; UPGMA) of the ERIC-PCR profiles of virulence gene carrying E. coli isolates (n=45).

120 kbp, and typical astA-encoding plasmids are 55 – 65 MDa (Johnson and Nolan, 2009). The plasmids of the STI gene-carrying strains, excluding the stx2 strains (n=3), were analysed (n=12). Both the sizes and the number of plasmids amongst the STI-carrying strains were variable (Figure 8.6). The STI-carrying strains from Slaughterhouse B (Groups 3 and 4; Figure 8.5) seemed to have larger sized plasmids and more complicated plasmid profiles than those from Slaughterhouse C. The four isolates from Pig 4 of Slaughterhouse C showed identical plasmid profiles. However, the ERIC-genotypes of these four isolates showed lower similarities (from 58% to 89%; Group 2, Figure 8.5). This suggests that the carriage of the plasmids may not correlate with their genomic structures. The plasmids of astA-carrying strains were also investigated (n=8). In the example shown in Figure 8.7, two astA-positive EAggEC strains isolated from the anus of Pig 7, P7-1 and P7-5, have the same plasmid profile. However, an isolate (anus P7-4) genotypically identical with anus P7-5 lacks the plasmids. This suggests that the strain the strain anus P7-5 is the same strain as P7-4 but having obtained the plasmids from a plasmid-carrying strain in the same pig (eg. a strain P7-1).

### 8.3.4 Use of DNA microarrays to detect virulence determinants and antimicrobial resistance genes

Five isolates were selected for screening using the IdentiBac microarrays (Table 8.4). The isolates 10BP1, 2BP3, and 8C7 were chosen because in a cooperative project with Dr. Jon Hobman's group using strains from this study, these strains were identified as Tn21-positive, a transposon which is related to

(a)




Lane 1: 1Kbp DNA marker; lane 2 to 7: $STI^+$ isolates from Slaughterhouse C; lane 8 to 13: $STI^+$ isolates from Slaughterhouse B; lane 14 : control strain ETEC H10407. Strains of Slaughterhouse C were isolated from caecal samples. Plasmid profiles were resolved by electrophoresis using 0.7% TAE agarose gel (45V for 8 h).

(b)

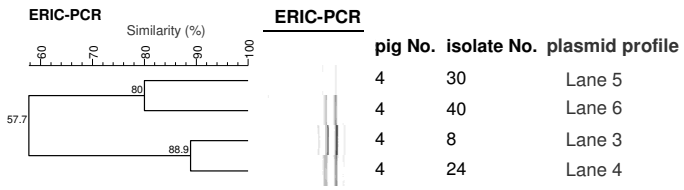
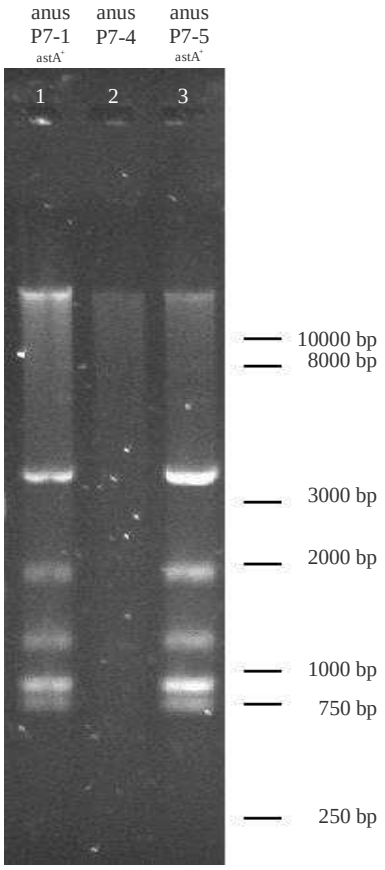



Figure 8.6. (a) Plasmid profiles of STI-carrying E. coli isolates, and (b) analysis of ERIC-PCR profiles for the four isolates from Pig 4, Slaughterhouse C.

(a)




Lane 1: Isolate 1 (astA$^+$); lane 2: Isolate 4; lane 3: Isolate 5 (astA$^+$). All isolates were from Pig 7, Slaughterhouse B. Plasmid profiles were resolved by electrophoresis using 0.7% TAE agarose gel (45V for 8 h).

(b)

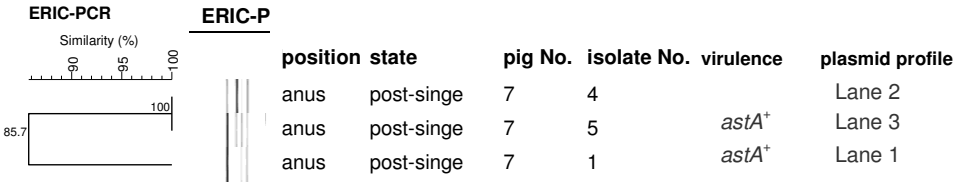



Figure 8.7. (a) Plasmid profiles of astA carrying E. coli isolates and (b) analysis of ERIC-PCR profiles for the three isolates from Pig 7, Slaughterhouse B.

the dissemination of antibiotic resistance genes in Gram-negative bacteria. The strains 2TP3 and 5G14 were chosen because of multi-virulence factors (stx2e/STI and stx2e/STI/astA, respectively) detected by PCR. For isolates 10BP1 and 5G14, astA was found by both methods. In general, most of the output from the chip analysis confirmed the PCR detection of virulence genes and also detected associated genes, such as the detection of stx2 subunit A and B in the stx2-carrying strain 10BP1, and the detection of both the STIa and STIb variants in the STI-carrying strain 2AP3 (Table 8.4). The analysis showed that the STI-carrying strain 2AP3 also carried the STII toxin gene. However, differences between these two methods were also observed. Isolate 8C7 carried the astA gene, as detected by PCR, but not by the array. On the other hand, K88 (F4 fimbrial gene) was potentially detected by the array (ambiguous) but not by PCR.

The tir gene codes for the translocated intimin receptor protein, which is essential for intimate attachment in vitro and is normally found in EPEC and EHEC as part of the LEE pathogenicity island. The tir-O111 gene was found on 2AP3 and 2TP3, which were not identified as EPEC or EHEC due to the absence of the eae gene. Thus, the reason why these are strains harbouring tir-O111 is not clear, but may suggest a defective LEE is present. Of particular interest was the finding of tir-O111 with Stx2 genes (subunit A and B detected) in the isolate 2TP3, as this could be an EHEC strain which has suffered a deletion event in the LEE region.

Table 8.4. Results for E. coli virulence and antimicrobial resistance (AMR) gene screening by DNA microarrays.

Strain 10BP1 ($astA^+$; Slaughterhouse B)

| **Virulence gene** | **Gene description** | |
|---|---|---|
| astA | EAST1 enterotoxin | positive |
| Ccl | cloacin bacteriocin plasmid | positive |
| F17-A | F17 fimbrial protein subunit A | positive |
| fedA | F107 fimbrial protein subunit A | positive |
| fedF | fimbrial adhesin precursor | positive |
| lpfA | long polar fimbriae | positive |
| toxB | toxin B plasmid | positive |
| Tsh | serine protease autotransporter protein | positive |
| IroN | enterobactin siderophore receptor protein | positive |
| Iss | increased serum survival (plasmid) | positive |
| mchF | ATP binding cassette transporter protein | positive |
| cdtB | cytolethal distending toxin B | ambiguous |
| Cma | colicin M- resembles β-lactam | ambiguous |
| **AMR gene** | **Antimicrobial resistance** | |
| aadA1 | aminoglycosides (amikacin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin, and apramycin) | positive |
| aadA2 | aminoglycosides | positive |
| cmlA1 | chloramphenicol | positive |
| intl1 | class 1 integrase | positive |
| intl2 | class 2 integrase | positive |
| sul3 | sulphonamides | positive |
| tetB | tetracycline | positive |
| tem1 | β-lactam antibiotics (penicillins, cephamycins, and carbapenems) | positive |
| tetA | tetracycline | ambiguous |
| ereB | erythromycin | ambiguous |
| oxa2 | β-lactam antibiotics | ambiguous |
| blaCMY | ampicillin and β-lactam antibiotics | ambiguous |

Table 8.4. continued.

Strain 8C7 (astA$^{+}$; Slaughterhouse C)

| **Virulence gene** | **Gene description** | |
|---|---|---|
| cdtB | cytolethal distending toxin B | positive |
| F17-A | F17 fimbrial protein subunit A | positive |
| lpfA | long polar fimbriae | positive |
| Tsh | serine protease autotransporter protein | positive |
| IroN | enterobactin siderophore receptor protein | positive |
| Iss | increased serum survival (plasmid) | positive |
| mchF | ATP binding cassette transporter protein | positive |
| mchB | microcin H47 synthesis gene | ambiguous |
| Ccl | cloacin bacteriocin plasmid | ambiguous |
| virF | transcriptional activator | ambiguous |
| nleC | non-LEE encoded effector C | ambiguous |
| K88 | F4 fimbrial subunit gene | ambiguous |
| **AMR gene** | **Antimicrobial resistance** | |
| aadA1 | aminoglycosides | positive |
| aadA2 | aminoglycosides | positive |
| catIII | chloramphenicol | positive |
| cmlA1 | chloramphenicol | positive |
| Intl1 | class 1 integrase | positive |
| sul2 | sulphonamides | positive |
| sul3 | sulphonamides | positive |
| tem1 | β-lactam antibiotics | positive |
| tetA | tetracycline | positive |
| tetB | tetracycline | positive |
| ereB | erythromycin | positive |

Table 8.4. continued.

Strain 2AP3 ($STI^+$; Slaughterhouse B)

| **Virulence gene** | **Gene description** | |
|---|---|---|
| cba | colicin B- pore forming gene | positive |
| Ccl | cloacin bacteriocin plasmid | positive |
| Cma | colicin M- resembles β-lactam | positive |
| stb | heat stable enterotoxin II | positive |
| sta1 | heat-stable enterotoxin ST-Ia | positive |
| sta2 | heat-stable enterotoxin ST Ib | positive |
| tir O111 | translocated intimin receptor protein | positive |
| **AMR gene** | **Antimicrobial resistance** | |
| aadA1 | aminoglycosides | positive |
| aadA2 | aminoglycosides | positive |
| cmlA1 | chloramphenicol | positive |
| intl1 | class 1 integrase | positive |
| sul3 | sulphonamides | positive |
| tetB | tetracycline | positive |
| vatE | aminoglycosides | ambiguous |

Table 8.4. continued.

Strain 2TP3 (stx2e$^{+}$/STI$^{+}$; Slaughterhouse B)

| **Virulence gene** | **Gene description** | |
|---|---|---|
| cba | colicin B- pore forming gene | positive |
| cdtB | cytolethal distending toxin B | positive |
| Cma | colicin M- resembles β-lactam | positive |
| stxA2 | shiga toxin 2 subunit A | positive |
| stxB2 | shiga toxin 2 subunit B | positive |
| tirO111 | translocated intimin receptor protein | positive |
| sta1 | heat-stable enterotoxin ST-Ia | positive |
| toxB | toxin B plasmid | positive |
| lpfA | long polar fimbriae | ambiguous |
| **AMR gene** | **Antimicrobial resistance** | |
| aac6 | aminoglycosides | positive |
| tetA | tetracycline | positive |
| tetE | tetracycline | positive |
| sul2 | sulphonamides | positive |
| ermB | erythromycin | ambiguous |
| oxa2 | β-lactam antibiotics | ambiguous |

Strain 5G14 (stx2e$^{+}$/STI$^{+}$/ astA$^{+}$; Slaughterhouse C)

| **Virulence gene** | **Gene description** | |
|---|---|---|
| stb | heat stable enterotoxin II | positive |
| sta1 | heat-stable enterotoxin ST-Ia | positive |
| stxA2 | shiga toxin 2 subunit A | positive |
| Iss | increased serum survival (plasmid) | ambiguous |
| astA | EAST1 enterotoxin | ambiguous |
| **AMR gene** | **Antimicrobial resistance** | |
| aadA1 | aminoglycosides | positive |
| tetA | tetracycline | positive |
| dfrA14 | trimethoprim | positive |
| intl1 | class 1 integrase | ambiguous |
| sul1 | sulphonamides | ambiguous |

All of the isolates tested harboured AMR genes and the numbers of AMR genes carried by individual isolates ranged from 5 to 12. Two isolates (2AP3 and 10BP1) from Slaughterhouse B were carrying six AMR genes in common (aadA1, aadA2, cmlA1, intl1, sul3, and tetB) whereas 2TP3 was not carrying any of these genes. The results indicated that all of these E. coli strains could be resistant to certain antibiotics, and the carriage of AMR genes differs from pig to pig.

## 8.4 DISCUSSION

In the present study the virulence determinants associated with ETEC, EPEC, VTEC, and EAggEC pathotypes were identified in the porcine E. coli isolates using PCR methods. The fact that about a quarter (27%) of all of the isolates (n=1065) were carrying virulence factors, and that neither of the two slaughterhouses were free from any pathotypes tested for, may suggest that the prevalence of pathogenic E. coli is a common problem in UK slaughterhouses. Furthermore, only 5 out of the 57 sampled pigs yielded isolates without virulence genes suggesting a high prevalence of the virulence determinants screened for in this study within healthy slaughtered pig herds.

The most common virulence determinant was astA. Although different detection methods may be applied, it has been reported that the prevalence of the EAST1 toxin gene in E. coli isolated from diarrhoeal pigs was high, ranging from 22.7% to 79.7% (Choi et al., 2001; Frydendahl, 2002; Veilleux and Dubreuil, 2006; Wang et al., 2010). However, in the present study the

reason for astA being relatively common on the carcasses of healthy pigs remains unclear. One possible explanation could be that the presence of EAST1 toxin alone may cause no symptoms in the colonised pigs (Zajacova et al., 2012). Therefore, the astA-carrying strains may persist and spread in the pig herds without detection and then enter the slaughterlines.

In the dendrogram of ERIC-types, distribution of the virulence-factor carrying isolates strongly reflected the slaughterhouse source. Some genetic characters of the bacterial isolates from farm animals could be geographically associated (Hoelzer et al., 2010). This can be seen with the stx2 carrying isolates. Although the same stx2 gene was detected amongst all the stx-carrying strains, the stx2 carrying isolates from slaughterhouse C were clustered in one group (Figure 8.5), and also harboured the astA gene, which was not seen in slaughterhouse B strains in different genogroups. The results suggest different farms or slaughterhouses may have their own predominant pathotypes.

The stx2 is usually carried on a prophage integrated into the chromosome. It is expected that all these stx2e-positive strains within a herd are the same genotype (such as Slaughterhouse C strains). Finding a variety in one herd therefore suggests the prophage has been acquired by different strains.

The LT, STI, and F4 genes are important ETEC virulence factors which are associated with strains which cause porcine post-weaning diarrhoea (Nagy et al., 1990; Nagy and Fekete, 1999; Do et al., 2005). In an Australian study of porcine diarrhoeal ETEC, the strains harbouring F4/STI/STII/LT with serotype

O149 were most prevalent in neonatal, under 3 week old, and weaned pigs (Do et al., 2005). In the present study, only the STI gene was detected amongst the collection of pig isolates. Because pigs being subjected to slaughter are supposed to be healthy and free from diarrhoea, the major diarrhoeal factor genes may not be found frequently. Besides, the adhesive attributes of F4 are typically associated with the gene for LT production (Nagy et al., 1997), so the absence of F4 correlates with the absence of the LT gene. One explanation may be that the STI toxin gene is located on a plasmid and is part of a transposon, Tn1681 (So et al., 1980). These features would allow transfer of this toxin gene between strains. A non-ETEC strain may obtain the STI gene through conjugative transfer of plasmids or other routes and then becomes an STI carrying strain. The high genotypic variety of the 16 STI carrying isolates (Figure 8.5) also supports this point of view.

Nineteen eae-carrying isolates were detected and most of them were recovered from Slaughterhouse B. The presence of the eae gene is typical of EPEC strains but also of EHEC strains which in combination with the presence of the Verotoxin gene cause severe bloody diarrhoea. However, the eae-positive isolates collected in the present study did not harbour verotoxin genes, suggesting the eae carrying strains found were not EHEC but EPEC strains. The sequence alignment results of the eae gene (Table 8.3) suggested that the three strains tested belonged to EPEC-2 subgroup. Whittam and McGraw (1996) conducted a genetic study to classify a collection of strains of EPEC and EHEC based on multilocus enzyme electrophoresis. According to their identification, EPEC-1 is composed of serotypes with the H6 and H34 antigens,

and EPEC-2 of serotypes possessing H2 antigen. The EPEC-2 subgroup also carried the Beta type of intimin (Trabulsi et al., 2002). The Beta type of intimin does not belong to serogroups frequently associated with EHEC strains and are probably less pathogenic for humans (Loukiadis et al., 2006). If the pig strains of the present study belong to the EPEC-2 subgroup and are H-antigen 2 serotype and carrying the Beta type intimin, then they may be less frequently associated with severe diseases. Using IdentiBac, two isolates carrying the tir- O111 but not carrying eae gene were noted, suggesting that these strains have a defective or truncated LEE island.

Bovet et al. (2002) suggested that the potential danger of pork consumption to public health was low since, although 15% of carcass and pork samples were PCR-positive for stx genes, none of these isolates harboured other VTEC/EHEC virulence genes such as eae, ehx and uidA genes. In addition, a high percentage (89%) of the stx positive isolates carried the variant stx2e. The variant stx2e-carrying E. coli is recognised as a pig pathogen which only causes mild diarrhoea or asymptomatic infections in humans, due to the specific adherence of the toxin to pig intestinal epithelial cells (Sonntag et al., 2005; Bielaszewska et al., 2006; Zweifel et al., 2006). Although stx2e was the only verotoxin variant detected in the present study, the overall results did not suggest these stx2e carrying strains were safe for humans. The recent outbreak in Germany associated with bean sprouts has shown that combinations of verotoxin and other virulence genes can give rise to pathovars giving severe disease (Bielaszewska et al., 2011). In that case, combinations of different virulence genes might produce new pathovars, as seen in this study.

The stx/astA was the most frequent pathotype combination found in this study because all of the stx2 carrying isolates found in Slaughterhouse C also harboured astA. Since astA is a plasmid-coded gene, it is possible that those VTEC strains obtain the astA gene via plasmid transfer (Lopes et al., 2005). In a similar way, the combinations of eae/astA, STI/astA, stx/STI, and STI/stx/astA may be results of plasmid transfer too. Plasmid transfer appeared to be evident with the genotyping analysis. In Group1 (Fig. 8.5), the STI/stx/astA isolate from Pig 5 showed high similarity to other stx/astA strains also from Pig 5. Within the same group, an STI/astA strain was also isolated from the same pig suggesting plasmid transfer between strains.

Some combinations of E. coli virulence determinants have been reported in other studies. Fekete et al. (2003) detected ETEC/VTEC from weaned pigs, which possessed STII, stx2e, and F18 genes. Cheng et al. (2005) isolated ETEC/VTEC strains from diarrhoeal weaned pigs which harboured enterotoxin genes and stx2e. In a Korean study a complex combination of a ETEC/VTEC/EPEC/EAggEC isolated from diarrhoeal piglets was observed, which harboured F18/eae/LT/STI/STII/stx2e genes (Kim et al., 2010). These combinations suggest that the evolution of pathogenic E. coli is happening within livestock and new pathovars are constantly produced. In examining the plasmids of the STI- and astA-carrying strains in the present study, it was found that strains could have the same plasmid profile but different genotypes, or different plasmid profiles with the same genotypes. This demonstrates the mobility of plasmids between strains and conjugative plasmid transfer is

associated with the change of pathovars (Lopes et al., 2005; Stecher et al., 2012).

Analysis using the IdentiBac microarray gave more detailed results relating to the virulence and AMR of E. coli strains because it screens for a total of 126 virulence genes and 72 antimicrobial resistance genes. The DNA microarray gave a confirmation of the PCR results found of the key virulence genes, and also demonstrated these pathogenic strains harboured other virulence genes which had not been examined in this study. The results also demonstrated some AMR genes frequently detected in the porcine E. coli strains. All of isolates tested (n=5) possessed AMR genes which would give resistance to tetracycline and aminoglycosides, two of the antibiotics frequently used in pigs in the UK (DEFRA, 2011a), suggesting that antimicrobial resistance may reflect the use of antimicrobial agents in pig production (Hammerum and Heuer, 2009).

Self-transmissible DNA elements such as plasmids, transposons, integrons, and bacteriophage, can facilitate the acquisition and dissemination of antibiotic resistance genes (Nagachinta and Chen, 2009). In a parallel study of metal resistance of animal strains of E. coli conducted by Dr. Jon Hobman in the University of Nottingham, the transposon Tn21 was found on the isolates 10BP1, 2BP3, and 8C7. Tn21 is related to the dissemination of mercury resistance, and carried In2, an integron primarily responsible for resistance to several classes of antibiotics in Gram-negative bacteria (Liebert et al., 1999; Carattoli, 2001). The results confirmed that Tn21-positive strains harboured several relevant AMR genes (aadA1, aadA2, cmlA1, intl1, and sul3) associated

with this transposon.

Since these AMR strains were obtained in different slaughterhouses, the prevalence of the multi-antibiotic resistant strains could be common in the UK. Enteric bacteria (such as E. coli, Salmonella, and Campylobacter) found resistant to antimicrobials were present on a high proportion of UK pigs (Taylor et al., 2008; Miller et al., 2011) The major concern regarding anti-microbial resistant E. coli carriage by pigs is the impact on public health (Hammerum and Heuer, 2009). In a study in Denmark, resistance in E. coli isolates from pigs was strongly and significantly correlated with resistance in isolates from humans, especially for ampicillin, aminoglycosides, and fluoroquinolone resistance (Vieira et al., 2011).

In conclusion, the results demonstrated the presence of a number of virulence genes in porcine E. coli isolates from healthy UK pigs. Although most of the virulence factor-carrying strains are unlikely to cause severe human diseases, healthy slaughtered pigs cannot be excluded as a potential source of human infection with these pathogenic E. coli. Mixed types of virulence genes suggest that conjugative plasmids associated with ETEC or EAggEC strains can be transferred within the population of E. coli within the pig. Although it is not possible to trace back the origin of the AMR strain-carrying pigs, the results still suggests that the control of antibiotic usage on pig farms may require more effort.

# CHAPTER 9 GENERAL DISCUSSION

It is unavoidable that microbial contamination occurs at different stages during the slaughter and dressing process. The procedures involved in the slaughter of pigs for meat provides an opportunity for bacteria to contaminate the carcass surface. The detection and quantification of microorganisms during slaughter operations is an important aspect in ensuring pork safety. Bacterial numbers on the carcass surface can serve as a monitor of hygienic practice (Roberts et al., 1980). The numbers of bacteria can be assessed using different media and incubation temperatures in order to present a general bacteriological index or the potential presence of presumptive pathogens (Ingram and Roberts, 1976; Roberts et al., 1980). The different bacterial counts examined provide comparable trends between different stages of the process as well as on different sites on the carcass. With the introduction of HACCP, there has been a move away from routine counting but counts are still useful in understanding the process and its problems. Across the slaughter processes, the TACs and ECCs showed relatively high bacterial contamination at the beginning (post-bleeding and pre-scalding) with subsequent reduction of contamination by the scalding and the singeing processes, and an increase of contamination after the polishing process. These base-line data provide the slaughterhouses with an overview of bacterial levels over their production lines, and are useful for monitoring and enhancing the control of CPs or CCPs. The TACs and ECCs also reflected the relative effectiveness of the singe operation on different sites on the carcass. This results suggest that bacterial counts do reflect the microbial population changes which occur within the slaughter

process, and can still provide valuable data to improve the control of contamination.

TBX agar was chosen in the present study as E. coli O157:H7 does not give typical colonies on this medium because it is ß-glucuronidase negative. This suggested that an ACDP category 3 E. coli strain would not be selected from it. However, the virulence gene examination results showed that VTEC strains (stx-carrying) were isolated from this medium. Hence, the assumption that TBX agar would avoid the selection of more serious pathogenic types is not correct. Moreover, the fact that one isolate turned out to be an Aeromonas species demonstrated that isolation of E. coli could not depend only on the colony characteristics on this selective medium, and that additional confirmation testings were needed.

In the present work, further study of many of the E. coli isolates could not be carried out once they were established to be carrying stx gene as ACDP3 facilities were not available. It would be of interest to establish the serotype of the strains to see if they are known VTEC serovars. Moreover, since only isolates from typical E. coli colonies on TBX agar were selected and further characterised for the pathogenicity in the present work, E. coli O157 strains were excluded at an early step due to their lack of beta-glucuronidase. This suggests that use of an alternative isolation procedure might increase the number of virulence factor carrying isolates. Using an alternative isolation method to include O157 and other beta glucuronidase-negative strains may provide data which more closely demonstrated the real prevalence of the

pathogenic strains.

Frequent detection of E. coli contamination on carcasses along the slaughter line and the use of this organism as an indicator of enteric pathogens made E. coli the primary microorganism investigated in the present study. Both counts and strain characterisation were used for the analysis of E. coli populations following different slaughter processes. However, the strain characterisation could give detailed changes of the flora which were not seen in the enumeration results. Enumerating total aerobic and specified organism counts from food samples is a typical method to determine the microflora (Tebbutt, 2007). However, this does not provide sufficient data to elucidate the origins of pathogens (Warriner et al., 2002). Counts of indicator organisms would demonstrate the gross changes throughout the processing line but not the subtle flora changes as revealed by applying molecular typing (Warriner et al., 2002). The genotyping analysis using ERIC-PCR revealed that sometimes the diversity of the flora decreased when the counts just reduced slightly, and when counts were significantly reduced the flora composition did not necessarily change substantially. This demonstrates that changes in numbers may not truly reflect the full impact on the community of processing events, and this impact needs the detailed understanding of flora changes which molecular typing techniques allow.

The ERIC-PCR genotyping data in the present study suggested that a high diversity of E. coli types can be found on pig carcasses during slaughter. The findings corroborate previous studies which reported similar high diversities of

E. coli genotypes throughout the slaughter process (Warriner et al., 2002; Namvar and Warriner, 2006). The diversity of E. coli types may indicate that the population of various types carried by the carcasses are mixed at given processing stages, and provides evidence of carcass-to-carcass cross-contamination.

E. coli has been widely used as a measure of the hygienic characteristics of red meat production with many studies simply applying the detection or the numbers of E. coli to indicate faecal contamination and therefore process safety (Jordan et al., 2007; Delhalle et al., 2008; Antic et al., 2010; Yuan et al., 2010). Despite the application of HACCP systems at slaughter and during processing, Salmonella contamination is still a significant biological hazard associated with pork products (Letellier et al., 2009). E. coli is used as an indicator for enteric pathogens (including Salmonella) because the direct identification of cross-contamination with such pathogens is difficult due to their low numbers and sporadic occurrence during slaughter. However, there are little objective data showing the relationship between detection of E. coli and these pathogens. Ghafir et al. (2008) suggested that E. coli may be considered as a good indicator for enteric zoonotic agents such as Salmonella for beef, pork, and poultry samples based on the bacterial monitoring data of slaughterhouses from the official Belgian surveillance plan from 2000 to 2003. In the present study, a significant positive correlation was found between the number of E. coli on the samples and the detection of Salmonella during the slaughter process. Moreover, the genotyping results revealed a similar trend of diversity changes between both microorganisms. These results provide sound and direct evidence

that E. coli is a competent indicator for Salmonella.

Due to the extremely high temperature used, singeing is the last operation, after scalding, that actually reduces microbiological contamination (James et al., 2007). The reduction of bacterial contamination by the singeing process has been reported in many studies (Gill and Bryant, 1992; Berends et al., 1996; Berends et al., 1997; Spescha et al., 2006). However, the uniformity of the singeing process has been considered less often. Pearce et al. (2004) investigated bacterial contamination during pork production using sampling at three sites (ham, belly and neck). In their results the coliform counts after singeing showed similar levels of reduction at all three sites when compared with after dehairing. However, this does not mean that the singeing process is uniform. Tinker et al. (2007) observed that the temperature around the trotter and the anus area dropped quickly post-singeing suggesting that variations in thermal stress occurred during the process. Based on the variations detected using thermal imaging, the present results did show differences in level of microbial reduction between designated "cold-spot" and "hot-spot" sites. The relatively high ECC of the anus area post-singeing suggested that singeing may be less effective in this area. The genotyping results supported this point of view, and the finding that several genotypes were shared by pre-singeing and post-singeing carcass populations also suggested the existence of strains which were surviving the process.

Although singeing operations show a strong bactericidal effect, the polishing process re-introduces contamination to the carcasses. An increase in levels of

contamination after polishing was seen in several parts of the present study. Additionally, the fact that genotypes found on the polisher and the polished carcasses were the same indicated cross-contamination occurred during this process. Previously unseen genotypes were frequently found post-polishing suggesting that new sources of contamination were introduced at this stage. The present work has shown possible sources of the new contaminants. The failure to clean the equipment is one source as the strains isolated at start of the day were later found on the carcasses. Berends et al. (1997) inferred that following the singeing process, bacteria hidden in folds, orifices or hair follicles may be spread in the subsequent polishing operation. The survival of isolates through singeing in the "cold-spot" supports this point of view. Faecal leakage is another possible source of contamination. In the present study the genotypic correlation between E. coli isolates recovered from caecal contents and from the polisher and the final carcasses indicated that faecal leakage is one of the major sources of contamination.

In the slaughterhouses, several places could be contaminated from the pig GI tract: pens where pigs are kept before slaughtering, the scalding tank, knives, and workers hands (Malakauskas et al., 2006). It is possible that pigs carry the contaminants into the slaughterline during production. In the present study it was noticed that on the individual pig carcass, the caecal genotypes from the pig itself did not comprise the majority of the genotypes found on the final carcass. More genotypes found on the final carcass originated from other carcasses than the pig's own caecal flora. These results indicated that cross-contamination occurred more frequently than self-contamination through

the slaughter process.

Processing operations such as dehairing and polishing may generate aerosols. Pearce et al. (2006) indicated that the air within an abattoir contained organisms such as Salmonella and E. coli and suggested that air may be an important source of carcass contamination. Yuan et al. (2010) genotyped E. coli in the aerosols collected in pig houses and found that identical E. coli types could be recovered in aerosols, faeces, and the surroundings of these areas, suggesting an airborne contamination. Therefore, studies in the future to examine carcasses with additional sampling of the slaughter environment and the aerosols may be helpful to track further contamination sources and to allow the design of effective measures to eliminate bacterial dissemination.

Thermal inactivation is the main measure to control bacterial contamination within the pig slaughterline. The interventions employing heat (such as scalding, singeing, steaming, and hot water spraying) stress can eventually kill the bacteria on the carcasses (Williams and Ingham, 1998). However, the survival of some genotypes was observed in the present study. The investigation of the heat tolerance of the porcine E. coli isolates was to examine the relationship between the heat-resistance and the genotypic characteristics. The results revealed that the E. coli isolates which had similar ERIC profiles demonstrated a similar heat tolerance whether they were recovered before or after the heat-related stages. It provided evidence that some strains are sufficiently heat tolerant to survive the slaughterline processing, and also explained the presence of identical strains found before and after the

scalding or the singeing processes (if present on a “cold spot”). Study of RpoS induction suggested that the expression of the rpoS gene may play an important role in the survival of these porcine E. coli strains in slaughterhouses. Growth phase may therefore be one selective effect for strains surviving on carcasses since the stress tolerance caused by RpoS expression is normally present whilst cells are in stationary phase.

Studying both commensal and pathogenic E. coli is important to gain a better understanding of their ecological niches (Bettelheim et al., 2005). The carriage of pathogenicity factors of the porcine E. coli isolates was therefore examined. Pigs are a vehicle for pathogenic E. coli strains since several virulence factor-positive strains have been isolated from pork, and some pork products involved in human infections (Bouvet et al., 2002b; Jakobsen et al., 2010; Xia et al., 2011). The presence of virulence factor genes in strains from the pig carcasses and the environmental samples simply indicates contamination by potentially pathogenic E. coli strains during slaughter and not necessarily the expression of these genes. In the present study a surprisingly high percentage of E. coli were carrying virulence factors. Since samples were taken from different slaughterhouses and therefore from different pig farms, it suggests a high prevalence of pathogenic E. coli. Although isolation of the virulence factor gene carrying strains does not indicate that the products of the slaughtered pigs will lead to an outbreak, it suggests that current risk control systems require more effort for setting an effective hygiene strategy.

The most frequently detected virulence factors were astA, a plasmid-encoded

enterotoxin gene, suggesting that this gene may frequently transfer between E. coli strains. The heat-stable enterotoxin gene and the antimicrobial resistance genes were plasmid-coded and could therefore be horizontally transferred (Yamamoto and Yokota, 1983; Lopes et al., 2005; Nagachinta and Chen, 2009). These transferable virulence factors produced different combinations of pathovars. As evidenced by the new combination of virulence factors in EHEC O104 which caused a major outbreak in Germany in the Spring of 2011, the recombination of virulence factors produces diverse pathotypes and may increase strain pathogenicity (Aurass et al., 2011; Bielaszewska et al., 2011). An isolate carrying genes associated with ETEC/VTEC/EAggEC found suggested that gene transfer is common within the porcine flora and the monitoring and control of these new types will require considerable effort.

In summary the present work highlights possible sources of microbiological contamination (such as the equipment and faecal leakage) and helped identify insufficient performance of particular operations (scalding and singeing) in pork slaughter processing. The contamination levels as well as the bacterial communities were constantly changing during processing. The results in the present study have demonstrated that the singeing process was not equally effective over the whole length of a carcass; the polishing process provided considerable opportunity for cross-contamination for strains from various sources in the slaughterline. The heat-tolerant genotypes could survive the heat-involved processes in a slaughterline, and may be selected for survival onto the final carcass. Salmonella and pathogenic E. coli were present on the final carcasses demonstrating that the employment of HACCP does not lead to

production of totally safe meat. Finally, the study reinforces the need to study bacterial contamination in depth and demonstrates the value of molecular typing of strains for this purpose.

# APPENDIX

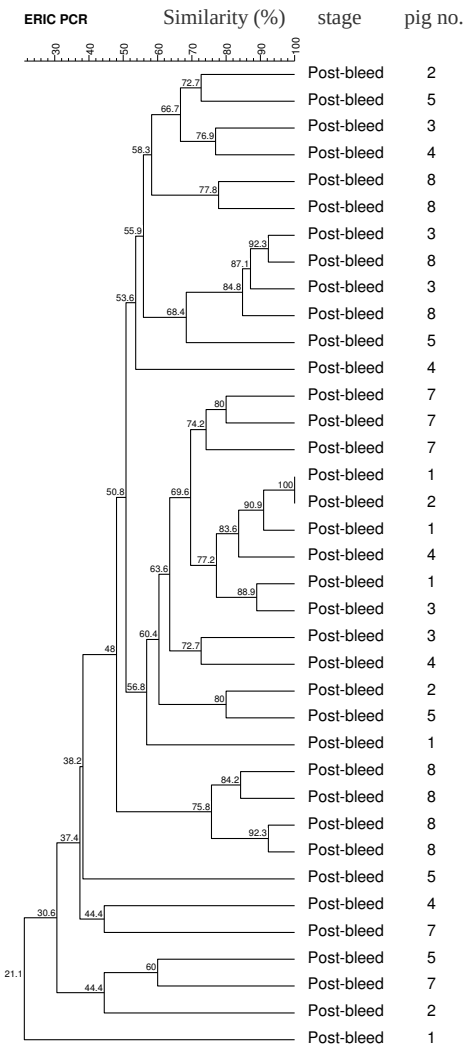
## Appendix 3.1 Process line of Slaughterhouse A

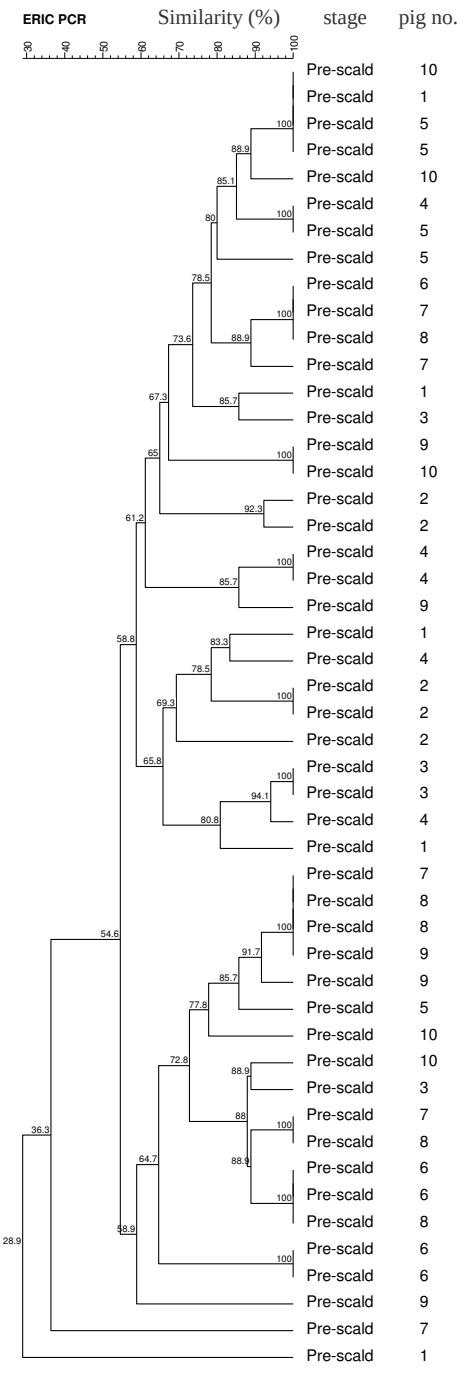
| Abattoir Process | Notes |
|---|---|
| **1. Lairage** | Estimate 2.5m x 10m pens, no bedding, nipple drinkers, sprinklers. Pressure hose cleaning as feasible during production. Deep cleaning when empty. |
| **2. Stunning** | CO2 stunner with 2-3 pigs to a cradle approx.75s cycle delivering 2-3pigs every 26s to single leg chain hang attachment table. Electric stun as backup. |
| **3. Sticking** | - |
| **4. Bleeding** | 11.5s between carcasses. |
| **5. Scalding** | Vertical condensing steam scalding modules. |
| **6. Dehairing** | 5m long spiral type dehairer. Outside reared, and winter pigs are more difficult to dehair. Carcass surface temperatures at exit 39.5-41.4°C (3 measurements). Pigs arrive at gambrelling table at 9.5, 10.3, 14.5s intervals measured. |
| **7. Gambrel** | - |
| **8. Ultrasonic back fat grader** | Pull through ultrasonic back fat grader (SFK) being trialed for correlation to other grading techniques. |
| **9. Dry polishing** | Carcass buffer at inlet to 1 set of vertical whip flail polishers, 1m total length. Direct exit into singer. |
| **10. Singeing (CCP)** | 1m long, vertical, intermittent gas flaming with 2 ranks of gas burners. Singeing lasts approximately 8s. Carcass surface temperature at exit 61.5-63.5°C (3 measurements). Temperature in singe 68-270°C measured where visible. 1s added to singe time in summer. Swing entry doors rub on each carcass. |
| **11. Wet polishing** | 4 m long polishers consisting of 2 vertical and 2 horizontal whip flails. Carcass surface temperature at exit 38.8-39.2°C (3 measurements). Swing exit doors rub on each carcass. |

(Provided by D. Tinker, 2007)

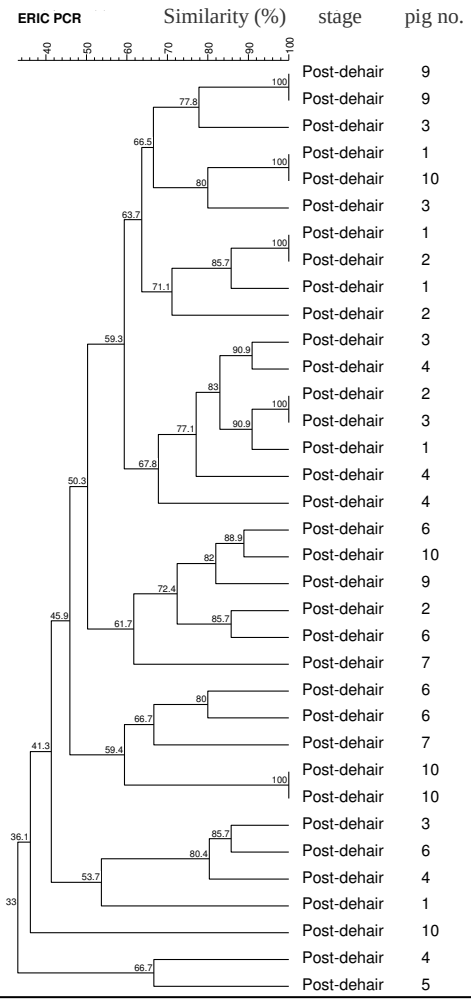
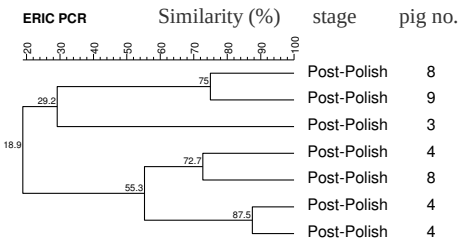
Appendix 4.1. Overall similarity of E. coli population at each process stage. Dendrogram obtained from cluster analysis (Dice coefficient; UPGMA).

| (a) Post-bleeding | (b) pre-scalding |
|---|---|



Appendix 4.1. Continued

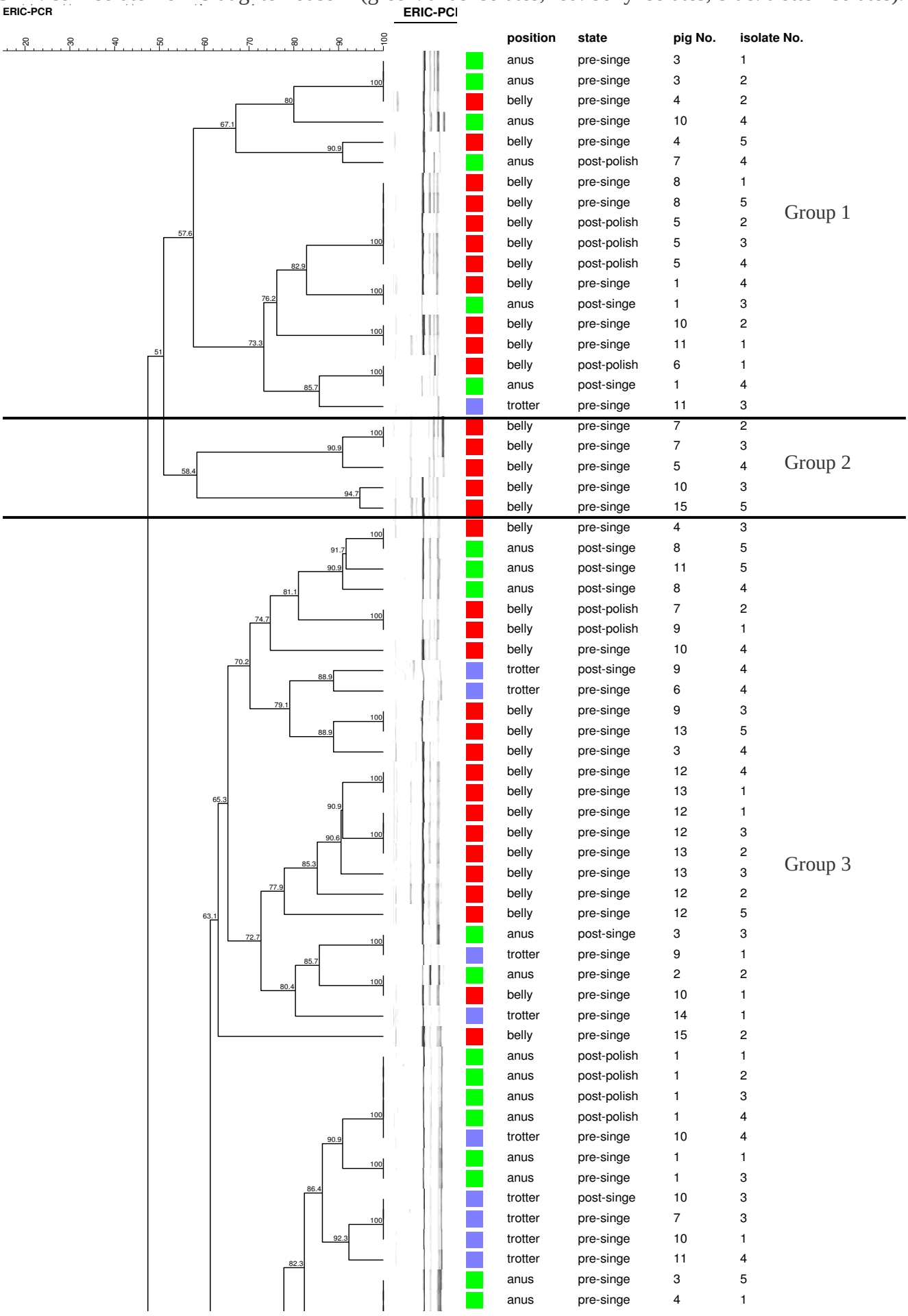
| (c) post-dehairing | (d) post-polishing |
|---|---|
|  |  |

Appendix 5.1 Process line of Slaughterhouse B

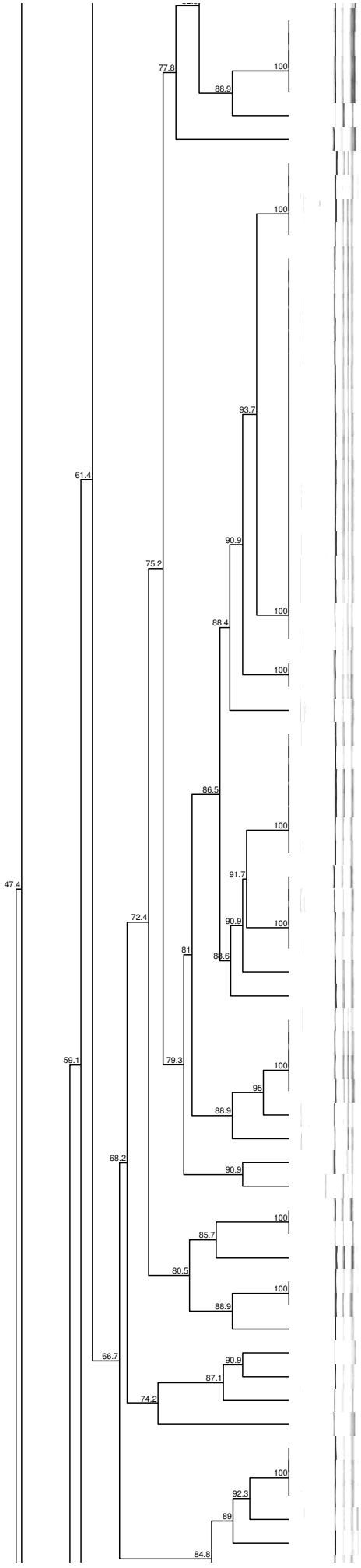
| Abattoir Process | Notes |
|---|---|
| **12. Lairage and Races.** | Estimated 2.5m x 15m pens. Concrete floor. Nipple drinkers. Sprinklers generally on (if air temp warm enough) for pig cooling, washing and aiding with electrical contact on stun. |
| **13. Stun** | 5.9s. 4s @ 400Hz then 2s @ 90Hz. 250-300V, 1.5A |
| **14. Shackle** | Single leg chain hang. |
| **15. Stick** | - |
| **16. Bleed** | 6 minutes |
| **17. Scalding** | 4 x 5m vertical steam scalding modules. Digital readout on each module (61.5/64.1/63.4/66.3°C). Separate transport line through scalder with rehang at each end. Carcass surface temperatures on outlet 48.6/53.4/52.2°C (3 samples). When installed was more cost effective than water based scald – current economics unknown. Strip doors contact each carcass on inlet and out of scalder. 16.2s between carcasses. |
| **18. Dehairer** | Drop from chain into spiral dehairer. Carcasses ejected at variable intervals 11/5/13/26s measured. Carcass surface temperatures on outlet 42.8/33.5/42.8°C (3 samples). |
| **19. Gambrels.** | Including manual scrape if required |
| **20. Singeing (CCP)** | Sarcophagus type with single base flame. Heavy singe (16s) to give safety margin on colour and live brine curing. Carcass surface temperatures on inlet 38.2°C (1 sample). Carcass surface temperatures on outlet 70.2/93.5/69.5°C (3 samples). Head temps at outlet 70.4/86.3/109.1°C (3 samples). . |
| **21. Black Scrapers.** | 3m vertical scraper fins. |
| **22. White Polishers** | 10m fixed head brushes and scapers with controlled carcass orientation. Carcass surface temperatures on outlet 15.8 - 20.1°C (3 samples). |

(Provided by D. Tinker, 2007)

Appendix 5.2. Dendrogram obtained from cluster analysis (Dice coefficient; UPGMA) of ERIC-PCR types of 437 E. coli isolate from Slaughterhouse B (green: anus isolates; red: belly isolates; blue: trotter isolates).

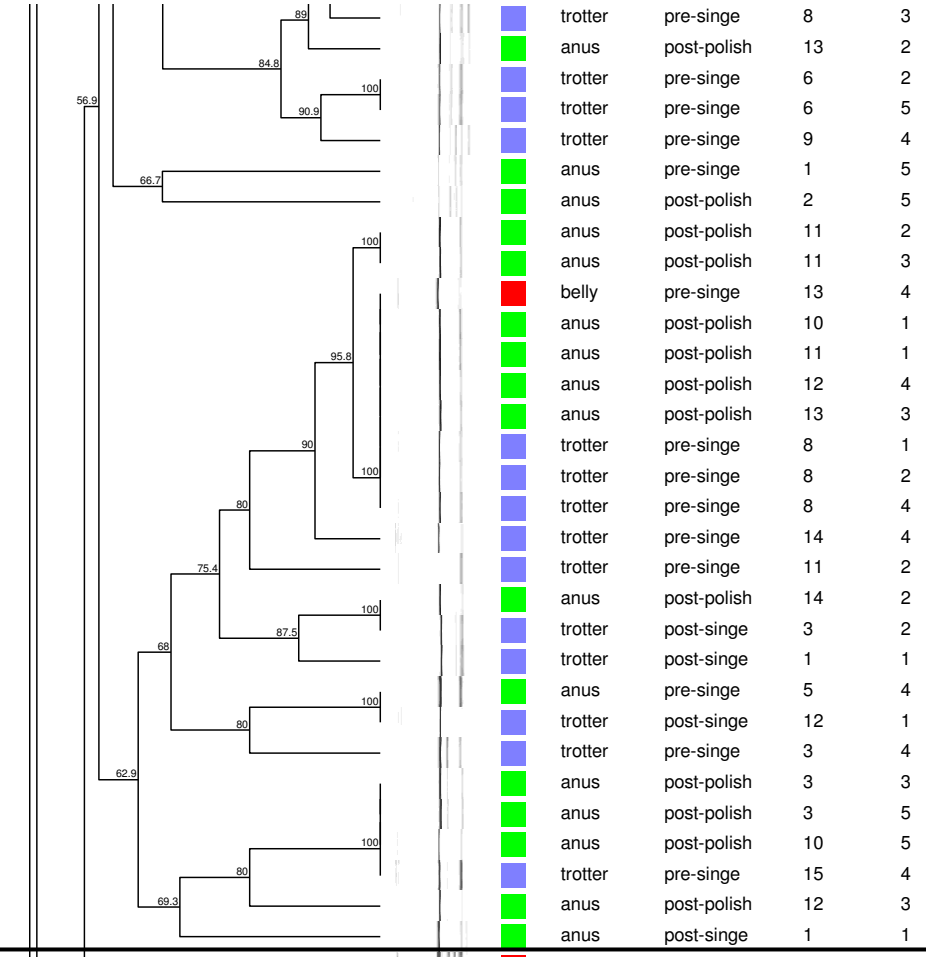



| position | state | pig No. | isolate No. |
|---|---|---|---|
| anus | pre-singe | 3 | 1 |
| anus | pre-singe | 3 | 2 |
| belly | pre-singe | 4 | 2 |
| anus | pre-singe | 10 | 4 |
| belly | pre-singe | 4 | 5 |
| anus | post-polish | 7 | 4 |
| belly | pre-singe | 8 | 1 |
| belly | pre-singe | 8 | 5 |
| belly | post-polish | 5 | 2 |
| belly | post-polish | 5 | 3 |
| belly | post-polish | 5 | 4 |
| belly | pre-singe | 1 | 4 |
| anus | post-singe | 1 | 3 |
| belly | pre-singe | 10 | 2 |
| belly | pre-singe | 11 | 1 |
| belly | post-polish | 6 | 1 |
| anus | post-singe | 1 | 4 |
| trotter | pre-singe | 11 | 3 |
| belly | pre-singe | 7 | 2 |
| belly | pre-singe | 7 | 3 |
| belly | pre-singe | 5 | 4 |
| belly | pre-singe | 10 | 3 |
| belly | pre-singe | 15 | 5 |
| belly | pre-singe | 4 | 3 |
| anus | post-singe | 8 | 5 |
| anus | post-singe | 11 | 5 |
| anus | post-singe | 8 | 4 |
| belly | post-polish | 7 | 2 |
| belly | post-polish | 9 | 1 |
| belly | pre-singe | 10 | 4 |
| trotter | post-singe | 9 | 4 |
| trotter | pre-singe | 6 | 4 |
| belly | pre-singe | 9 | 3 |
| belly | pre-singe | 13 | 5 |
| belly | pre-singe | 3 | 4 |
| belly | pre-singe | 12 | 4 |
| belly | pre-singe | 13 | 1 |
| belly | pre-singe | 12 | 1 |
| belly | pre-singe | 12 | 3 |
| belly | pre-singe | 13 | 2 |
| belly | pre-singe | 13 | 3 |
| belly | pre-singe | 12 | 2 |
| belly | pre-singe | 12 | 5 |
| anus | post-singe | 3 | 3 |
| trotter | pre-singe | 9 | 1 |
| anus | pre-singe | 2 | 2 |
| belly | pre-singe | 10 | 1 |
| trotter | pre-singe | 14 | 1 |
| belly | pre-singe | 15 | 2 |
| anus | post-polish | 1 | 1 |
| anus | post-polish | 1 | 2 |
| anus | post-polish | 1 | 3 |
| anus | post-polish | 1 | 4 |
| trotter | pre-singe | 10 | 4 |
| anus | pre-singe | 1 | 1 |
| anus | pre-singe | 1 | 3 |
| trotter | post-singe | 10 | 3 |
| trotter | pre-singe | 7 | 3 |
| trotter | pre-singe | 10 | 1 |
| trotter | pre-singe | 11 | 4 |
| anus | pre-singe | 3 | 5 |
| anus | pre-singe | 4 | 1 |



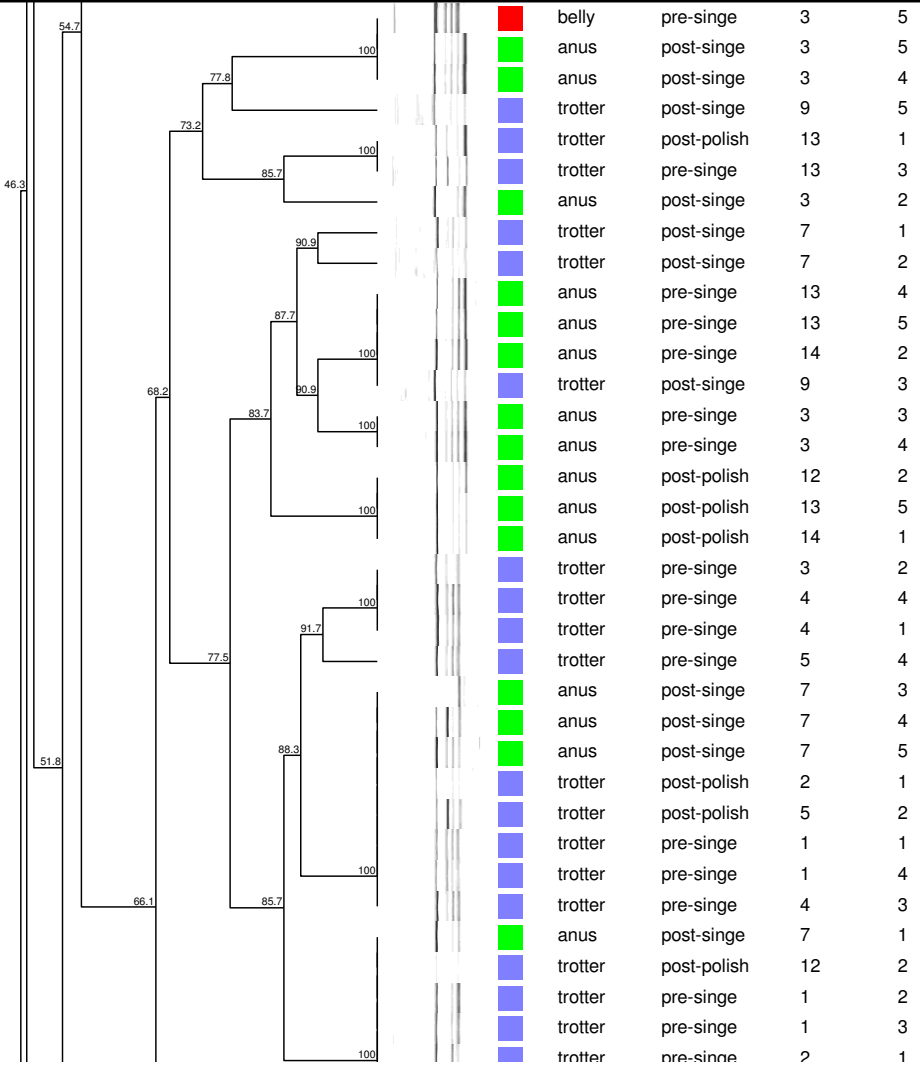

| | | | |
|---|---|---|---|
| anus | pre-singe | 3 | 5 |
| anus | pre-singe | 4 | 1 |
| anus | pre-singe | 4 | 2 |
| anus | pre-singe | 4 | 3 |
| trotter | post-polish | 6 | 1 |
| anus | pre-singe | 2 | 1 |
| trotter | post-singe | 3 | 1 |
| trotter | pre-singe | 12 | 3 |
| trotter | post-singe | 3 | 5 |
| trotter | pre-singe | 7 | 5 |
| anus | post-polish | 1 | 6 |
| anus | post-polish | 2 | 1 |
| anus | post-polish | 2 | 2 |
| anus | post-polish | 2 | 3 |
| anus | post-polish | 2 | 4 |
| anus | post-polish | 3 | 1 |
| anus | post-polish | 3 | 2 |
| anus | post-polish | 4 | 1 |
| anus | post-polish | 4 | 2 |
| anus | post-polish | 4 | 3 |
| anus | post-polish | 4 | 4 |
| anus | post-polish | 4 | 5 |
| anus | post-polish | 5 | 5 |
| anus | post-polish | 7 | 1 |
| anus | post-polish | 7 | 2 |
| trotter | pre-singe | 7 | 1 |
| trotter | pre-singe | 11 | 1 |
| belly | post-polish | 9 | 2 |
| belly | post-polish | 9 | 3 |
| trotter | pre-singe | 14 | 2 |
| anus | post-polish | 5 | 4 |
| anus | post-polish | 8 | 2 |
| trotter | post-polish | 7 | 1 |
| trotter | post-polish | 7 | 2 |
| trotter | post-polish | 10 | 1 |
| trotter | pre-singe | 12 | 4 |
| anus | pre-singe | 8 | 1 |
| trotter | pre-singe | 13 | 5 |
| trotter | post-polish | 6 | 2 |
| trotter | pre-singe | 8 | 5 |
| trotter | post-polish | 6 | 3 |
| trotter | post-polish | 6 | 5 |
| anus | post-polish | 10 | 4 |
| trotter | post-polish | 5 | 3 |
| trotter | post-polish | 5 | 4 |
| trotter | post-polish | 8 | 1 |
| trotter | pre-singe | 15 | 5 |
| trotter | pre-singe | 13 | 1 |
| anus | post-singe | 8 | 1 |
| anus | post-polish | 15 | 5 |
| anus | pre-singe | 5 | 3 |
| anus | post-polish | 8 | 4 |
| trotter | post-polish | 6 | 4 |
| trotter | post-polish | 9 | 2 |
| trotter | post-polish | 12 | 5 |
| anus | post-polish | 11 | 5 |
| anus | pre-singe | 1 | 4 |
| anus | post-polish | 11 | 4 |
| trotter | pre-singe | 10 | 3 |
| anus | pre-singe | 4 | 4 |
| anus | post-polish | 5 | 1 |
| anus | post-polish | 5 | 2 |
| anus | post-polish | 5 | 3 |
| trotter | pre-singe | 8 | 3 |
| anus | post-polish | 13 | 2 |

Group 3



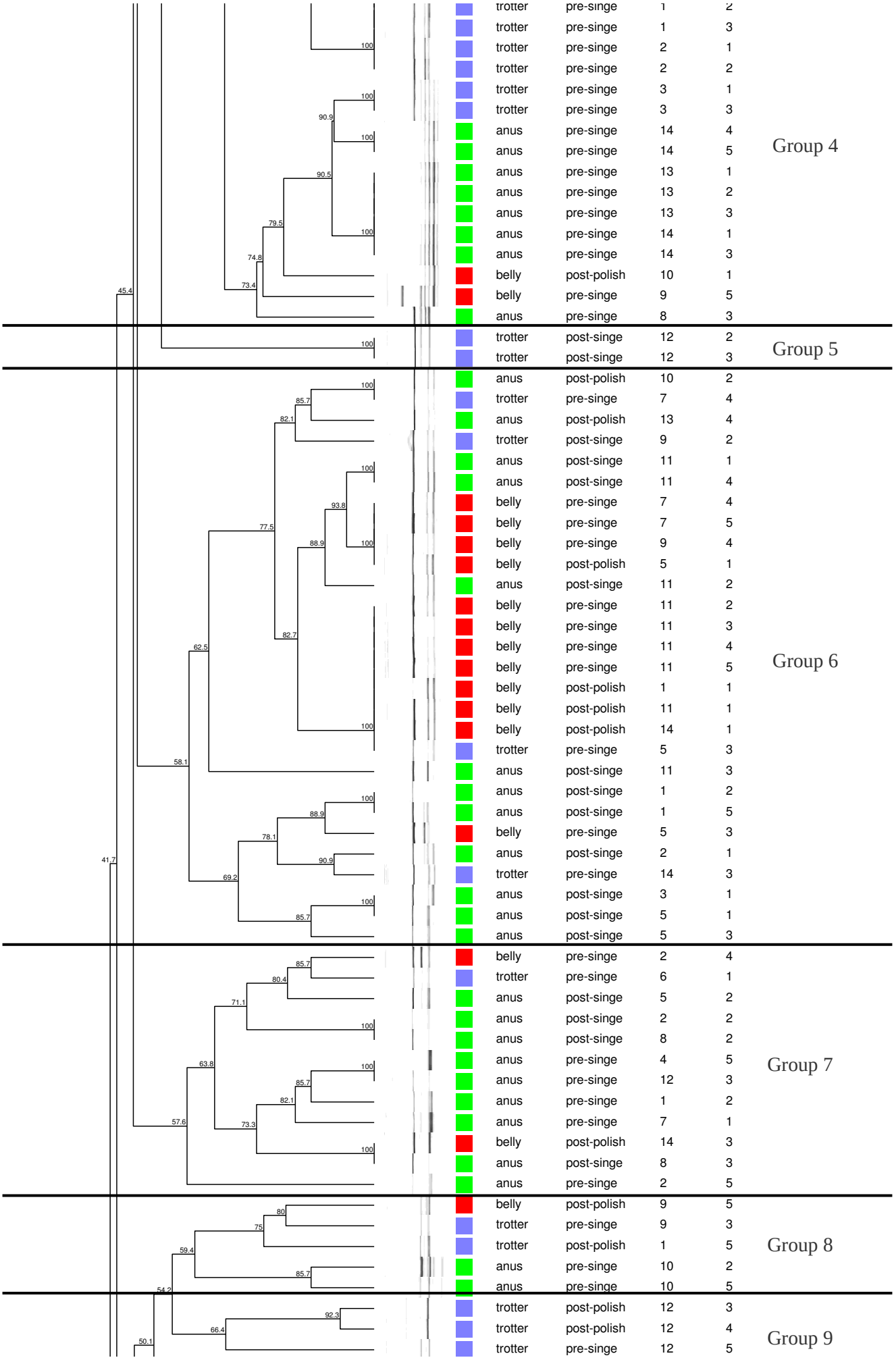

| | | | |
|---|---|---|---|
| trotter | pre-singe | 8 | 3 |
| anus | post-polish | 13 | 2 |
| trotter | pre-singe | 6 | 2 |
| trotter | pre-singe | 6 | 5 |
| trotter | pre-singe | 9 | 4 |
| anus | pre-singe | 1 | 5 |
| anus | post-polish | 2 | 5 |
| anus | post-polish | 11 | 2 |
| anus | post-polish | 11 | 3 |
| belly | pre-singe | 13 | 4 |
| anus | post-polish | 10 | 1 |
| anus | post-polish | 11 | 1 |
| anus | post-polish | 12 | 4 |
| anus | post-polish | 13 | 3 |
| trotter | pre-singe | 8 | 1 |
| trotter | pre-singe | 8 | 2 |
| trotter | pre-singe | 8 | 4 |
| trotter | pre-singe | 14 | 4 |
| trotter | pre-singe | 11 | 2 |
| anus | post-polish | 14 | 2 |
| trotter | post-singe | 3 | 2 |
| trotter | post-singe | 1 | 1 |
| anus | pre-singe | 5 | 4 |
| trotter | post-singe | 12 | 1 |
| trotter | pre-singe | 3 | 4 |
| anus | post-polish | 3 | 3 |
| anus | post-polish | 3 | 5 |
| anus | post-polish | 10 | 5 |
| trotter | pre-singe | 15 | 4 |
| anus | post-polish | 12 | 3 |
| anus | post-singe | 1 | 1 |

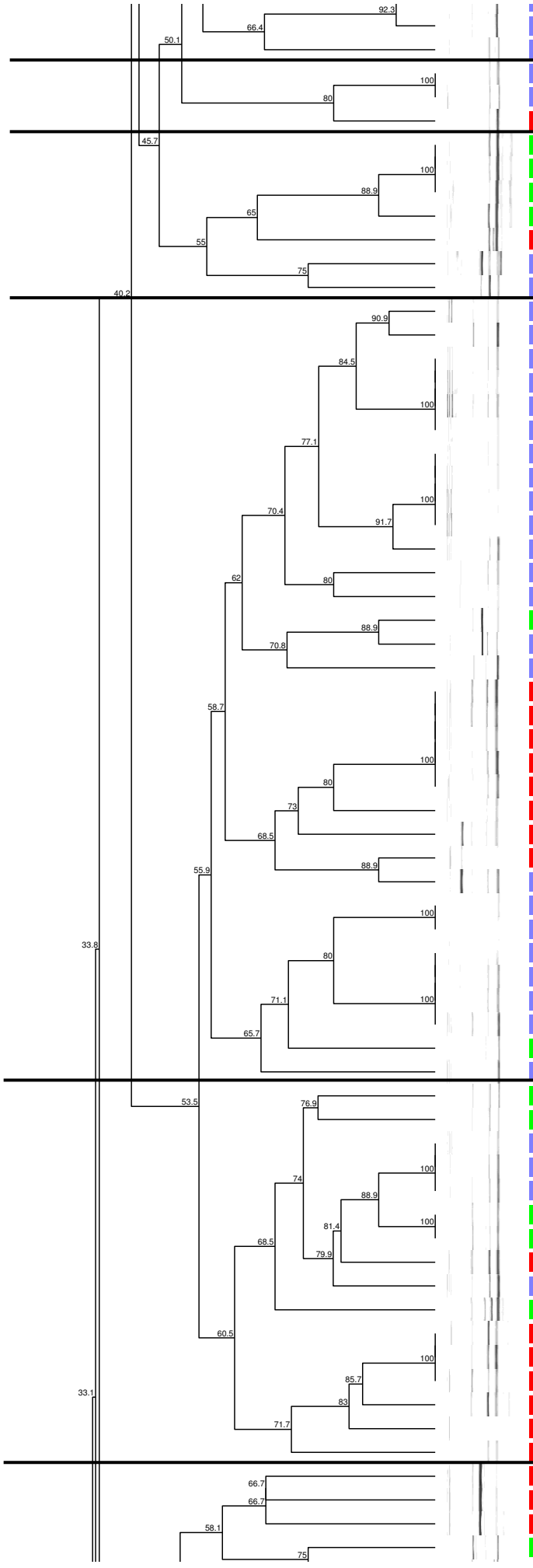
Group 3



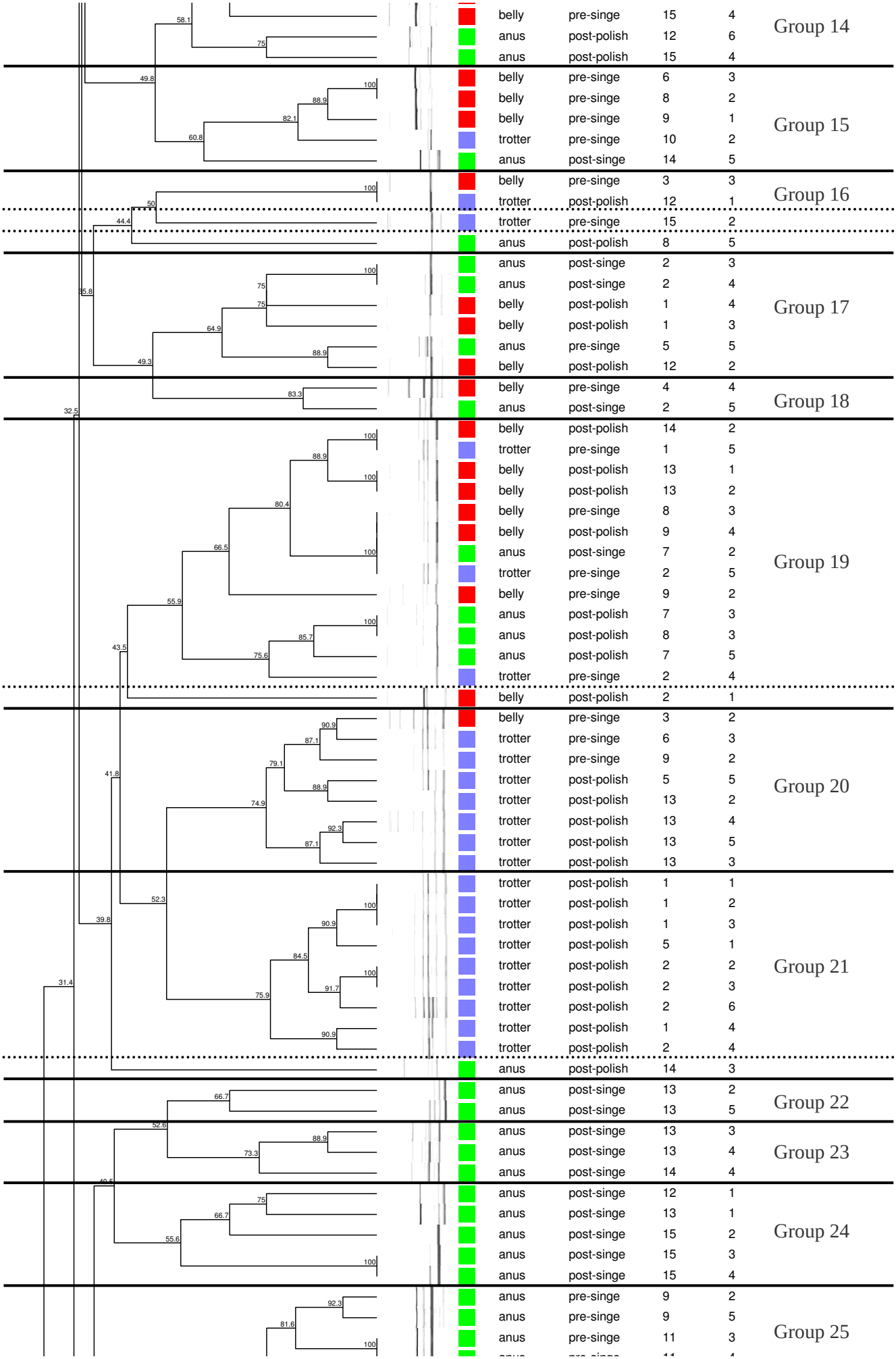

| | | | |
|---|---|---|---|
| belly | pre-singe | 3 | 5 |
| anus | post-singe | 3 | 5 |
| anus | post-singe | 3 | 4 |
| trotter | post-singe | 9 | 5 |
| trotter | post-polish | 13 | 1 |
| trotter | pre-singe | 13 | 3 |
| anus | post-singe | 3 | 2 |
| trotter | post-singe | 7 | 1 |
| trotter | post-singe | 7 | 2 |
| anus | pre-singe | 13 | 4 |
| anus | pre-singe | 13 | 5 |
| anus | pre-singe | 14 | 2 |
| trotter | post-singe | 9 | 3 |
| anus | pre-singe | 3 | 3 |
| anus | pre-singe | 3 | 4 |
| anus | post-polish | 12 | 2 |
| anus | post-polish | 13 | 5 |
| anus | post-polish | 14 | 1 |
| trotter | pre-singe | 3 | 2 |
| trotter | pre-singe | 4 | 4 |
| trotter | pre-singe | 4 | 1 |
| trotter | pre-singe | 5 | 4 |
| anus | post-singe | 7 | 3 |
| anus | post-singe | 7 | 4 |
| anus | post-singe | 7 | 5 |
| trotter | post-polish | 2 | 1 |
| trotter | post-polish | 5 | 2 |
| trotter | pre-singe | 1 | 1 |
| trotter | pre-singe | 1 | 4 |
| trotter | pre-singe | 4 | 3 |
| anus | post-singe | 7 | 1 |
| trotter | post-polish | 12 | 2 |
| trotter | pre-singe | 1 | 2 |
| trotter | pre-singe | 1 | 3 |
| trotter | pre-singe | 2 | 1 |

Group 4

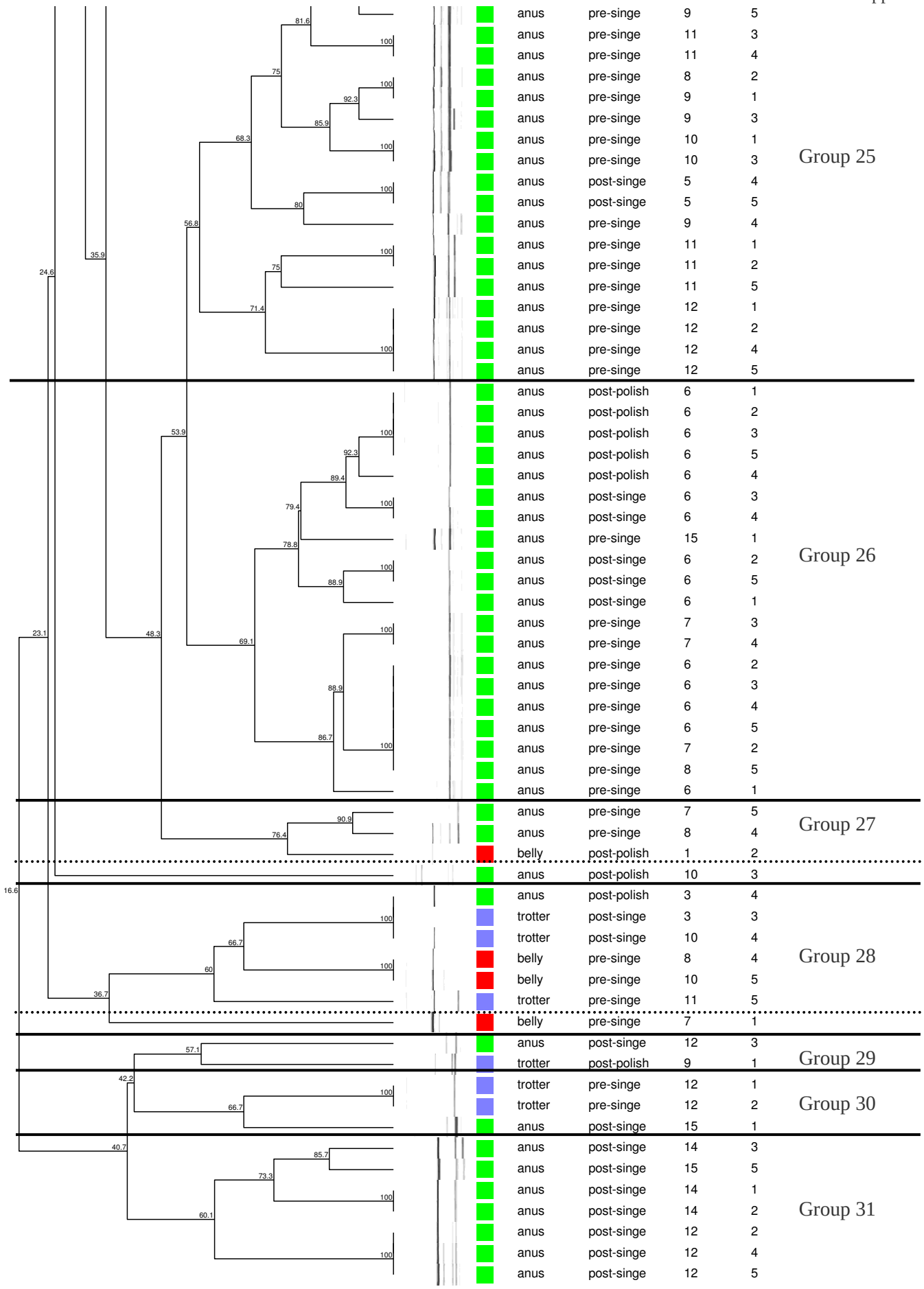

trotter pre-singe 1 2
trotter pre-singe 1 3
trotter pre-singe 2 1
trotter pre-singe 2 2
trotter pre-singe 3 1
trotter pre-singe 3 3
anus pre-singe 14 4
anus pre-singe 14 5
anus pre-singe 13 1
anus pre-singe 13 2
anus pre-singe 13 3
anus pre-singe 14 1
anus pre-singe 14 3
belly post-polish 10 1
belly pre-singe 9 5
anus pre-singe 8 3
Group 4
trotter post-singe 12 2
trotter post-singe 12 3
Group 5
anus post-polish 10 2
trotter pre-singe 7 4
anus post-polish 13 4
trotter post-singe 9 2
anus post-singe 11 1
anus post-singe 11 4
belly pre-singe 7 4
belly pre-singe 7 5
belly pre-singe 9 4
belly post-polish 5 1
anus post-singe 11 2
belly pre-singe 11 2
belly pre-singe 11 3
belly pre-singe 11 4
belly pre-singe 11 5
belly post-polish 1 1
belly post-polish 11 1
belly post-polish 14 1
trotter pre-singe 5 3
anus post-singe 11 3
anus post-singe 1 2
anus post-singe 1 5
belly pre-singe 5 3
anus post-singe 2 1
trotter pre-singe 14 3
anus post-singe 3 1
anus post-singe 5 1
anus post-singe 5 3
Group 6
belly pre-singe 2 4
trotter pre-singe 6 1
anus post-singe 5 2
anus post-singe 2 2
anus post-singe 8 2
anus pre-singe 4 5
anus pre-singe 12 3
anus pre-singe 1 2
anus pre-singe 7 1
belly post-polish 14 3
anus post-singe 8 3
anus pre-singe 2 5
Group 7
belly post-polish 9 5
trotter pre-singe 9 3
trotter post-polish 1 5
anus pre-singe 10 2
anus pre-singe 10 5
Group 8
trotter post-polish 12 3
trotter post-polish 12 4
trotter pre-singe 12 5
Group 9



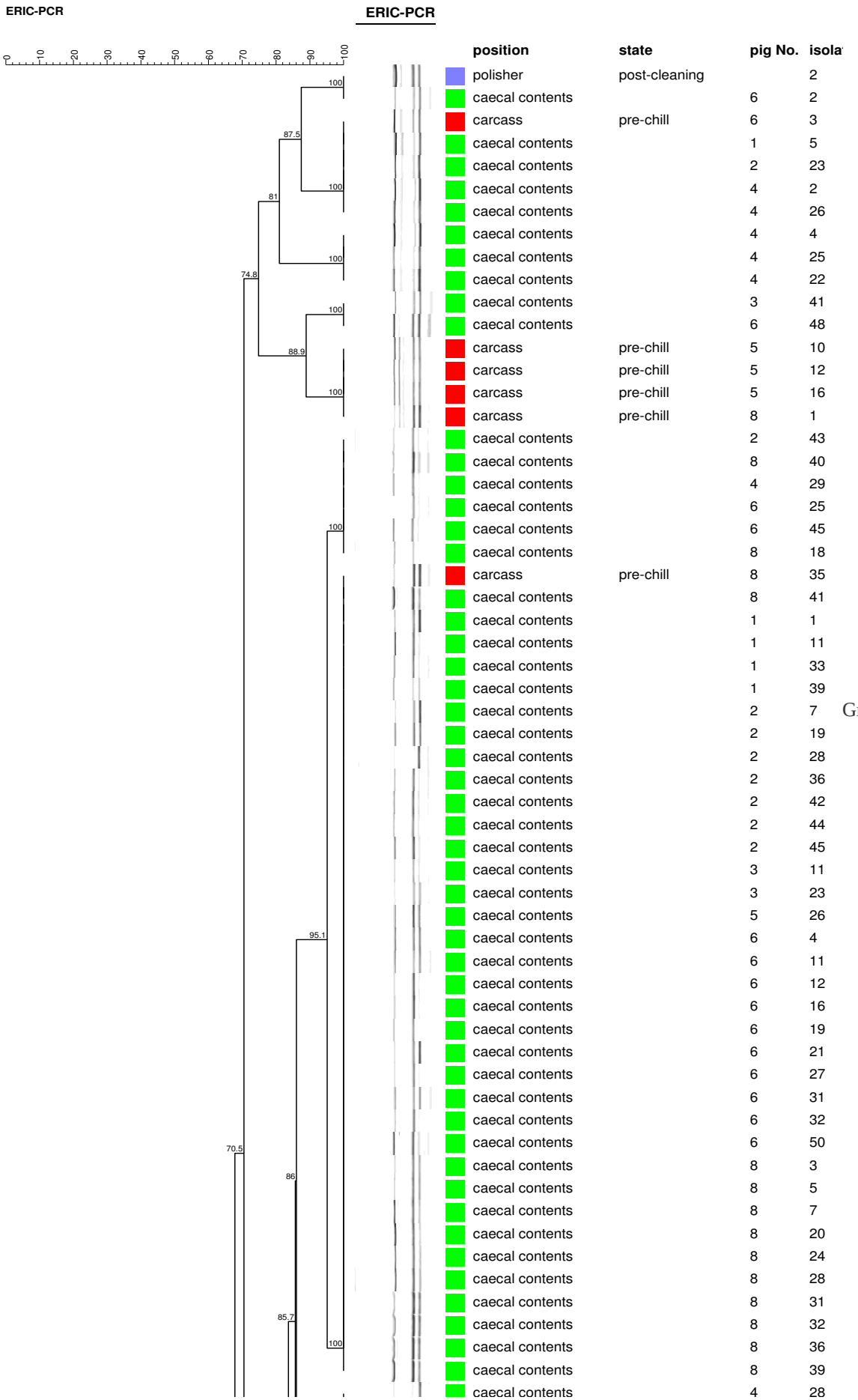
| | | | | |
|---|---|---|---|---|
| trotter | post-polish | 12 | 4 | Group 9 |
| trotter | pre-singe | 12 | 5 | |
| trotter | pre-singe | 3 | 5 | |
| trotter | pre-singe | 13 | 2 | Group 10 |
| belly | post-polish | 12 | 1 | |
| anus | pre-singe | 15 | 2 | |
| anus | pre-singe | 15 | 3 | |
| anus | pre-singe | 15 | 4 | |
| anus | pre-singe | 15 | 5 | |
| belly | pre-singe | 2 | 2 | Group 11 |
| trotter | post-singe | 9 | 1 | |
| trotter | pre-singe | 2 | 3 | |
| trotter | post-singe | 10 | 5 | |
| trotter | post-singe | 11 | 1 | |
| trotter | post-singe | 4 | 1 | |
| trotter | post-singe | 4 | 3 | |
| trotter | post-singe | 4 | 4 | |
| trotter | post-singe | 10 | 2 | |
| trotter | post-singe | 4 | 5 | |
| trotter | post-singe | 5 | 1 | |
| trotter | post-singe | 5 | 2 | |
| trotter | post-singe | 5 | 3 | |
| trotter | pre-singe | 13 | 4 | |
| trotter | pre-singe | 7 | 2 | |
| trotter | pre-singe | 9 | 5 | |
| anus | post-polish | 13 | 1 | |
| trotter | post-singe | 3 | 4 | |
| trotter | post-singe | 14 | 1 | |
| belly | pre-singe | 1 | 1 | Group 12 |
| belly | pre-singe | 1 | 2 | |
| belly | pre-singe | 1 | 3 | |
| belly | pre-singe | 2 | 1 | |
| belly | pre-singe | 2 | 5 | |
| belly | pre-singe | 1 | 5 | |
| belly | pre-singe | 6 | 4 | |
| belly | pre-singe | 3 | 1 | |
| trotter | pre-singe | 5 | 5 | |
| trotter | pre-singe | 4 | 5 | |
| trotter | pre-singe | 5 | 1 | |
| trotter | post-singe | 10 | 1 | |
| trotter | pre-singe | 4 | 2 | |
| trotter | pre-singe | 5 | 2 | |
| trotter | pre-singe | 15 | 3 | |
| anus | post-polish | 8 | 1 | |
| trotter | pre-singe | 14 | 5 | |
| anus | post-polish | 12 | 1 | |
| anus | post-polish | 15 | 3 | |
| trotter | post-singe | 5 | 4 | |
| trotter | post-singe | 5 | 5 | |
| trotter | pre-singe | 10 | 5 | |
| anus | post-polish | 15 | 1 | |
| anus | post-polish | 15 | 2 | |
| belly | pre-singe | 5 | 2 | Group 13 |
| trotter | pre-singe | 15 | 1 | |
| anus | pre-singe | 2 | 3 | |
| belly | pre-singe | 4 | 1 | |
| belly | pre-singe | 6 | 5 | |
| belly | pre-singe | 14 | 2 | |
| belly | pre-singe | 5 | 5 | |
| belly | pre-singe | 14 | 1 | |
| belly | pre-singe | 6 | 1 | |
| belly | pre-singe | 15 | 1 | |
| belly | pre-singe | 15 | 3 | |
| belly | pre-singe | 15 | 4 | Group 14 |
| anus | post-polish | 12 | 6 | |



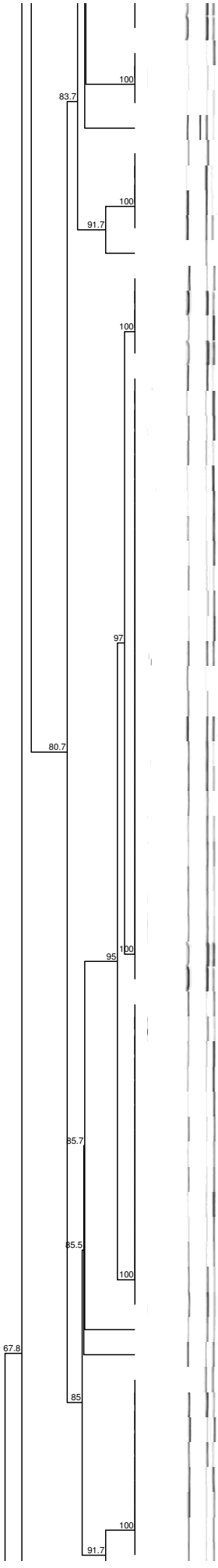
| | | | | |
|---|---|---|---|---|
| belly | pre-singe | 15 | 4 | Group 14 |
| anus | post-polish | 12 | 6 | |
| anus | post-polish | 15 | 4 | |
| belly | pre-singe | 6 | 3 | Group 15 |
| belly | pre-singe | 8 | 2 | |
| belly | pre-singe | 9 | 1 | |
| trotter | pre-singe | 10 | 2 | |
| anus | post-singe | 14 | 5 | |
| belly | pre-singe | 3 | 3 | Group 16 |
| trotter | post-polish | 12 | 1 | |
| trotter | pre-singe | 15 | 2 | |
| anus | post-polish | 8 | 5 | |
| anus | post-singe | 2 | 3 | Group 17 |
| anus | post-singe | 2 | 4 | |
| belly | post-polish | 1 | 4 | |
| belly | post-polish | 1 | 3 | |
| anus | pre-singe | 5 | 5 | |
| belly | post-polish | 12 | 2 | |
| belly | pre-singe | 4 | 4 | Group 18 |
| anus | post-singe | 2 | 5 | |
| belly | post-polish | 14 | 2 | Group 19 |
| trotter | pre-singe | 1 | 5 | |
| belly | post-polish | 13 | 1 | |
| belly | post-polish | 13 | 2 | |
| belly | pre-singe | 8 | 3 | |
| belly | post-polish | 9 | 4 | |
| anus | post-singe | 7 | 2 | |
| trotter | pre-singe | 2 | 5 | |
| belly | pre-singe | 9 | 2 | |
| anus | post-polish | 7 | 3 | |
| anus | post-polish | 8 | 3 | |
| anus | post-polish | 7 | 5 | |
| trotter | pre-singe | 2 | 4 | |
| belly | post-polish | 2 | 1 | Group 20 |
| belly | pre-singe | 3 | 2 | |
| trotter | pre-singe | 6 | 3 | |
| trotter | pre-singe | 9 | 2 | |
| trotter | post-polish | 5 | 5 | |
| trotter | post-polish | 13 | 2 | |
| trotter | post-polish | 13 | 4 | |
| trotter | post-polish | 13 | 5 | |
| trotter | post-polish | 13 | 3 | |
| trotter | post-polish | 1 | 1 | Group 21 |
| trotter | post-polish | 1 | 2 | |
| trotter | post-polish | 1 | 3 | |
| trotter | post-polish | 5 | 1 | |
| trotter | post-polish | 2 | 2 | |
| trotter | post-polish | 2 | 3 | |
| trotter | post-polish | 2 | 6 | |
| trotter | post-polish | 1 | 4 | |
| trotter | post-polish | 2 | 4 | |
| anus | post-polish | 14 | 3 | Group 22 |
| anus | post-singe | 13 | 2 | |
| anus | post-singe | 13 | 5 | |
| anus | post-singe | 13 | 3 | Group 23 |
| anus | post-singe | 13 | 4 | |
| anus | post-singe | 14 | 4 | |
| anus | post-singe | 12 | 1 | Group 24 |
| anus | post-singe | 13 | 1 | |
| anus | post-singe | 15 | 2 | |
| anus | post-singe | 15 | 3 | |
| anus | post-singe | 15 | 4 | |
| anus | pre-singe | 9 | 2 | Group 25 |
| anus | pre-singe | 9 | 5 | |
| anus | pre-singe | 11 | 3 | |


anus pre-singe 9 5
anus pre-singe 11 3
anus pre-singe 11 4
anus pre-singe 8 2
anus pre-singe 9 1
anus pre-singe 9 3
anus pre-singe 10 1
anus pre-singe 10 3
Group 25
anus post-singe 5 4
anus post-singe 5 5
anus pre-singe 9 4
anus pre-singe 11 1
anus pre-singe 11 2
anus pre-singe 11 5
anus pre-singe 12 1
anus pre-singe 12 2
anus pre-singe 12 4
anus pre-singe 12 5
anus post-polish 6 1
anus post-polish 6 2
anus post-polish 6 3
anus post-polish 6 5
anus post-polish 6 4
anus post-singe 6 3
anus post-singe 6 4
anus pre-singe 15 1
anus post-singe 6 2
Group 26
anus post-singe 6 5
anus post-singe 6 1
anus pre-singe 7 3
anus pre-singe 7 4
anus pre-singe 6 2
anus pre-singe 6 3
anus pre-singe 6 4
anus pre-singe 6 5
anus pre-singe 7 2
anus pre-singe 8 5
anus pre-singe 6 1
anus pre-singe 7 5
Group 27
anus pre-singe 8 4
belly post-polish 1 2
anus post-polish 10 3
anus post-polish 3 4
trotter post-singe 3 3
trotter post-singe 10 4
belly pre-singe 8 4
Group 28
belly pre-singe 10 5
trotter pre-singe 11 5
belly pre-singe 7 1
anus post-singe 12 3
Group 29
trotter post-polish 9 1
trotter pre-singe 12 1
trotter pre-singe 12 2
Group 30
anus post-singe 15 1
anus post-singe 14 3
anus post-singe 15 5
anus post-singe 14 1
anus post-singe 14 2
Group 31
anus post-singe 12 2
anus post-singe 12 4
anus post-singe 12 5

Appendix 6.1. Dendrogram obtained from cluster analysis (Dice coefficient; UPGMA) of ERIC-PCR types of 538 E. coli isolates from pig carcasses caecal contents and polisher samples (red: carcass isolates; green: caecal isolates; blue: polisher isolates).

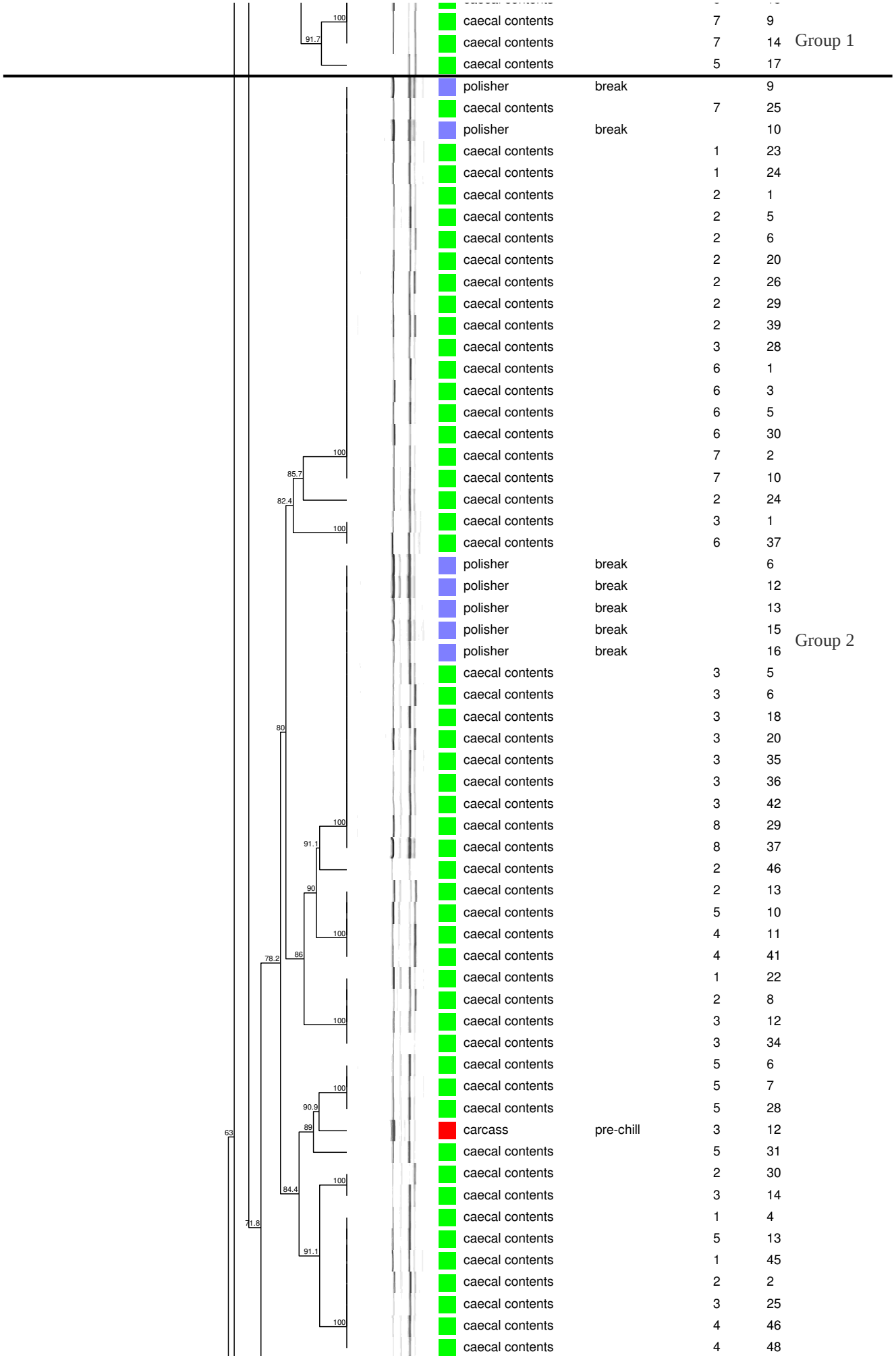



Group 1




| | | | |
|---|---|---|---|
| caecal contents | | 8 | 39 |
| caecal contents | | 4 | 28 |
| caecal contents | | 8 | 25 |
| caecal contents | | 6 | 18 |
| carcass | pre-chill | 4 | 1 |
| caecal contents | | 6 | 29 |
| caecal contents | | 6 | 41 |
| caecal contents | | 6 | 39 |
| caecal contents | | 6 | 40 |
| caecal contents | | 6 | 38 |
| caecal contents | | 3 | 40 |
| caecal contents | | 8 | 44 |
| caecal contents | | 4 | 1 |
| caecal contents | | 8 | 33 |
| carcass | pre-chill | 1 | 5 |
| carcass | pre-chill | 4 | 6 |
| caecal contents | | 1 | 2 |
| caecal contents | | 1 | 34 |
| caecal contents | | 1 | 43 |
| caecal contents | | 2 | 38 |
| caecal contents | | 3 | 16 |
| caecal contents | | 3 | 32 |
| caecal contents | | 3 | 39 |
| caecal contents | | 3 | 44 |
| caecal contents | | 3 | 50 |
| caecal contents | | 5 | 8 |
| caecal contents | | 6 | 17 |
| caecal contents | | 6 | 28 |
| caecal contents | | 6 | 49 |
| caecal contents | | 8 | 2 |
| caecal contents | | 8 | 4 |
| caecal contents | | 8 | 9 |
| caecal contents | | 8 | 13 |
| caecal contents | | 8 | 14 |
| caecal contents | | 8 | 17 |
| caecal contents | | 8 | 19 |
| caecal contents | | 8 | 21 |
| caecal contents | | 8 | 34 |
| caecal contents | | 8 | 43 |
| caecal contents | | 1 | 10 |
| caecal contents | | 8 | 27 |
| caecal contents | | 1 | 32 |
| caecal contents | | 1 | 37 |
| caecal contents | | 2 | 10 |
| caecal contents | | 2 | 18 |
| caecal contents | | 3 | 37 |
| caecal contents | | 3 | 48 |
| caecal contents | | 4 | 12 |
| caecal contents | | 7 | 1 |
| caecal contents | | 7 | 11 |
| caecal contents | | 8 | 23 |
| caecal contents | | 8 | 26 |
| caecal contents | | 8 | 12 |
| caecal contents | | 5 | 11 |
| caecal contents | | 1 | 25 |
| caecal contents | | 8 | 1 |
| caecal contents | | 2 | 11 |
| caecal contents | | 3 | 26 |
| caecal contents | | 3 | 27 |
| caecal contents | | 6 | 15 |
| caecal contents | | 7 | 9 |
| caecal contents | | 7 | 14 |

Group 1


100
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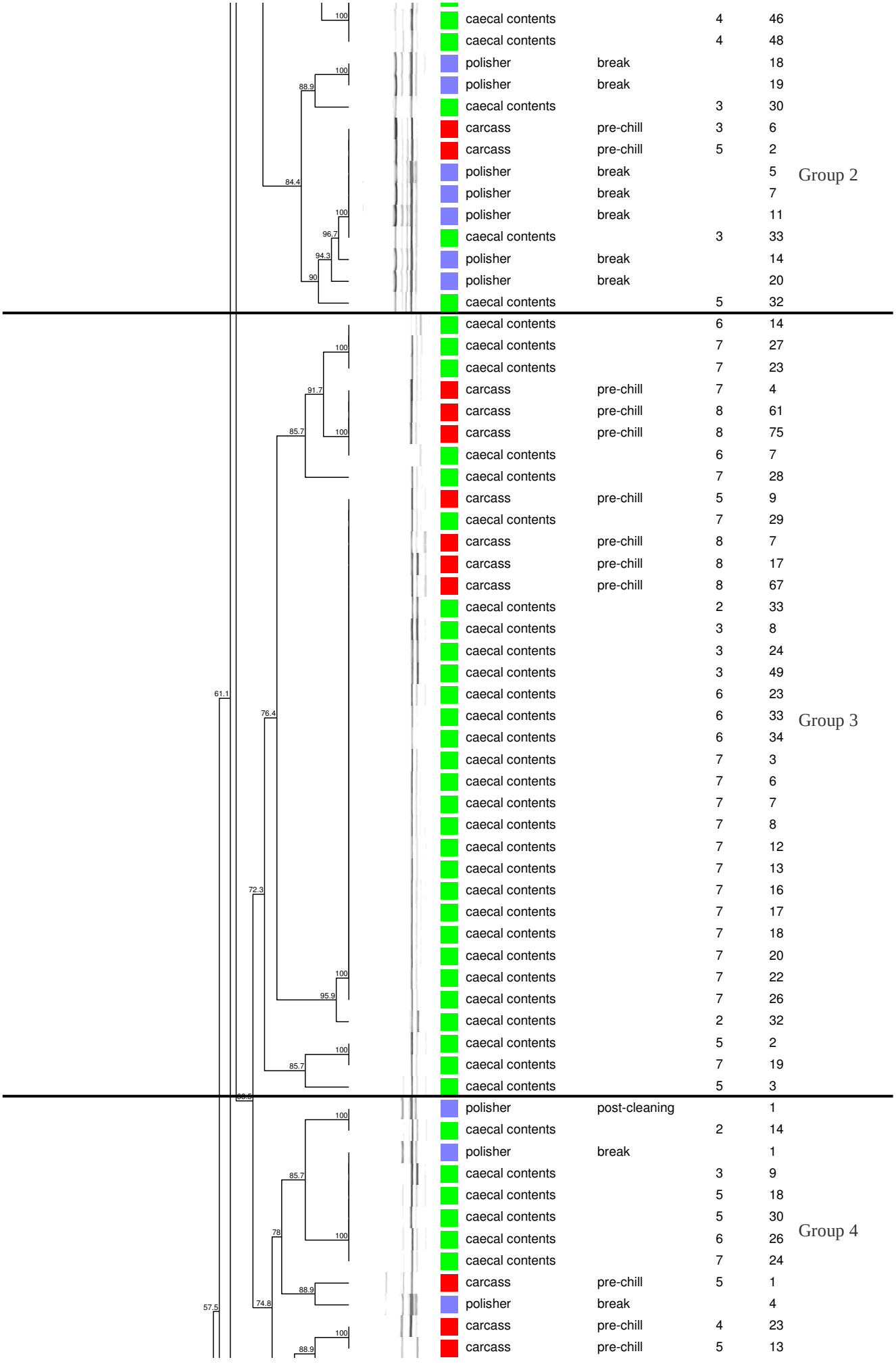
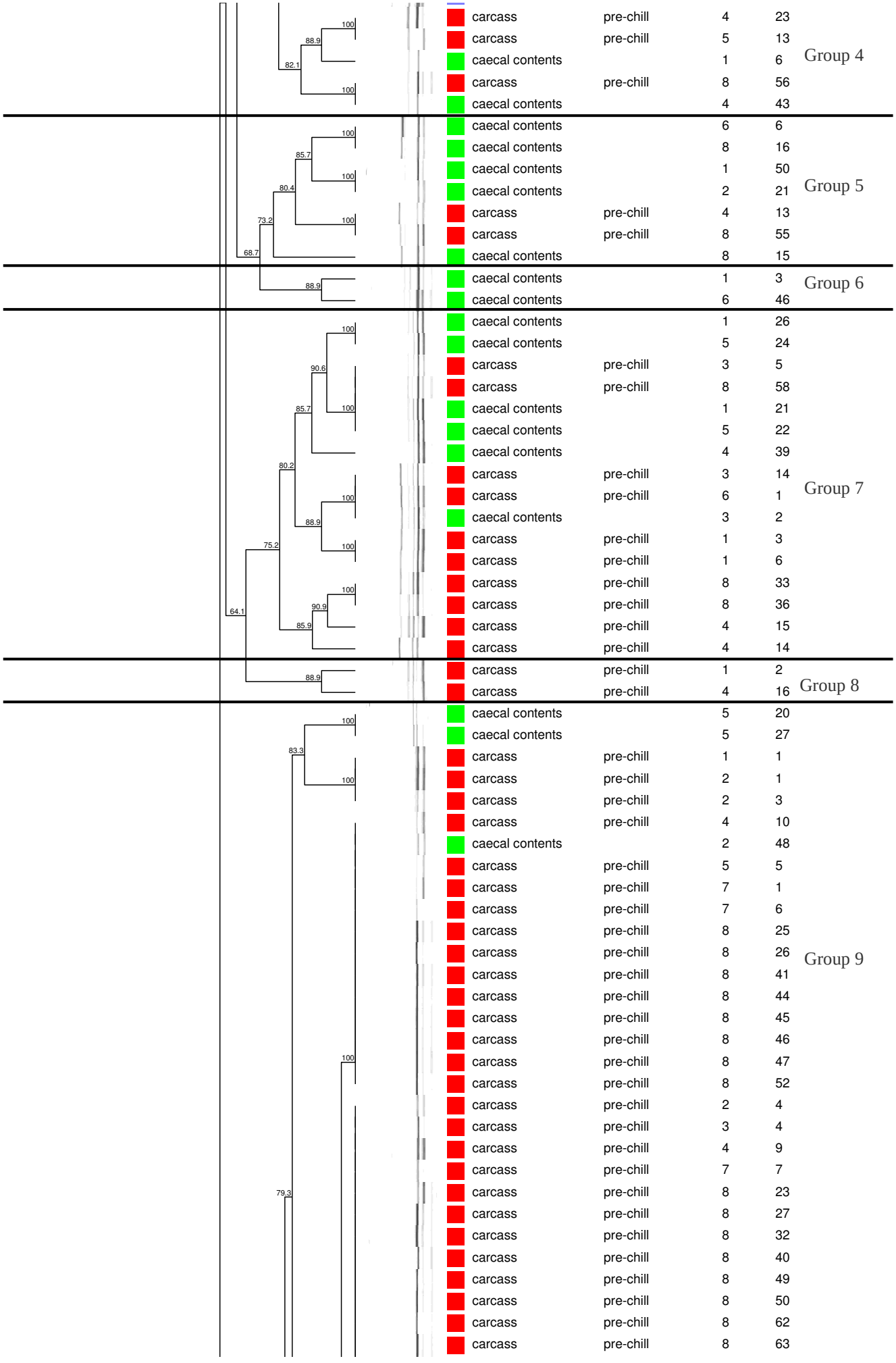
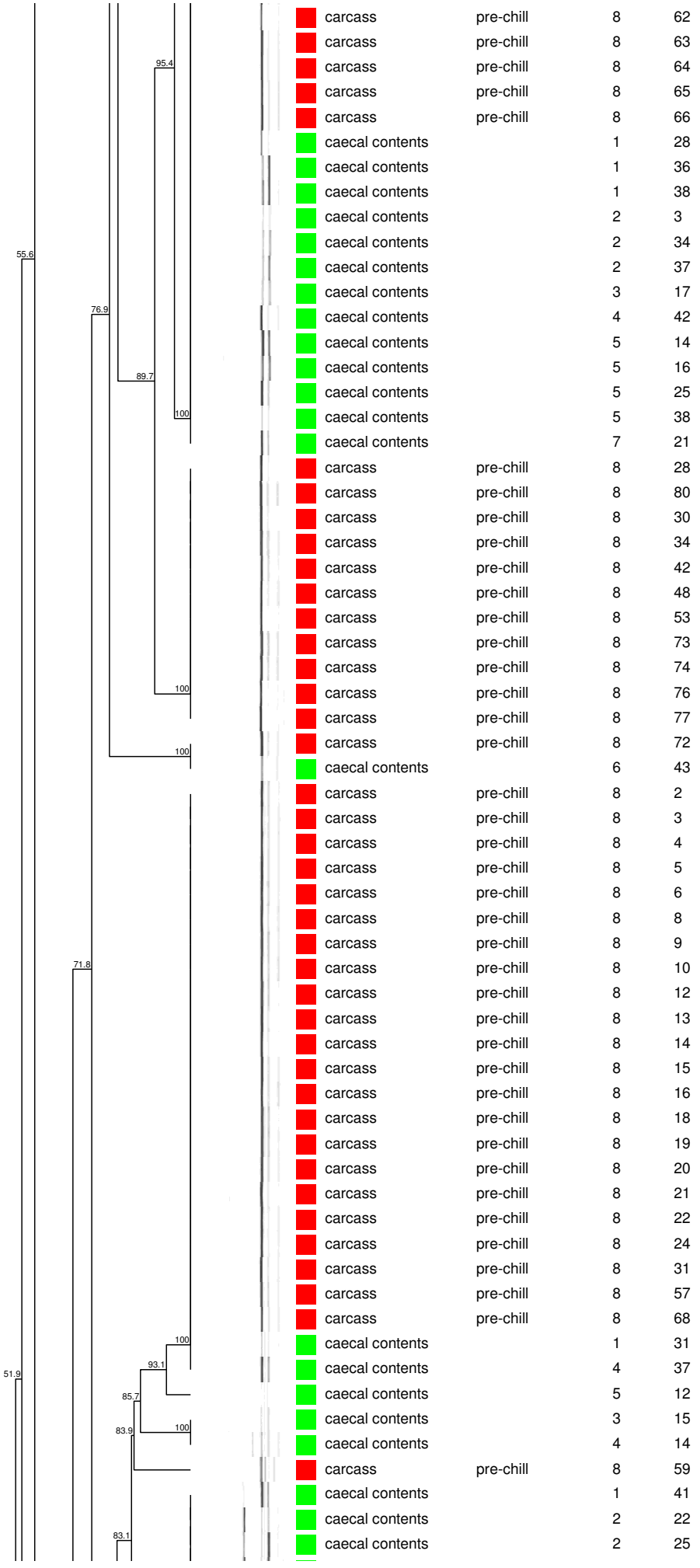
100
85.7
82.4
100
80
100
91.1
90
100
78.2
86
100
100
90.9
89
63
84.4
100
71.8
91.1
100
caecal contents 7 9
caecal contents 7 14
caecal contents 5 17
polisher break 9
caecal contents 7 25
polisher break 10
caecal contents 1 23
caecal contents 1 24
caecal contents 2 1
caecal contents 2 5
caecal contents 2 6
caecal contents 2 20
caecal contents 2 26
caecal contents 2 29
caecal contents 2 39
caecal contents 3 28
caecal contents 6 1
caecal contents 6 3
caecal contents 6 5
caecal contents 6 30
caecal contents 7 2
caecal contents 7 10
caecal contents 2 24
caecal contents 3 1
caecal contents 6 37
polisher break 6
polisher break 12
polisher break 13
polisher break 15
polisher break 16
caecal contents 3 5
caecal contents 3 6
caecal contents 3 18
caecal contents 3 20
caecal contents 3 35
caecal contents 3 36
caecal contents 3 42
caecal contents 8 29
caecal contents 8 37
caecal contents 2 46
caecal contents 2 13
caecal contents 5 10
caecal contents 4 11
caecal contents 4 41
caecal contents 1 22
caecal contents 2 8
caecal contents 3 12
caecal contents 3 34
caecal contents 5 6
caecal contents 5 7
caecal contents 5 28
carcass pre-chill 3 12
caecal contents 5 31
caecal contents 2 30
caecal contents 3 14
caecal contents 1 4
caecal contents 5 13
caecal contents 1 45
caecal contents 2 2
caecal contents 3 25
caecal contents 4 46
caecal contents 4 48
Group 1
Group 2

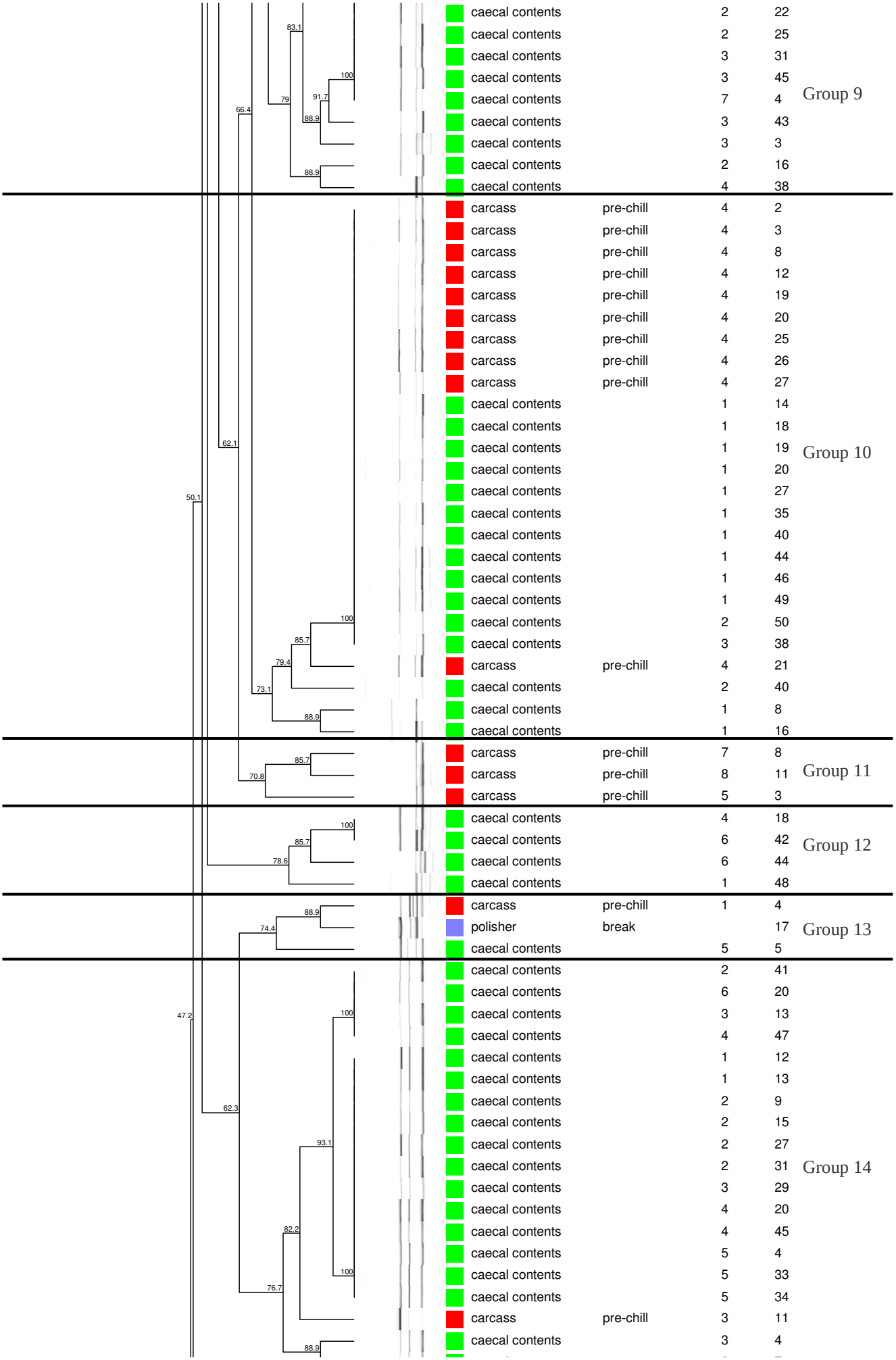

100
100
88.9
84.4
100
96.7
94.3
90
61.1
76.4
72.3
100
95.9
100
85.7
100
85.7
91.7
100
85.7
100
78
88.9
74.8
57.5
100
88.9
caecal contents 4 46
caecal contents 4 48
polisher break 18
polisher break 19
caecal contents 3 30
carcass pre-chill 3 6
carcass pre-chill 5 2
polisher break 5
polisher break 7
polisher break 11
caecal contents 3 33
polisher break 14
polisher break 20
caecal contents 5 32
caecal contents 6 14
caecal contents 7 27
caecal contents 7 23
carcass pre-chill 7 4
carcass pre-chill 8 61
carcass pre-chill 8 75
caecal contents 6 7
caecal contents 7 28
carcass pre-chill 5 9
caecal contents 7 29
carcass pre-chill 8 7
carcass pre-chill 8 17
carcass pre-chill 8 67
caecal contents 2 33
caecal contents 3 8
caecal contents 3 24
caecal contents 3 49
caecal contents 6 23
caecal contents 6 33
caecal contents 6 34
caecal contents 7 3
caecal contents 7 6
caecal contents 7 7
caecal contents 7 8
caecal contents 7 12
caecal contents 7 13
caecal contents 7 16
caecal contents 7 17
caecal contents 7 18
caecal contents 7 20
caecal contents 7 22
caecal contents 7 26
caecal contents 2 32
caecal contents 5 2
caecal contents 7 19
caecal contents 5 3
polisher post-cleaning 1
caecal contents 2 14
polisher break 1
caecal contents 3 9
caecal contents 5 18
caecal contents 5 30
caecal contents 6 26
caecal contents 7 24
carcass pre-chill 5 1
polisher break 4
carcass pre-chill 4 23
carcass pre-chill 5 13
Group 2
Group 3
Group 4

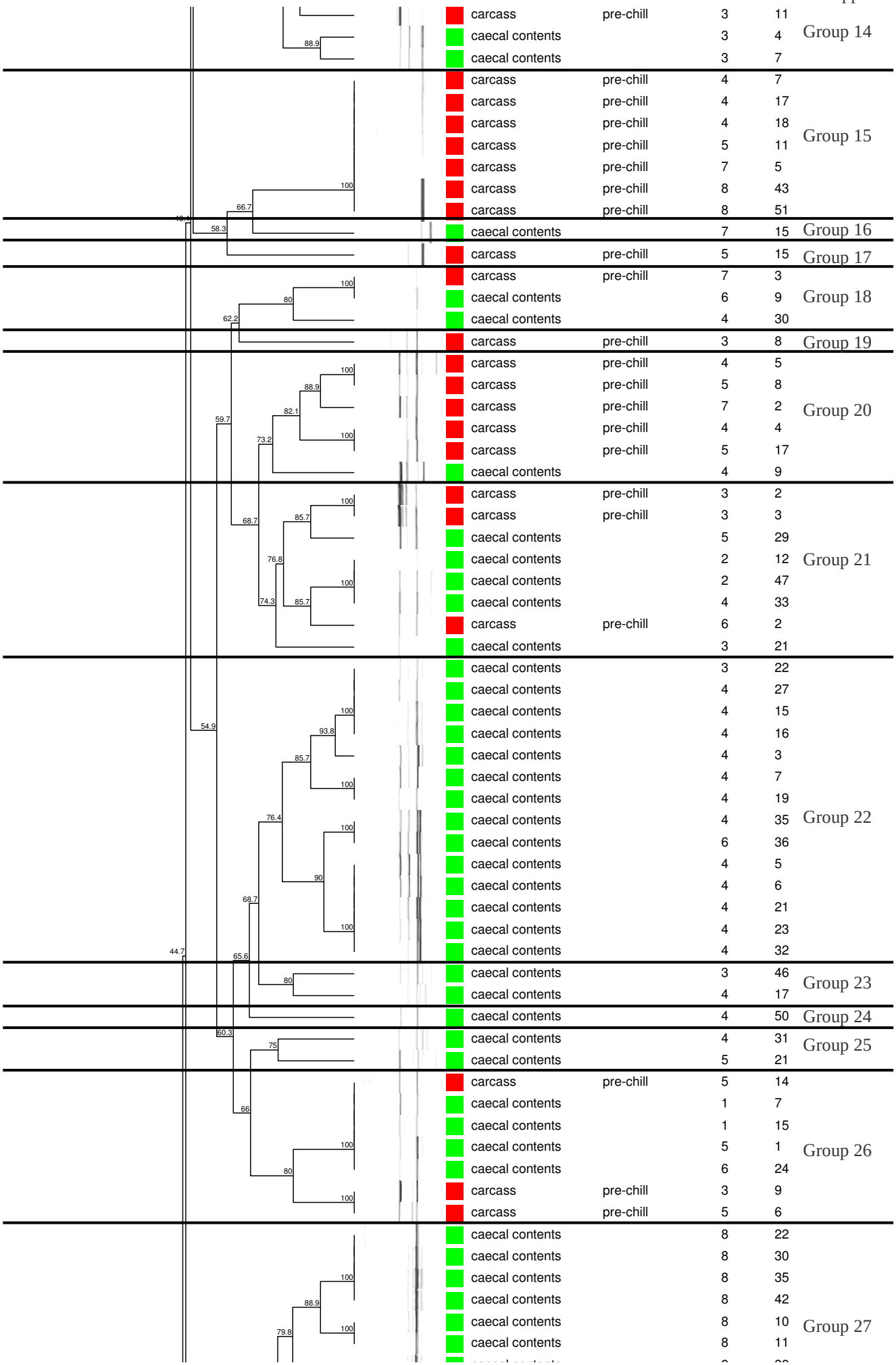


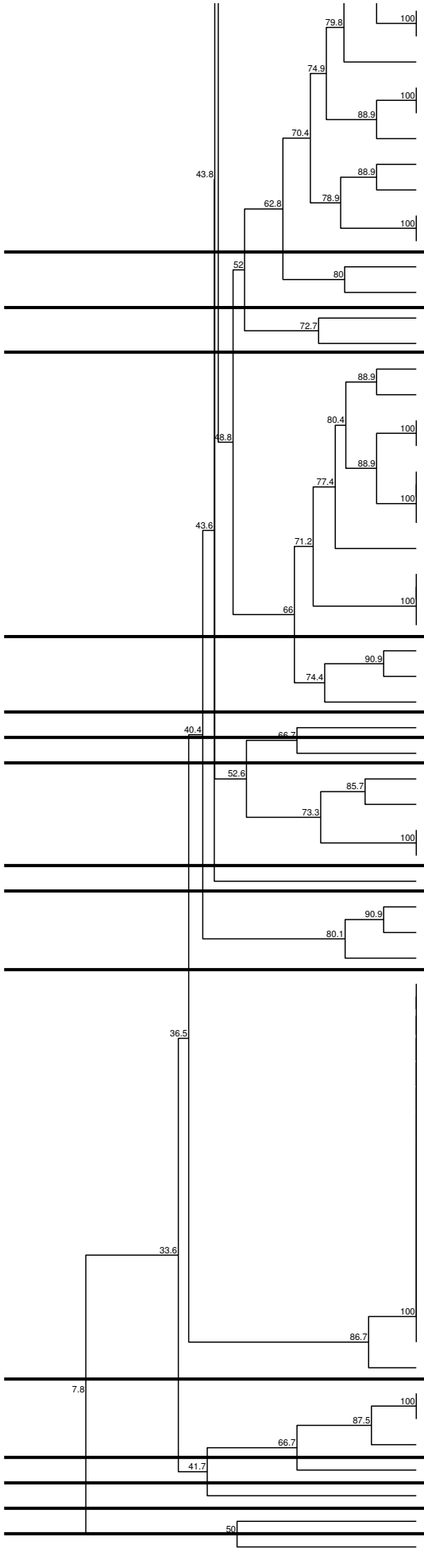

| | | | | |
|---|---|---|---|---|
| carcass | pre-chill | 4 | 23 | |
| carcass | pre-chill | 5 | 13 | |
| caecal contents | | 1 | 6 | Group 4 |
| carcass | pre-chill | 8 | 56 | |
| caecal contents | | 4 | 43 | |
| caecal contents | | 6 | 6 | |
| caecal contents | | 8 | 16 | |
| caecal contents | | 1 | 50 | |
| caecal contents | | 2 | 21 | Group 5 |
| carcass | pre-chill | 4 | 13 | |
| carcass | pre-chill | 8 | 55 | |
| caecal contents | | 8 | 15 | |
| caecal contents | | 1 | 3 | Group 6 |
| caecal contents | | 6 | 46 | |
| caecal contents | | 1 | 26 | |
| caecal contents | | 5 | 24 | |
| carcass | pre-chill | 3 | 5 | |
| carcass | pre-chill | 8 | 58 | |
| caecal contents | | 1 | 21 | |
| caecal contents | | 5 | 22 | |
| caecal contents | | 4 | 39 | |
| carcass | pre-chill | 3 | 14 | |
| carcass | pre-chill | 6 | 1 | Group 7 |
| caecal contents | | 3 | 2 | |
| carcass | pre-chill | 1 | 3 | |
| carcass | pre-chill | 1 | 6 | |
| carcass | pre-chill | 8 | 33 | |
| carcass | pre-chill | 8 | 36 | |
| carcass | pre-chill | 4 | 15 | |
| carcass | pre-chill | 4 | 14 | |
| carcass | pre-chill | 1 | 2 | |
| carcass | pre-chill | 4 | 16 | Group 8 |
| caecal contents | | 5 | 20 | |
| caecal contents | | 5 | 27 | |
| carcass | pre-chill | 1 | 1 | |
| carcass | pre-chill | 2 | 1 | |
| carcass | pre-chill | 2 | 3 | |
| carcass | pre-chill | 4 | 10 | |
| caecal contents | | 2 | 48 | |
| carcass | pre-chill | 5 | 5 | |
| carcass | pre-chill | 7 | 1 | |
| carcass | pre-chill | 7 | 6 | |
| carcass | pre-chill | 8 | 25 | |
| carcass | pre-chill | 8 | 26 | |
| carcass | pre-chill | 8 | 41 | Group 9 |
| carcass | pre-chill | 8 | 44 | |
| carcass | pre-chill | 8 | 45 | |
| carcass | pre-chill | 8 | 46 | |
| carcass | pre-chill | 8 | 47 | |
| carcass | pre-chill | 8 | 52 | |
| carcass | pre-chill | 2 | 4 | |
| carcass | pre-chill | 3 | 4 | |
| carcass | pre-chill | 4 | 9 | |
| carcass | pre-chill | 7 | 7 | |
| carcass | pre-chill | 8 | 23 | |
| carcass | pre-chill | 8 | 27 | |
| carcass | pre-chill | 8 | 32 | |
| carcass | pre-chill | 8 | 40 | |
| carcass | pre-chill | 8 | 49 | |
| carcass | pre-chill | 8 | 50 | |
| carcass | pre-chill | 8 | 62 | |
| carcass | pre-chill | 8 | 63 | |


95.4
55.6
76.9
89.7
100
100
100
71.8
51.9
100
93.1
85.7
83.9
100
83.1
carcass pre-chill 8 62
carcass pre-chill 8 63
carcass pre-chill 8 64
carcass pre-chill 8 65
carcass pre-chill 8 66
caecal contents 1 28
caecal contents 1 36
caecal contents 1 38
caecal contents 2 3
caecal contents 2 34
caecal contents 2 37
caecal contents 3 17
caecal contents 4 42
caecal contents 5 14
caecal contents 5 16
caecal contents 5 25
caecal contents 5 38
caecal contents 7 21
carcass pre-chill 8 28
carcass pre-chill 8 80
carcass pre-chill 8 30
carcass pre-chill 8 34
carcass pre-chill 8 42
carcass pre-chill 8 48
carcass pre-chill 8 53
carcass pre-chill 8 73
carcass pre-chill 8 74
carcass pre-chill 8 76
carcass pre-chill 8 77
carcass pre-chill 8 72
caecal contents 6 43
carcass pre-chill 8 2
carcass pre-chill 8 3
carcass pre-chill 8 4
carcass pre-chill 8 5
carcass pre-chill 8 6
carcass pre-chill 8 8
carcass pre-chill 8 9
carcass pre-chill 8 10
carcass pre-chill 8 12
carcass pre-chill 8 13
carcass pre-chill 8 14
carcass pre-chill 8 15
carcass pre-chill 8 16
carcass pre-chill 8 18
carcass pre-chill 8 19
carcass pre-chill 8 20
carcass pre-chill 8 21
carcass pre-chill 8 22
carcass pre-chill 8 24
carcass pre-chill 8 31
carcass pre-chill 8 57
carcass pre-chill 8 68
caecal contents 1 31
caecal contents 4 37
caecal contents 5 12
caecal contents 3 15
caecal contents 4 14
carcass pre-chill 8 59
caecal contents 1 41
caecal contents 2 22
caecal contents 2 25

Group 9

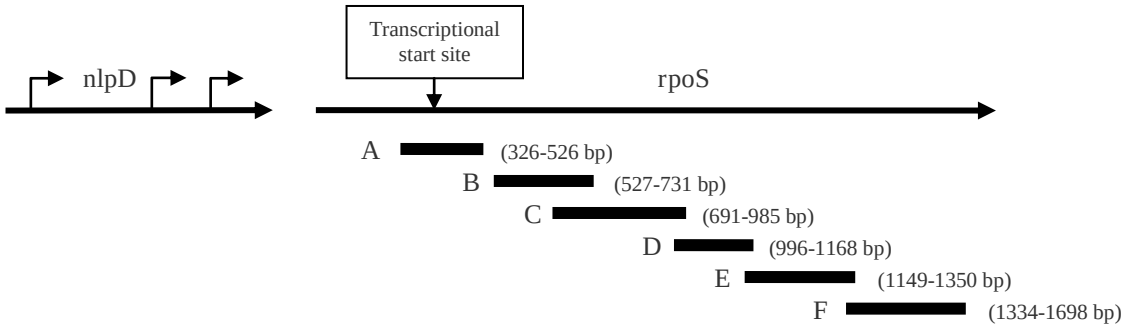

caecal contents 2 22
caecal contents 2 25
caecal contents 3 31
caecal contents 3 45
caecal contents 7 4
caecal contents 3 43
caecal contents 3 3
caecal contents 2 16
caecal contents 4 38
Group 9
carcass pre-chill 4 2
carcass pre-chill 4 3
carcass pre-chill 4 8
carcass pre-chill 4 12
carcass pre-chill 4 19
carcass pre-chill 4 20
carcass pre-chill 4 25
carcass pre-chill 4 26
carcass pre-chill 4 27
caecal contents 1 14
caecal contents 1 18
caecal contents 1 19
caecal contents 1 20
caecal contents 1 27
caecal contents 1 35
caecal contents 1 40
caecal contents 1 44
caecal contents 1 46
caecal contents 1 49
caecal contents 2 50
caecal contents 3 38
carcass pre-chill 4 21
caecal contents 2 40
caecal contents 1 8
caecal contents 1 16
Group 10
carcass pre-chill 7 8
carcass pre-chill 8 11
carcass pre-chill 5 3
Group 11
caecal contents 4 18
caecal contents 6 42
caecal contents 6 44
caecal contents 1 48
Group 12
carcass pre-chill 1 4
polisher break 17
caecal contents 5 5
Group 13
caecal contents 2 41
caecal contents 6 20
caecal contents 3 13
caecal contents 4 47
caecal contents 1 12
caecal contents 1 13
caecal contents 2 9
caecal contents 2 15
caecal contents 2 27
caecal contents 2 31
caecal contents 3 29
caecal contents 4 20
caecal contents 4 45
caecal contents 5 4
caecal contents 5 33
caecal contents 5 34
carcass pre-chill 3 11
caecal contents 3 4
Group 14


carcass pre-chill 3 11
caecal contents 3 4
caecal contents 3 7
Group 14
carcass pre-chill 4 7
carcass pre-chill 4 17
carcass pre-chill 4 18
carcass pre-chill 5 11
carcass pre-chill 7 5
carcass pre-chill 8 43
carcass pre-chill 8 51
Group 15
caecal contents 7 15
Group 16
carcass pre-chill 5 15
Group 17
carcass pre-chill 7 3
caecal contents 6 9
caecal contents 4 30
Group 18
carcass pre-chill 3 8
Group 19
carcass pre-chill 4 5
carcass pre-chill 5 8
carcass pre-chill 7 2
carcass pre-chill 4 4
carcass pre-chill 5 17
caecal contents 4 9
Group 20
carcass pre-chill 3 2
carcass pre-chill 3 3
caecal contents 5 29
caecal contents 2 12
caecal contents 2 47
caecal contents 4 33
carcass pre-chill 6 2
caecal contents 3 21
Group 21
caecal contents 3 22
caecal contents 4 27
caecal contents 4 15
caecal contents 4 16
caecal contents 4 3
caecal contents 4 7
caecal contents 4 19
caecal contents 4 35
caecal contents 6 36
caecal contents 4 5
caecal contents 4 6
caecal contents 4 21
caecal contents 4 23
caecal contents 4 32
Group 22
caecal contents 3 46
caecal contents 4 17
Group 23
caecal contents 4 50
Group 24
caecal contents 4 31
caecal contents 5 21
Group 25
carcass pre-chill 5 14
caecal contents 1 7
caecal contents 1 15
caecal contents 5 1
caecal contents 6 24
carcass pre-chill 3 9
carcass pre-chill 5 6
Group 26
caecal contents 8 22
caecal contents 8 30
caecal contents 8 35
caecal contents 8 42
caecal contents 8 10
caecal contents 8 11
Group 27




| | | | | |
|---|---|---|---|---|
| caecal contents | | 8 | 10 | |
| caecal contents | | 8 | 11 | |
| caecal contents | | 8 | 38 | |
| caecal contents | | 4 | 13 | |
| caecal contents | | 5 | 36 | Group 27 |
| caecal contents | | 4 | 36 | |
| carcass | pre-chill | 2 | 2 | |
| caecal contents | | 1 | 42 | |
| caecal contents | | 2 | 4 | |
| caecal contents | | 2 | 17 | |
| caecal contents | | 6 | 13 | Group 28 |
| caecal contents | | 8 | 6 | |
| caecal contents | | 5 | 9 | Group 29 |
| caecal contents | | 6 | 22 | |
| caecal contents | | 4 | 8 | |
| caecal contents | | 4 | 24 | |
| polisher | break | | 8 | |
| caecal contents | | 3 | 47 | |
| polisher | break | | 3 | |
| caecal contents | | 3 | 10 | Group 30 |
| caecal contents | | 5 | 23 | |
| caecal contents | | 6 | 35 | |
| carcass | pre-chill | 4 | 24 | |
| caecal contents | | 4 | 40 | |
| caecal contents | | 6 | 10 | |
| caecal contents | | 5 | 19 | |
| caecal contents | | 5 | 35 | Group 31 |
| polisher | break | | 2 | |
| caecal contents | | 1 | 9 | Group 32 |
| caecal contents | | 1 | 47 | Group 33 |
| carcass | pre-chill | 4 | 22 | |
| caecal contents | | 5 | 37 | Group 34 |
| caecal contents | | 2 | 35 | |
| caecal contents | | 6 | 8 | |
| caecal contents | | 1 | 17 | Group 35 |
| carcass | pre-chill | 3 | 7 | |
| carcass | pre-chill | 3 | 10 | Group 36 |
| carcass | pre-chill | 3 | 13 | |
| carcass | pre-chill | 3 | 1 | |
| carcass | pre-chill | 4 | 11 | |
| carcass | pre-chill | 5 | 7 | |
| carcass | pre-chill | 7 | 9 | |
| carcass | pre-chill | 8 | 29 | |
| carcass | pre-chill | 8 | 37 | |
| carcass | pre-chill | 8 | 38 | |
| carcass | pre-chill | 8 | 39 | Group 37 |
| carcass | pre-chill | 8 | 54 | |
| carcass | pre-chill | 8 | 60 | |
| carcass | pre-chill | 8 | 69 | |
| carcass | pre-chill | 8 | 70 | |
| carcass | pre-chill | 8 | 71 | |
| carcass | pre-chill | 8 | 78 | |
| caecal contents | | 1 | 30 | |
| carcass | pre-chill | 8 | 79 | |
| caecal contents | | 3 | 19 | |
| caecal contents | | 6 | 47 | Group 38 |
| caecal contents | | 7 | 5 | |
| caecal contents | | 4 | 10 | Group 39 |
| carcass | pre-chill | 5 | 4 | Group 40 |
| caecal contents | | 4 | 44 | Group 41 |
| caecal contents | | 4 | 49 | Group 42 |

Appendix 7.1. The locations of the primers utilized for the amplification of the rpoS fragments and oligonucleotide sequences of the six primer sets for PCR detection (adopted from Jordan et al. 1999).




| **Primer code** | **Oligonucleotide sequences (5'-3')** | **Melting temperature (°C)** | **Amplified product (bp)** |
|---|---|---|---|
| rposAf | CGTAAACCCGCTGCGTTAT | 56.7 | 200 |
| rposAr | GGTTCCTCTTCACTCAAGGCTT | 60.3 | |
| rposBf | AGTGATAACGACCTGGCTGAAGAA | 61.0 | 204 |
| rposBr | GTTACTCTCAATCATGCGACGG | 60.3 | |
| rposCf | GAGAGTAACCTGCTGGTGGTA | 59.8 | 294 |
| rposCr | TATGCGACAACTCACGTGCG | 59.4 | |
| rposDf | CGCACGTGAGTTGTCGCATA | 59.4 | 202 |
| rposDr | TGTCTTCCGGACCGTTCTCTT | 59.8 | |
| rposEf | AGAGAACGGTCCGGAAGACA | 59.4 | 201 |
| rposEr | GCCTTCAACCTGAATCTGACG | 59.8 | |
| rposFf | AGATTCAGCTTGAAGGCCTGC | 59.8 | 364 |
| rposFr | CCTTGCCCGGGCTGTGCCGATGCAC | 72.8 | |

Appendix 7.2. Original data for D value calculation.

(a) Slaughterhouse A isolates, testing on stationary phase cultures

| | time | post-bleed 7-3 | pre-scald 10-3 | post-dehair 10-5 |
|---|---|---|---|---|
| Batch 1 | 0 | 7.40 | 7.58 | 6.90 |
| | 30 | 5.53 | 6.95 | 6.56 |
| | 60 | 5.14 | 5.68 | 6.53 |
| | 180 | 3.88 | 5.12 | 4.92 |
| | 300 | 3.31 | 4.79 | 4.36 |
| | 600 | 1.78 | 4.18 | 4.04 |
| $D_{55℃}$ (min) | | 2.01 | 6.41 | 1.87 |
| $r^2$ | | (0.97) | (0.96) | (0.95) |
| | | | | |
| Batch 2 | 0 | 6.81 | 6.62 | 6.53 |
| | 30 | 4.99 | 5.06 | 5.30 |
| | 60 | 4.69 | 4.77 | 4.83 |
| | 180 | 3.49 | 4.17 | 4.27 |
| | 300 | 3.25 | 3.62 | 3.97 |
| | 600 | 2.40 | 3.16 | 3.45 |
| $D_{55℃}$ (min) | | 2.49 | 5.75 | 6.94 |
| $r^2$ | | (0.91) | (0.91) | (0.99) |
| | | | | |
| Batch 3 | 0 | 6.99 | 7.26 | 6.87 |
| | 30 | 6.00 | 6.95 | 6.66 |
| | 60 | 5.70 | 6.19 | 6.65 |
| | 180 | 4.10 | 5.59 | 5.70 |
| | 300 | 3.36 | 5.14 | 5.31 |
| | 600 | 2.32 | 4.00 | 4.69 |
| $D_{55℃}$ (min) | | 2.60 | 3.55 | 3.09 |
| $r^2$ | | (0.90) | (0.92) | (0.96) |
| | | | | |
| D1 | | 2.01 | 6.41 | 1.87 |
| D2 | | 2.49 | 5.75 | 6.94 |
| D3 | | 2.60 | 3.55 | 3.09 |
| mean D | | 2.37 | 5.23 | 3.97 |
| sd | | 0.31 | 1.50 | 2.65 |

Appendix 7.2. continued.

(b) Slaughterhouse A isolates, testing on exponential phase cultures

| | time | post-bleed 7-3 | pre-scald 10-3 | post-dehair 10-5 |
|---|---|---|---|---|
| Batch 1 | 0 | 5.97 | 6.82 | 6.44 |
| | 30 | 1.50 | 1.50 | 4.46 |
| | 60 | ND† | ND | 2.85 |
| | 180 | ND | ND | 0.74 |
| | 300 | ND | ND | ND |
| | 600 | ND | ND | ND |
| $D_{55℃}$ (min) | | 0.11* | 0.09* | 0.73 |
| $r^2$ | | (1) | (1) | (0.94) |
| | | | | |
| Batch 2 | 0 | 6.51 | 7.26 | 7.21 |
| | 30 | 3.30 | 4.87 | 6.34 |
| | 60 | ND | 3.42 | 4.77 |
| | 180 | ND | ND | 3.00 |
| | 300 | ND | ND | ND |
| | 600 | ND | ND | ND |
| $D_{55℃}$ (min) | | 0.16* | 0.26 | 0.74 |
| $r^2$ | | (1) | (0.98) | (0.93) |
| | | | | |
| Batch 3 | 0 | 7.47 | 7.28 | 7.89 |
| | 30 | 4.90 | 5.00 | 4.81 |
| | 60 | 3.44 | 3.42 | 3.86 |
| | 180 | ND | ND | 1.90 |
| | 300 | ND | ND | ND |
| | 600 | ND | ND | ND |
| $D_{55℃}$ (min) | | 0.25 | 0.26 | 0.64 |
| $r^2$ | | (0.98) | (0.99) | (0.91) |
| | | | | |
| D1 | | 0.11 | 0.09 | 0.73 |
| D2 | | 0.16 | 0.26 | 0.74 |
| D3 | | 0.25 | 0.26 | 0.64 |
| mean D | | 0.17 | 0.20 | 0.70 |
| sd | | 0.07 | 0.10 | 0.05 |

† ND = not detected;

*The $D_{55℃}$ were generated by two available count values.

Appendix 7.2. continued.

(c) Slaughterhouse B isolates, testing on stationary phase cultures;

| | time | Post-polish 84 | Post-singe 84 | Pre-singe 83 |
|---|---|---|---|---|
| Batch 1 | 0 | 6.93 | 7.20 | 6.71 |
| | 30 | 5.55 | 6.04 | 5.25 |
| | 60 | 3.96 | 5.58 | 5.06 |
| | 180 | ND† | 4.98 | 4.20 |
| | 300 | ND | 4.49 | 3.74 |
| | 600 | ND | 4.05 | 3.08 |
| $D_{55℃}$ (min) | | 0.34 | 3.09 | 2.92 |
| $r^2$ | | (0.99) | (0.96) | (0.98) |
| | | | | |
| Batch 2 | 0 | 7.13 | 7.46 | 7.74 |
| | 30 | 5.29 | 6.56 | 6.40 |
| | 60 | 4.15 | 6.54 | 6.27 |
| | 180 | 2.12 | 5.34 | 5.06 |
| | 300 | ND | 3.32 | 4.25 |
| | 600 | ND | 2.19 | 3.31 |
| $D_{55℃}$ (min) | | 0.66 | 2.08 | 3.21 |
| $r^2$ | | 0.91 | 0.90 | 0.92 |
| | | | | |
| D1 | | 0.34 | 3.09 | 2.92 |
| D2 | | 0.66 | 2.08 | 3.21 |
| mean D | | 0.50 | 2.58 | 3.06 |
| sd | | 0.23 | 0.71 | 0.20 |

† ND = not detected

Appendix 7.2. continued.

(d) Slaughterhouse B isolates, testing on exponential phase cultures;

| | time | Post-polish 84 | Post-singe 84 | Pre-singe 83 |
|---|---|---|---|---|
| Batch 1 | 0 | 7.65 | 6.73 | 7.29 |
| | 30 | 4.49 | 6.60 | 6.51 |
| | 60 | 2.69 | 5.11 | 5.54 |
| | 180 | ND | 2.67 | 2.55 |
| | 300 | ND | 2.50 | 2.16 |
| | 600 | | | |
| $D_{55℃}$ (min) | | 0.20 | 0.71 | 0.63 |
| $r^2$ | | 0.98 | 0.97 | 0.99 |
| | | | | |
| Batch 2 | 0 | 6.28 | 6.99 | 7.04 |
| | 30 | 4.32 | 5.84 | 4.19 |
| | 60 | 2.10 | 5.27 | 3.40 |
| | 180 | ND | 3.46 | ND |
| | 300 | ND | 2.73 | ND |
| | 600 | | | |
| $D_{55℃}$ (min) | | 0.24 | 0.91 | 0.28 |
| $r^2$ | | 0.99 | 0.96 | 0.90 |
| | | | | |
| D1 | | 0.20 | 0.71 | 0.63 |
| D2 | | 0.24 | 0.91 | 0.28 |
| mean D | | 0.22 | 0.81 | 0.45 |
| sd | | 0.03 | 0.14 | 0.25 |

Appendix 8.1. Oligonucleotide sequences of primers for detection of virulence genes.

| Pathogenicity | Virulence determinants | Primer code | Oligonucleotide sequences (5'-3') | Melting temperature (°C) | Amplified product (BP) | reference |
|---|---|---|---|---|---|---|
| ETEC | LT | LTF | GCACACGGAGCTCCTCAGTC | 63.5 | 218 | (Vidal et al., 2004) |
| | | LTR | TCCTTCATCCTTTCAATGGCTTT | 57.1 | | |
| | ST | STA1 | TCTTTCCCCTCTTTAGTCAG | 55.3 | 166 | (Osek, 2001) |
| | | STA2 | ACAGGCCGGATTACAACAAAG | 57.9 | | |
| | F4 | F4F | GGTGATTTCAATGGTTCG | 51.4 | 782 | (Do et al., 2005) |
| | | F4R | ATTGCTACGTTCAGCGGAGCG | 61.8 | | |
| EPEC/ EHEC | eae | EAEF | TCAATGCAGTTCCGTTATCAGTT | 57.1 | 482 | (Stacy-Phipps et al., 1995) |
| | | EAER | GTAAAGTCCGTTACCCCAACCTG | 62.4 | | |
| VTEC/ EHEC | Stx | LIN3 | TTTGATTGTTACAGTCAT | 44.6 | 900 | (Lin et al., 1993) |
| | | LIN5 | GAACGAAATAATTTATATGT | 45.0 | | |
| | stx1 | LP30 | CAGTTAATGTGGTGGCGAAGG | 59.8 | 348 | (Cebula et al., 1995) |
| | | LP31 | CACCAGACAATGTAACCGCTG | 59.8 | | |
| | stx2 | LP43 | ATCCTATTCCCGGGAGTTTACG | 60.3 | 584 | (Cebula et al., 1995) |
| | | LP44 | GCGTCATCGTATACACAGGAGC | 62.1 | | |
| | Stx2e | SLTAv1 | CCTTAACTAAAAGGAATATA | 47.1 | 726 | (Pohl et. al., 1992) |
| | | SLTAv2 | CTGGTGGTGTATGATTAATA | 51.2 | | |
| EAggEC | EAST1 | astA-F | CCATCAACACAGTATATCCGA | 50.0 | 111 | (Jenkins et al., 2006) |
| | | astA-R | GGTCGCGAGTGACGGCTTTGT | 39.3 | | |

Appendix 8.2. List of E. coli virulence genes screened by Identibac DNA array (chip version: no. 03m).

| Name of the probe | Gene description | Genbank ID [binding site of probe] |
|---|---|---|
| K88ab_10 | K88/F4 protein subunit gene | AJ616236.1[61:86] |
| astA_consens_10 | heat-stable enterotoxin 1 | AB042002.1[46:67] |
| bfpA_10 | major subunit of bundle-forming pili | AB024946.1[2807:2832] |
| cba_10 | colicin B- pore forming | CP001232.1[123951:123974] |
| ccl_10 | Cloacin | X04466.1[2876:2898:r] |
| cdtB_40 | cytolethal distending toxin B | AJ508930.1[1199:1221] |
| cdtB_50 | cytolethal distending toxin B | AY423897.1[244:266] |
| cdtB_60 | cytolethal distending toxin B | AY423896.1[245:266] |
| celb_10 | endonuclease colicin E2 | D00021.1[156:179] |
| cfa_c_10 | colonisation factor antigen I | AF296132.1[2121:2145] |
| cma_20 | colicin M - resembles B-lactam | CP000971.1[83114:83136] |
| cnf1_20 | cytotoxic necrotizing factor | AM261284.1[17107:17134] |
| cofA_10 | longus type IV pilus | AB049751.1[3154:3177] |
| eae_consensus_10 | intimin | AF022236.1[25466:25492] |
| eae_consensus_20 | intimin | AF116899.1[1017:1042] |
| eae_consensus_30 | intimin | AF022236.1[25697:25724] |
| eae_consensus_40 | intimin | AJ705050.1[849:875] |
| espB_O157_20 | secreted protein B | AE005174.2[4660387:4660410:r] |
| espB_O26_40 | secreted protein B | AJ287768.1[372:393] |
| f17-A_40 | subunit A of F17 fimbrial protein | AF055306.1[473:494] |
| f17-A_50 | subunit A of F17 fimbrial protein | AF055308.1[472:493] |
| f17-A_60 | major fimbrial subunit (F17b-A) | L14318.1[781:803] |
| f17-G_20 | adhesin subunit of F17 fimbrial protein | AF022140.1[4334:4359] |
| fanA_10 | involved in biogenesis ofK99/F5fimbriae | X05797.1[537:563] |
| fasA_10 | fimbriae 987P/F6 subunit | M35257.1[395:421] |
| fedA_10 | fimbrial protein F107 subunit A | AM293592.1[99:125] |
| fedF10_ | fimbrialadhesinACprecursor( | AY9707821[117]. |
| fim41a_10 | mature Fim41a/F41 protein | M21788.1[619:645] |
| gad_10 | glutamate decarboxylase | AE005174.2[1995950:1995972] |
| hlyA_20 | (hlyA) haemolysin A | AB011549.2[16866:16892] |
| hlyE_10 | avian E. coli haemolysin | AF052225.1[284:309] |
| ipaD_10 | invasion protein Shigella flexneri | AF348706.1[102588:102610:r] |
| ipaH9.8_20 | invasion plasmid antigen | AE005674.1[1422658:1422680:r] |
| ireA_20 | siderophore receptor | AE014075.1[4936546:4936571] |
| iroN_10 | enterobactin siderophore receptor protein | AE014075.1[1206674:1206699:r] |
| iss_10 | increased serum survival | AE014075.1[1423127:1423151:r] |
| lngA_20 | longus type IV pilus | AF004308.1[599:623] |
| ltcA_20 | heat-labile enterotoxin A subunit | CP000795.1[17038:17062] |
| mchB_10 | microcin H47 part of colicin H | AE014075.1[1176953:1176976] |
| mchC_20 | MchC protein | AE014075.1[1177890:1177914] |
| mchF_10 | ABC transporter protein MchF | AE014075.1[1180831:1180855] |
| mcmA_10 | microcin M part of colicin H | AE014075.1[1183303:1183327] |
| nfaE_10 | diffuse adherence fibrillar adhesin gene | AF325672.1[1098:1120] |
| perA_10 | EPEC adherence cactor, transcriptional activator | AB024946.1[21465:21491] |
| perA_20 | EPEC adherence cactor, transcriptional activator | AF255770.1[1041:1065] |
| pet_20 | autotransporter enterotoxin | AF056581.1[2732:2758] |
| prfB_30 | P-related fimbriae regulatory gene | AE014075.1[3437791:3437815:r] |
| senB_20 | plasmid encoded enterotoxin | CP000038.1[2824468:2824493] |
| sfaS_10 | S fimbriae minor subunit | CP000243.1[1104674:1104698] |
| sta1_110 | heat-stable enterotoxin ST-Ia | AJ555214.1[1835:1862:r] |
| sta2_210 | heat-stable enterotoxin ST Ib | AY342058.1[135:161] |
| stb_10 | heat stable enterotoxin II | AJ555214.1[6226:6249] |
| stx1A_10 | shiga-like toxin 1 A-subunit | AB015056.1[395:421] |
| stx2A_10 | shiga-like toxin 2 A-subunit | AB015057.1[414:439] |
| virF_20 | virF transcriptional activator, ipaBCD positive regulator | AF348706.1[36555:36582:r] |
| hp_cif_611 | type III secreted effector | AB285204.1[22533:22558] |
| hp_eaaA_611 | (eaaC) SPATE | AB255744.1[6159:6186] |
| hp_eatA_611 | SPATE | AY163491.2[1529:1553] |
| hp_efa1_611 | EHEC factor for adherence | AF159462.2[4706:4732] |
| hp_epeA_611 | SPATE | AY258503.2[49008:49033] |
| hp_espA_Crod_611 | type III secretion system | AF311901.1[31925:31950] |
| hpespAO103H2611hp_espA__ | typeIIIsecretionsystemtype tion t | AF0544211[915:945].: |
| hp_espA_O119H6_611 | type III secretion system | AJ225016.1[19:48] |

Appendix 8.2. Continued.

| | | |
|---|---|---|
| hp_espA_O127H7_611 | type III secretion system | AF022236.1[30702:30732] |
| hp_espA_O157H11_611 | type III secretion system | AE005174.2[4662650:4662677:r] |
| hp_espA_O49H12_611 | type III secretion system | AJ303141.2[71426:71453:r] |
| hp_espA_O55H7_611 | type III secretion system | AJ225020.1[348:377] |
| hp_espA_O8_611 | type III secretion system | AJ633130.1[32164:32190] |
| hp_espC_611 | SPATE | AF297061.1[6363:6389] |
| hp_espF_611 | type III secretion system | AE005174.2[4658919:4658944:r] |
| hp_espF_612 | type III secretion system | AF041809.1[559:584] |
| hp_espF_Crod_611 | type III secretion system | AF311901.1[35480:35502] |
| hp_espF_O103H2_611 | type III secretion system | AF116900.1[548:575] |
| hp_espF_O103H2_612 | type III secretion system | AJ633130.1[35605:35630] |
| hp_espI_611 | SPATE | AJ278144.1[14583:14607:r] |
| hp_espJ_611 | prophage encoded type III ss effector | AE005174.2[2744307:2744334] |
| hp_espJ_612 | prophage encoded type III ss effector | AB303060.1[9417:9443:r] |
| hp_espP_611 | putative exoprotein-precursor | AB011549.2[81080:81104] |
| hp_etpD_611 | type II secretion protein | AB011549.2[3983:4008] |
| hp_iha_611 | adherence protein | AE005174.2[1106944:1106972:r] |
| hp_katP_611 | catalase peroxidase, plasmid encoded | AB011549.2[77111:77137] |
| hp_lpfA_611 | long polar fimbriae | AB198066.1[464:491] |
| hp_nleA_611 | non LEE encoded effector A | AY373261.1[293:323] |
| hp_nleA_612 | non LEE encoded effector A | AM421997.1[288:317] |
| hp_nleA_613 | non LEE encoded effector A | AB303062.1[8368:8398:r] |
| hp_nleA_614 | non LEE encoded effector A | AM422003.1[288:317] |
| hp_nleB_611 | non LEE encoded effector B | AE005174.2[3931067:3931090] |
| hp_nleB_O157H7_611 | non LEE encoded effector B | AB303062.1[2834:2861] |
| hp_nleB_Styp_611 | non LEE encoded effector B | AE008894.1[13149:13174] |
| hp_nleC_611 | non LEE encoded effector C | AE005174.2[927628:927658] |
| hppic611hp_pic_611 | SPATESPATE | AE0056741[30714723071500r]5674.1[30:00:r] |
| hp_rpeA_611 | SPATE | AY552473.1[2055:2079] |
| hp_saa_611 | auto agglutinating adhesin | AF399919.3[6816:6842] |
| hp_sat_611 | SPATE | AE014075.1[3457345:3457369:r] |
| hp_sepA_611 | SPATE | AY604009.1[174:199] |
| hp_sigA_611 | SPATE | AE005674.1[3062073:3062100] |
| hp_stxA2_611 | shiga toxin 2 subunit A | AB232172.1[457:479] |
| hp_stxA2_613 | shiga toxin 2 subunit A | AB232172.1[534:557] |
| hp_stxA2_614 | shiga toxin 2 subunit A | AB015057.1[508:536] |
| hp_stxA2_615 | shiga toxin 2 subunit A | AB012101.1[497:523] |
| hp_stxA2_616 | shiga toxin 2 subunit A | AM904726.1[284:311] |
| hp_stxA2_617 | shiga toxin 2 subunit A | AB048227.1[196:222] |
| hp_stxA2_618 | shiga toxin 2 subunit A | AB015057.1[218:245] |
| hp_stxB2_612 | shiga toxin 2 subunit B | AB012101.1[987:1016] |
| hp_stxB2_613 | shiga toxin 2 subunit B | DQ059012.1[1114:1137] |
| hp_stxB2_614 | shiga toxin 2 subunit B | AB028899.1[253:275] |
| hp_stxB2_615 | shiga toxin 2 subunit B | AB252836.1[1297:1325] |
| hp_subA_611 | subtilase toxin subunit | AF399919.3[14610:14636:r] |
| hp_tccP_611 | tir-cytoskeleton coupling protein | AB253550.1[84:107] |
| hp_tccP_612 | tir-cytoskeleton coupling protein | AB253537.1[183:209] |
| hp_tir_4051.6_611 | translocated intimin receptor protein | AB288103.1[1347:1373] |
| hp_tir_MPEC_611 | translocated intimin receptor protein | AB026719.1[228:253] |
| hp_tir_NTH19_611 | translocated intimin receptor protein | AB288104.1[22:46] |
| hp_tir_O103H2_611 | translocated intimin receptor protein | AF045568.1[642:668] |
| hp_tir_O111_611 | translocated intimin receptor protein | AF025311.1[1453:1480] |
| hp_tir_O157H45_611 | translocated intimin receptor protein | AB036053.1[246:270] |
| hp_tir_O157H7_611 | translocated intimin receptor protein | AE005174.2[4670414:4670442:r] |
| hp_toxB_611 | toxin B | AB011549.2[56953:56981] |
| hp_toxB_612 | toxin B | AB011549.2[65192:65217] |
| hp_toxB_613 | toxin B | AB011549.2[64985:65015] |
| hp_tsh_611 | SPATE | AF218073.1[5135:5158] |
| hp_vat_611 | SPATE | AE016756.1[75241:75264:r] |
| prob_ihfA_611 | control probe | CP000653.1[1880477:1880504] |
| prob_gapA_611 | control probe | CP000822.1[1712061:1712089:r] |

## Appendix 8.3. List of E. coli antimicrobial resistance (AMR) genes screened by Identibac DNA array (chip version: no. 05m).

| Gene description | Genbank ID [binding site of probe] |
|---|---|
| aadA aadA1 aminoglycoside; Pseudomonas aeruginosa | AB104852.1[2802:2827] |
| aadA2, aadA2a,aadA2b, aadA2c, aadA3 aadA8 aminoglycoside; Corynebacterium glutamicum | AB027715.1[5446:5469] |
| aadA4 aadA5 aminoglycoside; Acinetobacter baumannii | AF364344.1[1146:1173] |
| aadB gene cassette; Salmonella typhimurium | AB186118.1[301:328] |
| all blaCMY genes plasmidic AmpC; Citrobacter freundii | AB429270.1[107:131] |
| all blaFOX genes plasmidic AmpC; Aeromonas punctata | AF462690.1[1606:1629] |
| all blaFOX genes plasmidic AmpC; E. coli | AJ277535.1[116:140] |
| all blaMOX genes plasmidic AmpC; E. coli | AB061794.1[6143:6166] |
| all blaSHV beta-lactam; E. coli | AB023477.1[255:281] |
| all blaTEM genes beta-lactam; E. coli | AB038654.1[36:63] |
| known as aacA4 aac(6')-1b-cr aminoglycoside; Serratia marcescens | AB070224.1[2449:2472] |
| known as aacC1 aminoglycoside; Klebsiella pneumoniae | AF207065.1[975:998] |
| aminoglycoside; Actinobacillus pleuropneumoniae | AB109805.1[2007:2029] |
| aminoglycoside; Pseudomonas stutzeri | AJ493432.1[13:38] |
| AmpC beta-lactamase; Enterobacter asburiae | AJ311172.1[762:786] |
| beta-lactam; Enterobacter aerogenes | AF034958.3[4350:4375] |
| beta-lactamase OXA-16; Pseudomonas aeruginosa | AF043100.1[193:220] |
| beta-lactamase; Citrobacter youngae | AJ487978.2[94:119] |
| blaACC-1 plasmidic AmpC; Hafnia alvei | AF180952.1[2487:2510] |
| blaACC-1 plasmidic AmpC; Klebsiella pneumoniae | AJ133121.1[686:709] |
| blaCARB-1 -2 -3 -8 blaPSE-5 carbenicillinase; Salmonella typhimurium | AB126603.1[479:505] |
| blaCARB-1 -2 -3 -8 blaPSE-5 carbenicillinase; Vibrio cholerae | AF409092.1[1168:1195] |
| bla-CMY-1b-8-8b-9-10-11-19- and blaMOX-1 beta-lactam; Enterobacter aerogenes | AF357597.1[1008:1032] |
| blaCTX-M -1 -3 -10 -12 -15 -22 -23 –28 Fec-1 beta-lactam; Citrobacter koseri | AB059404.1[48:73] |
| blaCTX-M -2 -4 –5 -6 -7 -20 Toho-1 beta-lactam; E. coli | AB098539.1[109:132] |
| M-2-4–5-6-7-20Toho-1beta-Klebsiellapneumoniae | AB1765321[38]. |
| blaCTX-M –25-39-41 beta-lactam; E. coli | |
| blaCTX-M –8-40-63 beta-lactam; Klebsiella pneumoniae | AB205197.1[596:620] |
| blaDHA -1 -2 plasmidic AmpC; Morganella morganii | AF055067.1[1737:1761] |
| blaLEN-1 beta-lactam; Klebsiella pneumoniae | AF452105.1[255:279] |
| blaMOX-2, beta-lactamase; Klebsiella pneumoniae | AJ276453.1[5377:5400] |
| blaOXA –1 –30 -31 -33 beta-lactam; Salmonella enterica | AB218659.1[467:493] |
| blaOXA- -2 -3 -15 -20 -21 -22 -32 -34 -36 beta-lactam; Pseudomonas aeruginosa | AB188812.1[3087:3110] |
| catB3 catB4 chloramphenicol acetyltransferase; Salmonella typhimurium | AB186118.1[1043:1068] |
| cefotaximase; Shigella sonnei | AB284167.2[701:725] |
| chloramphenicol acetyltransferase; Klebsiella pneumoniae | AF227506.1[593:619] |
| chloramphenicol acetyltransferase; Mannheimia haemolytica | AJ249249.1[2525:2548] |
| chloramphenicol acetyltransferase; Photobacterium damselae | AB277723.1[9222:9245] |
| chloramphenicol/ florfenicol ; Vibrio cholerae | AB114188.1[11866:11890] |
| class 1 integrase; Corynebacterium glutamicum | AB027715.1[4127:4152:r] |
| class 2 integrase; E. coli | AJ001816.1[1602:1625:r] |
| class C beta-lactamase; E. coli | AY339625.2[4195:4218:r] |
| cmlA cmlA1 cmlA4 cmlA5 cmlA6 cmlA7 chloramphenicol exporter; E. coli | AB212941.1[1970:1993] |
| dfrA13 gene for dihydrofolate reductase; Salmonella enterica | AJ870926.1[5530:5553] |
| dfrA15 gene for dihydrofolate reductase; Vibrio cholerae | AB113114.1[449:476] |
| dihydrofolate reductase; E. coli | AB188269.1[364:387] |
| ereB, ereB type II erythromycin resistance; E. coli | AB207867.1[6812:6838] |
| erythromycin resistance; E. coli | AB089505.1[89:117] |
| extended-spectrum beta-lactamase; E. coli | AJ786366.1[313:336] |
| qnrA quinolone; Salmonella enteritidis | AY906856.1[88:112] |
| qnrA quinolone; Shewanella putrefaciens | AB325578.1[88:112] |
| quinolone; Citrobacter freundii | AB281054.1[505:532] |
| quinolone; Klebsiella pneumoniae | AJ971343.1[13776:13803:r] |
| quinolone; Shigella flexneri | AB178643.1[1918:1942] |
| sulphonamide; E. coli | AJ459418.2[3069:3095] |
| sulphonamide; Salmonella enterica | AB076707.2[183:207:r] |
| tetracycline; Aliivibrio salmonicida | AJ289103.1[4025:4050:r] |
| tetracycline; Citrobacter sp. TA3 | AB089599.1[265:289] |

Appendix 8.3. Continued.

| | |
|---|---|
| tetracycline; Vibrio cholerae | AB114188.1[16714:16738] |
| tetracycline; Gram-negative bacterium TA57 | AB089596.1[781:804] |
| tetracycline; Neisseria meningitidis | AB084245.1[819:844] |
| tetracycline;Pseudomonassptetracyc Pseudomonas sp. | AF1331391[2158:2182].:2182] |
| trimethoprim; Cloning vector pSB11 | AB027256.2[2867:2893:r] |
| trimethoprim; E. coli | AJ419170.1[163:188] |
| trimethoprim; Salmonella enteritidis | AB126604.1[143:169] |
| trimethoprim; Salmonella typhimurium | AF393510.1[260:285] |
| type I ereA, ereA2 erythromycin resistance; E. coli | AB188269.1[982:1006] |
| vatE-3-4-5-6-7-8, satG acetyltransferase; Enterococcus faecium | AF139725.1[372:399] |
| control probe; E. coli | AE005174.2[210552:210579] |
| control probe; Enterobacter sp. 638 | CP000653.1[1826614:1826642] |
| control probe; Salmonella enterica | AE008705.1[14591:14618] |

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