PULSED-FIELD GEL ELECTROPHORESIS: A TOOL FOR MOLECULAR EPIDEMIOLOGY

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ABSTRACT

Salmonella Brandenburg was the causative organism in abortion storms that occurred in 1998. Pulsed-Field Gel Electrophoresis was evaluated as a strain typing technique to determine whether one strain or several caused the outbreak. PFGE was successful for over 170 isolates, and it quickly became apparent that there was only one strain involved in the outbreak. PFGE was used to identify whether strains isolated from birds on case farms were the same as the outbreak strain. The outbreak strain had a particular fingerprint or pattern. This strain differed from the *S.* Brandenburg reference strain held by ESR (Environmental Science and Research) in Porirua, Wellington. The similarity between the outbreak strain and the reference strain was only 57.1%. This study showed the strain type found in all the animals that aborted was clonally derived (stemmed from a single ancestral type). Spread of the organism in future should be easier to control than if several strain types were observed. The outbreak strain type was seen throughout the laboratory-based investigation and the origins of this strain have been hypothesised.

Staphylococcus aureus is a major pathogen of bovine mastitis. Isolates previously cultured from farms in the North Island. This study examined the homogeneity between strain types of *S.* aureus. *S. aureus* strains were similar to each other indicating perpetuation of certain clones. One strain type dominated and indistinguishable patterns were isolated from four different animals on three different farms. Different strains were also isolated from the same quarter on subsequent sampling.

ACKNOWLEDGEMENTS

To all those who have put up with me: my supervisors, my colleagues, my friends and my loved ones.

I'm not entirely sure what to write. Having recently completed typing this missive, I may just be all run out of words. But apparently, this is the section where colloquialisms are allowed. However, short of something I can read off a Tele-prompt at a Hollywood award presentation thingy, I'd like to take this opportunity to say THANKYOU to you all.

CHEERS!

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1 INTRODUCTION

This study was conducted to examine the use of Pulsed-Field Gel Electrophoresis (PFGE), as a tool for molecular epidemiology, in two different situations.

Salmonella Brandenburg was the causative organism in abortion storms that occurred from 1996 to 1999. PFGE was used as the strain typing technique to determine whether the outbreak was caused by one strain or several. PFGE was successful, and it quickly became apparent that there was only one strain involved in the outbreak. Black-backed gulls were implicated in the spread of the organism, as several were seen by farmers and veterinarians further inland than previously noticed. PFGE was used to identify whether strains isolated from birds on case farms were the same as the outbreak strain.

Staphylococcus aureus is recognised worldwide as the most difficult mastitis pathogen to control. It is ubiquitous, contagious, able to evade the hosts natural defences and responds poorly to antimicrobial treatment. Infections tend to be subclinical, producing mild to moderate elevations in herd Somatic Cell Counts. PFGE was used to study isolates previously cultured from farms in the North Island. This study showed the homogeneity between strain types of *S. aureus*, indicating perpetuation of certain strains.

To summarise PFGE was a successful tool for studying the epidemiology of both a new disease, and in the investigation of an endemic disease.

2 PULSED FIELD GEL ELECTROPHORESIS

DNA is a polymer of deoxyribonucleotide monophosphates. These are joined as the following diagram illustrates.

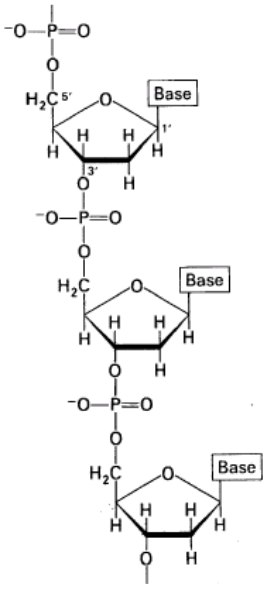


Figure 1: The phosphate-sugar backbone of DNA

Pulsed Field Gel Electrophoresis (PFGE) is a method of cutting, moving and separating large fragments of chromosomal DNA. The fragments are able to move in an electric field because of the negatively charged phosphate backbone. It will migrate toward the anode (positively charged) at a rate proportional to the net charge on the molecule, which is an inverse measure of the size of DNA. [1]

Small pieces of DNA will move through a gel matrix in a straight path. Larger molecules must migrate in a more indirect manner to find pores in the gel that are large enough to accommodate. [1] The gel is a matrix that consists of agarose molecules. If the concentration of the gel is decreased, larger DNA molecules are able to pass through it, although the resolution of the resultant bands will be poor and the gel will be difficult to manipulate. [1]

PFGE overcomes these difficulties by periodically altering the direction of the electrical current. The larger DNA molecules then have to realign to the new direction of the electrical field. This allows the separation of fragments based on

both the sieving capacity of the gel and the size related turning time of the DNA macromolecules. [1]

Contour Clamped Homogeneous Electric Field (CHEF) is the most popular apparatus for PFGE used in laboratories today. [2] A hexagonal array of 24 electrodes allows the field to switch through a fixed orientation angle of 120^{o} between pulses. To provide a uniform electrical field, the electrodes are "Clamped" to a particular voltage. This voltage is proportional to the migration rate and is dependent on the size range of fragments to be separated. The homogenous electrical field allows the resolution of large DNA molecules (> 5Mb) when combined with low field strengths, long switching times (times between direction change), and a low agarose concentration. [1]

Several models have attempted to explain the physical movement of the DNA molecules within the gel. Researchers have observed DNA movement by periodic removal of small pieces of the gel and using fluorescent microscopy. The U-model is the most popular of these, and shows the DNA forming a U shape around an obstruction in the gel (eg. a molecule of agarose). [3]

Segments of the DNA strand will then attempt to force their way into the nearby pores. One end, usually the longer of the two, will dominate and lead in the new direction. The longer the DNA molecule, the longer time it takes one end to dominate, hence the longer migration time. Thus, the U-model becomes the "bias-reptation" (reptation refers to the motion of a crawling snake) model that suggests that the larger pieces of DNA become trapped in the gel while the smaller pieces have already migrated further along the gel matrix. [2,4] The alternative model suggests the electric field alters the internal matrix structure of the gel, allowing the DNA to create pores or channels through which to travel. This theory has not been rejected to date. [2]

Electronic and software devices now control the parameters under which the gel is run. Refrigeration devices and pumps are also used. The gel chamber has manifolds that permit the recirculation of low ionic strength buffer that is cooled throughout the process. [2]

The pulse time determines the size range of the molecules separated. If the pulse time is shorter than the time required for molecular reorientation, the molecules will only experience the average of the two applied electric fields. This prevents separation due to molecular reorientation, as the DNA strands no longer change direction. Similarly, if the pulse time were much longer than the time needed for reorientation, then the migration would be independent of the molecular size (as in conventional steady-field electrophoresis). [4] It can be ramped linearly (a linear change from the minimum to the maximum) which produces a more regular migration pattern. [2] The voltage used is proportional to the migration rate, and is usually set at 6.0V/cm to separate molecules up to 1.5Mb in size. [2] Band sharpness decreases proportionally to increasing run time. A longer run time also increases the risk of DNA exposure to nucleases. [2]

The agarose concentration affects both the mobility and the separation of the DNA. At higher concentrations bands become sharper, and only smaller DNA molecules can be resolved. The temperature at which the gel is run affects both the migration rate and resolution. It is usually kept between 12-16^{o}C, which allows a compromise between both. [2]

Traditional methods of DNA preparation for gel electrophoresis involve manipulations that lead to the shearing of a large molecule. [2 3] To protect this, the DNA for PFGE is prepared in agarose plugs made of the same agarose used to make the gel. The bacterial cells are suspended in agarose, and treated to remove the cell wall and other cellular debris. [2 3] The DNA remains trapped within the agarose matrix of the plugs and is treated with rare-cutting restriction endonucleases leaving fragments of chromosomal DNA particular to that strain of bacteria. [3]

The plugs are introduced into the wells of the gel and electrophoresed under the desired conditions. Marker molecules are run alongside the experimental DNA as an indication of individual fragment size. Following electrophoresis (often 24 hours later) the gel is stained with Ethidium Bromide, and viewed with a transilluminator. [2,3] With the development of image capture and analysis software, an extensive database of strain types can be built and stored easily.

2.1 Examining Gels

Ethidium Bromide is a carcinogen that binds to the DNA and allows us to visualise the bands under UV light. When the gel is viewed it is photographed, and/or stored electronically.

The digital image that is captured can then be examined with the aid of computer programs designed to carry out specific tasks related to PFGE. These programs are often very expensive, making them less common. The markers run on each gel are a reference that allow the bands to be compared on the basis of position, which corresponds to DNA fragment size. This way it is possible to see whether a band in one lane is the same size as a band in another lane further away. With the help of the aforementioned computer programs, it is no longer necessary to manually translate the distances travelled in mm to base pairs. The program can provide this information after the size of the bands in the markers is input.

Gels contain several lanes, each of which contains the DNA from one bacterium (resulting from a single colony). By examining the fragment sizes, the gels allow us to determine whether there are genetic differences in the DNA of each isolate. The bacterial chromosome should be cleaved with a restriction endonuclease that gives a resolvable and informative number (5-60) DNA fragments. Appropriate rare-cutters are based on the GC content (proportion of the bases in the genome that are guanine and cytosine) and codon usage of the bacterial species and on the length of the recognition sequence of the restriction enzyme. [5] Bacteria with a GC content greater than 45% of all nucleotides are cleaved infrequently by endonucleases with recognition sites containing mostly A's and T's (adenine and thymidine). [2] The tetranucleotide CTAG is counterselected for in most genomes, particularly those with a high GC content, the four enzymes (*Spe*I, *Xba*I, *Nhe*I and *Avr*II) that recognise this sequence give the most informative PFGE profiles for these bacteria. [5] Genomes with a low GC content contain two rare trinucleotide sequences, CCG and CGG. Restriction endonucleases that recognise these sequences (*Sac*II, *Sma*I, *Nae*I, *Not*I, *Sfi*I, *Srf*I and *Asc*I) provide the most useful fingerprint for these organisms. [2]

If there are genetic differences between isolates, insertions, deletions, or point mutations may be apparent on the PFGE profile. Point mutations occur most

frequently in a genome, and often go unrecognised. Unless they occur in a gene coding area that alters the phenotypic behaviour of the isolate, or in a restriction site that can be visualised through restriction techniques. Insertions however, will be recognised, as a particular band will be larger, and therefore not travel as far along the gel as expected. Deletions are the opposite, and the band will move further along the gel, as the DNA strands are smaller than originally.

2.2 Mathematical formulae

The first parameter to be examined is the relationship and similarities between the lanes. These must be calculated before clustering methods can be of use. Similarity can be expressed mathematically as an Index that ranges from 0 to 1 (1 suggests indistinguishable isolates). There are several methods to calculate this, such as the Dice Coefficient, the Jaccard Coefficient, and the Euclidean Distance Squared. They differ in the weight of the number of positive and negative matches. Whether a distance or a similarity is calculated is of no importance as they can be transformed into each other. [6]

The three methods use the same numbers, merely in different ways. A mathematical equation that can be written as a vector is constructed. It represents the bands found in each lane. When comparing PFGE profiles, the vector S contains B elements (S=[s1, s2, s3…sB]), where B is the number of band types in the lane's band set.

S_i is 1 if the band type is found in the lane.
S_i is 0 if the band type is not found.

If S and T are two vectors representing two samples with lanes that are from epidemiologically related isolates then the similarity index can be calculated as described.

2.2.1 DICE COEFFICIENT

This coefficient was first called a coincidence index, measuring the chance of one species (band) occurring in the same sample (lane) as another, given the second species (band) is present. [7] The formula can be explained as "the sum of bands that

appear in both lanes divided by the sum of all bands in both lanes, multiplied by 200".

Similarity = 200 x (? (s_i, t_i)) / (? $(s_i + t_i)$)
Distance = 100 – similarity

2.2.2 JACCARD COEFFICIENT

This measure is generally used when chromosomes are being compared. Each locus is taken into account with the particular markers (probes) used. The formula is complex to understand until written. "The number of bands that are the same in both isolates divided by the number of bands that are individual to each isolate, multiplied by 100).

Similarity = 100 X (? (s_i, t_i)) / (? $(s_i + t_i)$ - (? (s_i, t_i))
Distance = 100 – similarity

2.2.3 EUCLIDEAN DISTANCE SQUARED

This distance matrix is used when clustering is being determined using the median, centroid or Ward methods. Here the formula of distance can be described as "the number of bands in one lane minus the number of bands in another lane".

Similarity = 100 x (B – similarity) / B
Distance = ? $(s_i – t_i)^2$

Each of these calculations essentially expresses the same thing. If the similarity index is equal to 100% then the isolates are identical.

There are also other ways of viewing the same information. They are more diagrammatic and involve algorithms that are based on phylogenetic research. The tree outcomes are schematic representations of lane similarity. There are several methods for calculating the similarity between the lanes, all slightly different.

Agglomerative hierarchical procedures start with clusters formed by a single object. With successive joins, a hierarchy is formed that can be represented as a tree or

dendrogram. Although there are several methods, most can be treated as variations of a single major technique. The general procedure is as follows. [6]

1. Begin with n clusters – one cluster for each sample.
2. Calculate the distance matrix d between the clusters.
3. Join the two clusters with the minimum distance into one cluster.
4. Compute the new distance matrix d including the cluster formed in step 3.
5. Repeat steps 3 and 4 until there is only one cluster. [6]

The difference between each method is based on the definition of minimum distance in step 3 and on the method of computing the new distance matrix in step 4 as follows.

2.2.4 CLUSTERING METHODS

The following definitions are used in the clustering methods described.

- P and Q are indices indicating two clusters to be joined to a single cluster.
- K is the index of the cluster formed by joining P and Q.
- I is the index of any remaining clusters other than P, Q or K.
- NP is the number of samples in the Pth cluster.
- NQ is the number of samples in the Qth cluster.
- N is the number of clusters in the Kth cluster formed by joining the Pth and Qth cluster (n=np+nq).
- Dpq is the distance between cluster P and Q. [6]

The algorithm is the same for each clustering method, only the formula determining the minimum distance differs. The space conserving cluster methods are ‘Single Linkage’, ‘Complete Linkage’, ‘UPGAMA’, ‘WPGAMA’, ‘Centroid’, ‘Median’, and ‘Ward’ Methods. Ward's method, UPGAMA, and WPGAMA give the most plausible clusters and are least affected by outliers. [6]

2.2.4.1 Single Linkage (Nearest Neighbor or Minimum Method)

$dk_i = \min (dp_i, dq_i)$

Two clusters are fused for which the distance (dp_i, dq_i in the equation) between the NEAREST (the term 'min') objects (p and q) is the least. This method is space

contracting (the chance that an object will be incorporated into a group g grows with increasing g) and it's chaining tendencies are strong. This is not a desirable property of a cluster algorithm. [6]

2.2.4.2 Complete Linkage (Furthest Neighbor or Maximum Method)

$dk_i = \max (dp_i, dq_i)$

This is the antithesis of Single Linkage. Two clusters are joined when the distance (dp_i, dq_i in the equation) between the FURTHEST (the term 'max') objects (p and q) is the smallest. This is a space dilating method (the union of an object with existing group g is made more difficult with increasing g) as the maximum distance between two objects is the criterion for fusion.

Single linkage and Complete linkage are good algorithms for indicating outlier clusters. [6]

2.2.4.3 Weighted Pair Group Method (WPGAMA)

$dk_i = 0.5 \times dp_i + 0.5 \times dq_i$

WPGAMA uses arithmetic averages (clusters are joined based on the result of half of the distance to p added to half the distance to q). This is also called Average Linkage. This method is the "mean" of both the single linkage and the complete linkage method. Therefore, it is space conserving (neither dilating nor contracting). It is a special case of UPGAMA that favours the most recent member clusters in forming new clusters.[6]

2.2.4.4 Unweighted Pair Group Method (UPGAMA)

$dk_i = (np/n) \times dp_i + (nq/n) \times dq_i$

This is also called Weighted Average Linkage. The foregoing method can be generalised by weighting the distances with the number of objects of the groups involved. The equation translates to the cluster being formed based on the distance to p and q proportional to the number of individual objects within p and q. The fusion of two groups depends on the least MEAN OF ALL POSSIBLE DISTANCES between the single objects. [6] As long as the rate of nucleotide substitution is constant, UPGAMA shows good performance. [8]

2.2.4.5 Ward

$dk_i = ((np + n_i)/(n + n_i)) \times dp_i + ((nq + n_i)/(n + n_i)) \times dqi - (n_i/(n + n_i)) \times dpq$

Ward attempts to minimise the information by describing a set of N samples using a fewer number of clusters. This results in a minimum increase of the total "within-group error sum of squares". It uses the Squared Euclidean distance matrix. [6]

2.2.4.6 Centroid

$dk_i = (np/n) \times dp_i + (nq/n) \times dq_i - (np \times nq)/n2dpq$

Every group can be characterised by its central vector. Two clusters are joined when the distance between their MEANS is the least. This also uses the Squared Euclidean distance matrix. [6]

2.2.4.7 Median

$dk_i = 0.5 \times dp_i + 0.5 \times dq_i - 0.25 \times dpq$

Slightly different from the Centroid method, rather than using the MEAN distance between two groups, it joins clusters by the least MEDIAN distance. [6]

Centroid and Median are similar to UPGAMA and WPGAMA, respectively, but the distance formula contains an additional third term. Centroid and Median methods are not monatomic hierarchical clustering algorithms. The similarity value between cluster k and any other cluster may be greater than the similarity between cluster p and cluster q. This occurs if the centroids (or medians) of the different clusters have approximately the same distance as the distances between the objects within the clusters.

The weakness of hierarchical methods such as these is that early decisions are permanent [6]The mathematical formulae are calculated within a computer algorithm contained within a program (GelCompar, Diversity Database etc). The output, rather than a series of numbers or tables, is a dendrogram. The information given in this format is a lot more intuitive than that in tables. A diagram represents the hierarchical evolutionary relationship between isolates of strains. Initially intended to determine the grand pattern of evolution and aid with taxonomy, these techniques

have been refined to the genetic level, where they can be used to differentiate between strains and hypothesise the evolution of different isolates. [6]

The tree output indicates quantitatively the relationships between different strains. It can be used in an outbreak situation or in surveillance systems. The problem with the latter is that PFGE typing may be too sensitive for extensive surveillance as it readily detects genomic rearrangements and requires the aforementioned detailed phylogenetic analysis to track subclonal evolution. [9] Also, the more divergent the genomic DNA patterns between strains, the less accurate the relatedness appears on interlaboratory evaluation.

However, in the case of an outbreak of a particular disease, PFGE has been proved a useful tool. It must be accompanied by an epidemiological study, as random sampling based on no prior knowledge is not only expensive, but may lead to incorrect conclusions and wrongful 'control' procedures.

2.3 Previous Uses of PFGE

2.3.1 INTRODUCTION

The method that offers the most promise for establishing a unified typing system for epidemiological investigations of most bacterial pathogens is digestion of total genomic DNA with restriction endonucleases. These enzymes cut the chromosome at infrequent intervals, producing relatively large fragments that can then be separated using Pulsed-Field Gel Electrophoresis (PFGE). A strain descriptor can be stored based on the sizes of fragments produced. These methods have been used and adopted for several medically important bacterial species [10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25] but have had limited application for veterinary pathogens. [26 27 28 29]

Accurate epidemiologic investigations require an assessment of relatedness between individuals with similar infections in order to determine if animal to animal spread has occurred. In order to accomplish this, one rapid laboratory approach taken has been to determine the presence or absence of genetic identity between microbial strains of the same genus and species affecting persons who may have had a common exposure. It is desirable to rapidly compare isolates of an organism in a

simple and accurate manner that can demonstrate the presence or absence of important epidemiologic associations (clonality). Chromosomal analysis provides a more fundamental measure of strain identity. Isolates from the same nosocomial environments that appear identical by PFGE except for a small number of bands should be considered clonally derived. The major issue with electrophoretic techniques is the lack of standard methods available for interpreting the results. When standardised methods and controls are published and maintained, then the same results will no longer be interpreted in different ways by different investigators. [30]

PFGE has been used in all areas of Molecular biology. It can be used to make long range physical maps of the genome in both prokaryotes and eukaryotes. It has enabled the mapping of genes and linkage groups to their chromosomal locations, which enabled the evolution of chromosomal libraries. It can be used more simply to provide information about the size and shape of the microbial genome. [3 2 1 31 32]

2.3.2 ORGANISMS

Pulsed Field Gel Electrophoresis has been used for several organisms in comparative studies, to investigate whether it should be used more routinely. A disadvantage of this method is the time consuming aspect of DNA preparation, however the discrimination between strains is much increased over other typing methods, both phenotypic and genotypic. The list below suggests the species for which PFGE is recommended as the typing system with the most discriminatory power and typeability. [33]

Acinetobacter
Clostridium difficile
Clostridium perfringens (REA typing (Restriction Endonuclease Analysis using frequent--cutting enzymes followed by electrophoresis of the small DNA fragments) equally as powerful)
Enterobacter (Ribotyping equally as powerful)
Enterococcus
Escherichia coli O157:H7
Campylobacter

Legionella
Listeria
Mycobacterium avium (Ribotyping equally as powerful)
Mycobacterium other than *tuberculosis*
Pseudomonas (PCR typing equally as powerful)
Stenotrophomonas (PCR typing equally as powerful)
Salmonella
Staphylococcus aureus, (MRSA)
Staphylococcus epidermidis

There are other bacterial organisms for which PFGE is not necessarily the best method for typing the organism but there is no preferred method of typing. PFGE in this case gives a high standard of discriminatory power and typeability. These organisms are as follows. [33]

Bacteroides
Haemophilus
Klebsiella
Mycobacterium tuberculosis (Southern Blot and PCR typing actually preferred)
Neisseria meningitidis
Neisseria gonorrhea
Proteus
Rickettsia
Serratia
Shigella

2.3.3 GEOGRAPHIC COMPARISONS

PFGE has been used to compare genomic DNA of enterococci from three distinct geographical locations. [34] Bacterial strains were recovered from the United States of America, Thailand and Chile during a previous study. The isolates were from patients within one hospital in each area over a 1-2 month period. The resulting restriction fragment length polymorphisms (RFLP) were found even in isolates from the same geographic location. None of the strain types from one country appeared in isolates from a different country. Some of the isolates within a country were

indistinguishable from each other, which does not prove a direct epidemiologic relationship unless epidemiologic links have been found concurrently to the microbiological similarities. [34]

2.3.4 EPIDEMIOLOGIC INVESTIGATIONS

PFGE has been used in several epidemiological studies worldwide. Several organisms have been examined and studied through this method. [1] The most popular is MRSA (Methicillin Resistant *Staphylococcus aureus*) which is a known cause of nosocomial infections in hospitals and small clustered outbreaks. Using PFGE, the origin of infection has often been pinpointed, and the patients determined to be related through either space or time.

It is strongly advised that in the face of nosocomial outbreaks, an epidemiologic investigation with subsequent epidemiologically directed environmental and personnel cultures is conducted. Initial culture surveys without prior epidemiologic investigation is discouraged as it may be misdirected, expensive and expend valuable laboratory resources. This situation applies to all microbiology techniques, phenotypic and genotypic typing systems available for identifying cause of infection. Advances in molecular typing have facilitated the identification of clonal and non-clonal strain outbreaks. Use of these results without an epidemiologic study can often lead to uninterpretable results. [35]

2.3.4.1 Outbreak Investigation

A genomic typing method must be highly discriminatory and generate sufficient data to be used quantitatively to observe relatedness. High-resolution techniques with an index of discriminatory power over 0.95 are appropriate for outbreak investigations, where it is necessary to type a limited number of isolates in a single experiment. Such methods include PFGE, PCR (amplification of a known gene that is particular to a strain type and can be probed for), RAPD (random amplified polymorphic DNA assay copying DNA using non-specific primers for any site in the chromosome) and serotyping. PFGE may be too sensitive for surveillance as it readily detects genomic rearrangements and requires detailed phylogenetic analysis to track subclonal evolution. [9]

2.3.5 TAXONOMY

Modern taxonomy assigns bacteria according to their ancestral relationships. Methods that use genetic relatedness are increasingly used in the area of taxonomy, including PFGE. Numerical scoring of REA typing fingerprints is hampered by the large signal-noise ratios of the bands. RFLP analysis scan few sites in the genome and exhibits less discriminatory power in this circumstance. PFGE combines a high degree of polymorphism with reproducible resolution of few bands. [32]

2.3.6 EXAMINATION OF HETEROGENEITY

The genomic DNA of *Yersinia enterocolitica* has been examined by PFGE. Phenotypic markers were unable to distinguish strains within a serotype, as there is a high degree of correlation between biotypes, serotypes and phage types. PFGE was used to investigate the degree of heterogeneity between European type strains. The technique unequivocally distinguished between the pathogenic and non-pathogenic strains of serotype O:5 suggesting the two types of bacteria belong to two clusters of strains. It was determined that PFGE could be used to ascertain the origin of a given isolate based on the appearance of the fingerprint, and could be adapted for use in outbreak conditions, nosocomial or otherwise. [20]

2.3.7 VACCINE STRAIN DETECTION

With obligatory vaccination of poultry, using a live vaccine, it is necessary to be certain that the vaccine strain can be differentiated from wild-type strains that may be isolated from animals. PFGE was used as a method to detect the vaccine strain of *Salmonella typhimurium* Zoosaloral H. This strain produced a unique fingerprint that was not shared by wild-type isolates from non-vaccinated or the vaccinated poultry in the study. [28]

2.4 Evaluation of PFGE as an Epidemiological Tool

In 1995 in Japan, Murase et al evaluated the use of PFGE as an epidemiologic tool, *Salmonella* strains from outbreaks of food poisoning and sporadic infection, and patients infected abroad were fingerprinted. All 28 isolates presented digestion patterns that were analysable. Those S. typhimurium isolates involved in various outbreaks were distinguished from one another. Some strains studied from one

phage type were clearly distinguishable by PFGE. The results suggested that PFGE is useful for epidemiological analysis of *Salmonella* outbreaks. [14]

A different panel of *Salmonella enteritica* serovar Enteritidis Phage Type 4 samples was tested to determine the potential of PFGE as a tool for epidemiologic surveillance. This time, strains were all of the same phage type (4) and were from poultry, humans, poultry feed and products. The isolates were taken over a 25-year time period. The resultant profiles fell into 9 distinct patterns with one predominant strain. Some bands (DNA fragments) were common to all strains. Of the eight secondary strains, six were identified in chickens and poultry products of which one was also found in a human isolate, two were identified in human isolates but no poultry isolates. Since the predominant strain was also exhibited in the reference strain for the bacteria, this was considered the ancestral genotype. The fingerprints of the other strains could be described by observing the band sizes and creating deletions and insertions so those bands appeared as they did in the profiles. The overall conclusion was that PFGE provides a means for the genetic characterisation of *Salmonella enteritidis* PT4 and may be of use for strain discrimination as required in epidemiological investigations. [36]

2.4.1 OUTBREAK INVESTIGATION

Two well-defined outbreaks of cholera have occurred in parts of Malaysia in recent years. Strains isolated from stools of infected individuals or environmental samples were used in this study. Isolates from sporadic cases in the same period and approximate geographic location were used for comparison. The data showed that isolates from areas where cholera is endemic are highly heterogeneous. Multiple clones appear to be endemic to Malaysia, and these cause the sporadic cases. Periodic outbreaks appear to be caused by a single clone or closely related clone with a similar pulsed-field pattern. The different patterns seen in the two outbreaks suggest that more than one clone can cause an outbreak of *V. cholerae*. PFGE was found a useful technique for differentiating between individual isolates. [19]

V. cholerae O139 was identified for the first time in 1993 in Thailand. It has since spread to several areas within the country and on to several other Asian countries besides. This demonstrates the pandemic potential of this serotype. 48 isolates taken

from patients at hospitals in Thailand over a 3-month period in 1993 were examined and compared to four strains from Bangladesh and India (the origin of the serotype). PFGE and ribotyping were the methods used to view each strain. Results indicated that PFGE was more discriminatory than ribotyping, several pulsed-field types were found in one ribotype. The four isolates from Bangladesh and India corresponded to one subtype in Thailand, while the other was not seen in the India/Bangladesh isolates. This indicated that most of the isolates from Thailand recently diverged from the India/Bangladesh common isolate. However, the strain is clearly evolving over time, leading to the evolution of subtypes particular to Thailand. This study suggested that PFGE could be used to follow the O139 epidemic as it progressed. It was able to differentiate among epidemiologically unrelated O139 strains that were indistinguishable with ribotyping. [37]

2.4.1.1 Multistate Foodborne Outbreak

Foodborne intoxication can be caused by enterotoxin producing *Staphylococci*. Until recently, the main culprit was *Staphylococcus aureus*, while *S. intermedius* was considered part of veterinary flora or a veterinary pathogen. A multi state outbreak that covered nine counties in California and Nevada in 1991 altered the perception that *S. intermedius* was only a veterinary concern. The relatedness of 15 outbreak-associated strains was examined and compared with those of veterinary origin and *S. aureus* strains. The outbreak-associated isolates were indistinguishable and pointed to a common source of contamination rather than post-process contamination of the food product to which the outbreak was linked. PFGE was invaluable as a method for tracking this outbreak, and confirmed the epidemiological outbreak study results. [38]

In 1994, a multistate foodborne outbreak of *E.coli* O157:H7 was linked to the consumption of ground beef produced over two days. Epidemiologists required laboratory confirmation that this was the case and PFGE and Phage typing were used in conjunction to affirm the findings. Twenty-six isolates taken from humans (outbreak-associated) were indistinguishable from the 27 isolates taken from samples of the uncooked meat. Only one of 102 pre outbreak (sporadic) samples had the same pulsed-field type. Fifteen sporadic isolates taken at the same time as the

outbreak showed the same pulsed-field type, although the origins of these infections were unidentified. The isolation of the strain two months post-outbreak suggested that transmission had continued. A random sample of bovine isolates was examined for relatedness, but not one of 33 samples looked like the outbreak strain type. [18]

Listeriosis is also a common form of food poisoning. While most infections are sporadic, outbreaks of *Listeria monocytogenes* have been recorded in association with contaminated food. When a voluntary recall of four products was announced, the health department enhanced passive surveillance to identify humans potentially associated with any of these products. A questionnaire for sporadic listeriosis cases was distributed in the areas where the recalled food products had been found. Individuals who were hospitalised or went to a doctor and had consumed one of the four products were included in the study. The strains that were isolated from four hospitalised patients were indistinguishable from those isolated from two of the products and the associated dairy environment of those products. PFGE was again useful in linking sporadic cases of illness with recalled products. [39]

2.4.1.2 Nosocomial Outbreak

PFGE was evaluated as a typing system using a nosocomial outbreak of *Candida rugosa* - a eukaryote. Nine isolates from patients in a burn ward, one from a patient in another ward and a further eight from a reference laboratory were karyotyped using PFGE. The nine isolates from the burn unit were indistinguishable, while the one isolate from the other ward in the same hospital showed a different pattern. Each isolate from the reference laboratory used for comparison showed a distinctly different pattern. The study showed that PFGE could be used as a tool for the discrimination of strains of *C. rugosa*, and would be useful as a marker for monitoring future *C. rugosa* infections. [40]

2.4.1.3 Pseudo-Outbreak

A pseudo-outbreak is a situation that arises when an organism is found in culture at a rate that is not exhibited clinically. *Bacillus cereus* is a systemic pathogen that is widely found in nature. In a paediatric unit in one hospital, there was an unusual frequency of isolation of *B. cereus*. On further consultation with each patient's

doctor, it was suggested that contamination was the reason behind the upsurge in isolation rather than true infection. Environmental samples were taken in this unit, different wards in the same hospital and from a different hospital altogether for comparison. Colonies of *B. cereus* were found in the paediatric unit on the settle plates that had been placed underneath the ventilation system. PFGE patterns of all the isolates could clearly identify between the epidemiologically-related strains and those that were unrelated. The isolates from the settle plates from the ventilation system showed the epidemic strain, although the source of contamination of the air filtration system could not be established. [12]

2.4.2 RETROSPECTIVE STRAIN TYPING

Nine MRSA isolates were cultured from different patients in a hospital burn unit over a 190-day period. PFGE produced patterns that were almost indistinguishable, the presence of one 'extra' band in some isolates, and the absence of one large band in one isolate. It was assumed that the nine MRSA isolates were clonal derivatives of the same strain. The results again note the usefulness of molecular techniques in the epidemiological analysis of nosocomial infections. [41]

Forty-five Vancomycin-resistant *E. faecium* isolates were obtained from 42 patients in several wards at one hospital over a 15-month period. The isolates had been frozen and stored and were subjected to PFGE later. A surprising diversity was seen when the isolates were observed with REA typing (two different enzymes) and PFGE. The two REA typing methods were discordant in detecting clonality. Overall, many isolates that were identified as clonal by PFGE and REA had strong clinical data to support this finding. [42]

Nineteen strains of *Campylobacter fetus* isolated from humans, and two strains isolated from animals, were analysed by PFGE. Most strains had different fingerprint patterns, although there were 18 types among 21 strains. The DNA type was not related to the serotype as determined by the Penner system. The DNA types among the strains isolated from the United States and Japan showed some variation although none that defined the geographic distribution of the bacterium. A dendrogram (neighbour joining method) that summarised the relationships among

the isolates was constructed. PFGE was more convenient for the epidemiological study of *C. fetus* infection than any other typing system. [16]

PFGE was used to strain type 35 Australian isolates of *Mycobacterium paratuberculosis.* Isolates were obtained from animals with Johne's disease. One enzyme produced all clonal PFGE profiles. Further cleavage with another enzyme divided the isolates into two groups, although the genetic difference was small (single or double band at one position on the gel). These two groups were irrespective of area or animal from which the isolate was cultured. The study demonstrated the presence of two PFGE types of *M. paratuberculosis* in ruminants in Australia, and suggests that this typing method may be of use in further epidemiologic studies on Johne's disease in Australia. [26]

2.4.3 MULTICENTRE STUDIES

Standardisation of molecular techniques remains an issue in medical microbiology. The only typing system to have been standardised so far is that for *Mycobacterium tuberculosis*, the IS 6110 method. 12 laboratories in nine countries took part in a study to compare fragment patterns of well documented MRSA isolates using a standard PFGE method as well as those used regularly in the laboratory (the "in-house" method). The differing results suggested that standardisation of PFGE typing for MRSA has not been achieved yet. The overall qualitative assessment of the results indicated that continued effort to decrease the variability of PFGE parameters would lead to acceptable numerical and methodological standardisation of PFGE procedure and analysis. [43]

2.4.4 ENDEMIC DISEASE INVESTIGATION

PFGE was used to compare the molecular characteristics of *Salmonella typhi* isolates from Malaysia, Thailand and Indonesia. All isolates were obtained from sporadic cases of typhoid fever within the same period (1987-1994). 120 isolates in total were analysed and clustered according to the UPGAMA method. Certain similar PFGE profiles were shared by isolates from all three countries. This implied mobility and movement of the strains in South East Asia. The differences between some strains indicated that *S. typhi* isolates from different parts of the world might

not have diverged from the same clone. If PFGE could be standardised, molecular typing of *S. typhi* strains could form the basis of an effective surveillance system. [15]

2.4.5 SURVEY OF BACTERIAL INFECTION

A survey of MRSA isolates from a 15-bed ICU was conducted over one month. The survey collected 175 isolates that were then analysed by lysotyping, plasmid typing and PFGE. The resulting patterns from this method were not influenced by the loss or acquisition of plasmids. The three methods appeared complementary but PFGE did offer greater polymorphism than the other two markers. PFGE was also much more stable than the other two methods, even after 40 subcultures of several strains of different pulsed-field types. PFGE was decided the best strain typing method for epidemiological studies of MRSA and possibly for other nosocomially-acquired bacterial infections. [25]

A survey of cows with evidence of clinical or subclinical mastitis was conducted. Samples were collected from 15 farms, 46 cows, over a 2-month period. Bacteria identified as *Streptococcus* spp. were then analysed using PFGE. The restricted epidemiological window in this study allowed an effective assessment of PFGE and enabled investigation of some features of streptococcal mastitis. Results were consistent with what might be expected of an obligate parasite of the bovine mammary gland in a closed herd with direct transmission between glands at milking. The discrimination between strains as evidenced in this study, is critical to a full understanding of the epidemiology of infections caused by *Streptococcus* spp. [29]

There has been an increase in the number and variety of ESBL (extended-spectrum ?-lactamase)-producing bacteria. *Klebsiella pneumoniae* has been found resistant to a number of cephalosporins. Molecular techniques have been used to analyse the epidemiology of 31 clinical bacterial isolates (resistant to a cephalosporin-based antimicrobial drug) from a hospital in Taiwan. PFGE of these isolates showed 16 genotypes, with 20 isolates contained in five clusters (greater than 80% similarity). The remaining 11 isolates showed a high level of genetic heterogeneity. The variation in the 31 isolates was minor with only five patterns being identified (the five clusters). This finding suggested that an increase in the number of ESBL-

producing *K. peneumoniae* isolates was due mainly to dissemination of resistance plasmids rather than that of an ESBL-producing strain. [23]

PFGE was used to determine differences between environmental strains of *Vibrio vulnificus* and clinical isolates. Seventy-eight environmental strains were collected from shellfish, seawater and sediment samples over a two-year period. Fifty-three clinical isolates were collected from hospitals, medical examiners, state and federal laboratories. There was a high level of diversity among the PFGE profiles of clinical and environmental isolates. Results did not show that clusters were related to clinical or environmental sources. A few strains that showed similarities in PFGE profiles were epidemiologically related but it was determined that for studies such as these, PFGE was too discriminatory to define clusters that distinguished clinical strains from environmental strains. [44]

A longitudinal survey of human *Campylobacter jejuni* infections in two different geographical locations (parts of Finland) was carried out. The PFGE profiles resulting from the study were compared to those seen in isolates of *C. jejuni* from Finnish chickens. Although most cases seemed to be sporadic, five predominant PFGE profiles covered 42 and 44% of the isolates in Area 1 (urban) and Area 2 (rural) respectively. Certain types predominated in certain times of the year. Chicken was suspected to be the source of infection for 11 patients, and eight patients showed PFGE profiles identical to those found in poultry. Four of the ten most common human isolates were never seen in chickens. PFGE provided new information about apparently sporadic infections and showed geographic differences. It also suggested epidemiologic associations in several small outbreaks. [10]

2.4.6 VACCINE STRAIN TEST

Brucella abortus strain 19 has long since been used to vaccinate cattle against brucellosis. Strain 19 contains a polysaccharide that impedes interpretation of the standard diagnostic tests for presence of brucellae infection. Strain RB51 did not produce the polysaccharide and was therefore a candidate for a new vaccine. It was necessary to determine whether PFGE could be used to differentiate between the proposed new vaccine strain RB51, other strains and other species. Vaccinated

cattle, wildlife (elk, bison, cattle) and laboratory strains of brucellae were compared to RB51. There were discernible differences between the profiles of *B. abortus, B. melitensis, B. suis, B. canis*, and *B. neotomae* strains. Strain RB51 was also different to *B. abortus* biovars 1, 2 and 4, and *B. suis* biovar 1 that had been obtained from cattle. Genetically engineered strain 19, vaccine strain 19 and the wildlife brucellae profiles were again different from that of strain RB51. It was noted that *B. abortus* strains do not show much variation between their PFGE fingerprints, suggesting evolutionary convergence. [27]

2.5 Advantages and disadvantages of PFGE compared with other typing techniques

Epidemiologic typing systems are used to study population dynamics and spread of organisms that undergo clonal reproduction (ie. bacteria). Typing systems must be assessed according to performance criteria: typeability, reproducibility, stability, discriminatory power, epidemiologic concordance and typing system concordance. [45]

2.5.1 CRITERIA FOR TYPING TECHNIQUES

Typeability (T): The proportion of strains that are assigned a type by the method [45] or the ability of a technique to assign an unambiguous type to each isolate. [46]. T should be as close to one as possible

To calculate T: Number of isolates given a type / Number of isolates tested.

Reproducibilty (R): The ability of a typing system to assign the same type to a strain tested on independent assays. R should be 0.95 or above. Poor reproducibility may reflect technical variation or biological variation.

To calculate R: Number of isolates assigned the same type on repeat testing / Number of isolates tested.

Stability (S): The ability of a typing system to recognise clonal ancestry of strains despite the phenotypic or genomic variation that may occur. (Suggested that 10 strains are studied after every 5th passage in an experiment of 50 passages/subcultures)

S is calculated as the Number of tests in which the same strains were correctly identified the same type on repeat testing / total number of tests.

Discriminatory Power (D): The average probability that the typing system will assign a different type to two unrelated strains randomly sampled. With genotypic methods, the D value should be calculated at the similarity level used for defining clonal groups. Also expressed as Simpson's Index of Diversity where

D = 1 - 1 / N (N-1) where N is the number of unrelated strains tested.

Epidemiologic concordance (E): The probability that epidemiologically related strains are determined to be similar enough to be assigned clonal relationship by the typing technique. E should be equal to one

E is calculated as the Number of strains assigned to epidemic clones / number of strains tested from well-defined outbreaks.

Typing system concordance: Epidemiologic relatedness tends to be the gold standard for evaluating typing system specificity. However, since co-transmission of different strains could occur, another typing system should be used to compare results. Isolates concordantly placed within the same group by several methods are more likely to be clonally related. Where polymorphism of the whole genome is evaluated, there is more weight to this method and its results, than there is when only variation at a single locus is explored. [45]

2.5.2 PHENOTYPIC METHODS

Phenotypic methods frequently show lower discriminatory power than newer molecular methods. The table below shows the phenotypic methods compared against one another in regards to the above criterion. Phenotypic markers (biotypes, antimicrobial sensitivities) are much more likely to change over time than are genotypic markers (such as Pulsed-Field Gel Electrophoresis) as they can be affected by extrachromosomal DNA ie. Antibiotic resistance genes carried on plasmids. Multi Locus Enzyme Electrophoresis is considered a phenotypic method as it is based on analysis of electrophoretic variants of housekeeping enzymes. It can be considered an indirect genotypic method. [30 45 46 33]

Table 1: Phenotypic Markers

	BIOTYPING[1]	**ANTI-BIOGRAM[2]**	**SERO-TYPING[3]**	**PHAGE TYPING[4]**	**MLEE[5]**
Proportion of Strains Typeable	All	All	Most	Variable	All
Reproducibility	Poor	Good	Good	Fair	Excellent
Discriminatory Power	Poor	Poor	Fair	Variable	Good
Ease of Interpretation	Moderate	Easy	Moderate	Poor	Excellent
Ease of Performance	Easy	Easy	Moderate	Poor	Good

[1]Biotyping includes a number of biochemical and immunological tests known to vary within a genus.

[2]Antibiograms observe the susceptibility of bacteria to a number of antimicrobial agents. Resistance is often associated with mobile genetic elements (transposons and plasmids) hence the lower discriminatory power.

[3]Serotyping determines the surface antigens of bacteria by using polyclonal or monoclonal antibodies applicable to a single genus or species. Maintaining stock antisera is the main limiting factor for this method.

[4]Phage Typing measures the susceptibility of the test organism to bacteriophages. Stocks are difficult to maintain.

[5]MLEE differentiates organisms by analysing the mobilities of numerous metabolic enzymes. This reflects variations in genetic loci.

2.5.3 GENOTYPIC METHODS

Genotypic methods are those that are based on analysis of the genetic structure of an organism. They are less subject to natural variation, although DNA changes (insertions, deletions, and random mutations) can have an effect on the resulting fingerprint. The following table compares each genotypic method according to the criteria established for deciding upon a typing system. [45] [46]

Table 2: Genotypic Markers

	PLASMID TYPING[1]	**RFLP[2]**			**PCR TYPING METHODS[6]**
		REA Analysis[3]	**PFGE[4]**	**IS- & Ribotyping[5]**	
Proportion of Strains Typeable	Most	All	All	All	All
Reproducibility	Good	Good	Excellent	Excellent	Good
Discriminatory Power	Good	Good	Excellent	Moderate to Excellent	Good
Ease of Interpretation	Moderate	Difficult	Moderate	Moderate	Moderate
Ease of Performance	Moderate	Moderate	Moderate	Difficult	Moderate

[1]Plasmid typing is based on restriction endonuclease analysis of plasmid DNA which is visualised by agarose gel electrophoresis.

[2]Restriction Fragment Length Polymorphism (RFLP) observes the changes in the genetic fingerprint/banding pattern between two isolates.

[3]REA typing observes the fragments of chromosomal DNA that result when a genome is cleaved by restriction enzymes into hundreds of small pieces and electrophoresed. Interpretation is very difficult with so many fragments that can be indistinct or overlapping.

[4]PFGE cut the chromosome into fewer pieces than REA typing by using rare cutting restriction enzymes, allowing banding patterns to be seen more clearly.

Electrophoresis takes longer due to the large size of the DNA fragments. Initial costs to the laboratory are large but once operational, this method can be applied to a number of bacteria with few alterations to the method. Has yet to be standardised.

[5]IS (Insertion Sequence)- & Ribotyping involve endonuclease digestions of genomic DNA being probed with labelled nucleic acids. As this method only observes sections of the genome, it is less discriminatory than other methods.

[6]PCR typing methods include arbitrarily primed PCR (AP PCR), randomly amplified polymorphic DNA (RAPD) or inter-repeat element PCR (rep-PCR). Primers are used to amplify sections of the genome, which are then electrophoresed and observed for relative position and size. AP PCR is more susceptible to technical variation. It is difficult to obtain reproducible patterns with this technique. Standard guidelines for interpretation of results are not available.

2.5.4 EXAMPLES OF COMPARATIVE TYPING STUDIES

2.5.4.1 Methicillin Resistant *Staphylococcus aureu*s (MRSA)

Identifying strains among the outbreak isolates is always a major step in determining the source of the outbreak and in designing control measures. Bacteriophage typing (BT) has been used to discriminate between outbreak strains of *Staphylococcus aureus*. Because of the advantages of PFGE over BT, including greater discriminatory power, universal typeability, ease of use and interpretation, Pulsed-Field Gel Electrophoresis has replaced BT as the method for strain typing used by the Centres of Disease Control and Prevention. [17]

Typing of MRSA strains has been difficult because most strains appear to be derived from few clones. Fifty-nine isolates of *S. aureus* and one *S. intermedius* were typed by 12 different methods in a study that compared the typing methods available. All were typeable by antibiogram, biotype, immunoblot analysis, MLEE, ribotype analysis, PFGE and Field-Inversion Gel Electrophoresis (FIGE). Phage typing and plasmid analyses were the least successful methods. Insertion Sequence (IS) typing was also evaluable for some of the strains. Duplicating strains measured reproducibility. In one duplicate set of isolates, PFGE, antibiograms and plasmid restriction all showed slight variations between the duplicates. However in the other set, all typing methods identified the duplicates as identical. Among the genotypic methods, FIGE correctly identified 28 of the 29 organisms in an outbreak, but

incorrectly included a further seven isolates in the outbreak. All but one technique tested included some unrelated isolates, while many failed to correctly identify those that were outbreak-associated. Plasmid typing was the most specific and included no false positives. Ribotyping using *Cla*I was the most sensitive and identified all of the outbreak related strains, but included seven false positives. There were significant differences in the ease of use and interpretation of all the techniques. Antibiograms and biotypes were the easiest to carry out and interpret. Immunoblotting required the most complex interpretation to those unacquainted with protein analysis, MLEE required the most sophisticated computer software, Ribo-, RFLP- and IS-typing procedures were highly labour intensive and time consuming. PFGE and FIGE both required expensive equipment initially but produced DNA fingerprints that were easy to interpret. While no method clearly prevailed in all circumstances, it was decided that a combination of two methods would be the best typing tool. One method that would be sensitive enough to include all potential patients for screening and a second for detailed strain differentiation later in an epidemiologic study. [30]

A collection of 239 MRSA strains isolated over a 2-year period was classified according to PFGE. Eighty-one of these isolates were then analysed by ribotyping to compare the two methods. Ribotyping did not allow subsets to be characterised within the PFGE profiles that had already been determined. PFGE appeared to be more discriminating than ribotyping. [24]

Twenty-six strains of MRSA with different PFGE profiles were tested by random amplified polymorphic DNA assay (RAPD). The Dice coefficient was established for the resulting profiles and dendrograms constructed by the UPGAMA method described previously. Although three sets of primers were used with the RAPD assay, and the three were combined to construct an overall dendrogram, RAPD was still less discriminatory than PFGE. [47]

Plasmid profiles of nine isolates of MRSA cultured from patients in a burn unit of a hospital showed that there could be three groups of organisms. In contrast, FIGE showed one-band differences between different groups of the isolates. This suggested the strains were clonally derived. FIGE was determined an economical and powerful technique for strain differentiation of nosocomial organisms. [41]

2.5.4.2 *Salmonella* spp

Salmonella enteritica Enteritidis can be subdivided by phage typing and currently 44 different phage types (PT's) have been identified. Types of different PT's were further characterised by IS200-, ribo- and PFGE typing. Pulsed-Field Gel Electrophoresis showed 10 different patterns and the most common pattern was seen in 22 of the 33 isolates. It was the discriminatory method. PFGE is of potential use for outbreaks of *S enteritica* Enteritidis either in combination with another typing method, or alone. [48]

A marked increase in the number of *Salmonella* Brandenburg isolates sent to the Reference Laboratory in Switzerland was seen in 1992 although there was no geographical clustering of cases, and no epidemiological study was carried out. During the outbreak, several isolates from meat, meat products, chicken carcasses and fattening herds of pigs were also submitted for serotyping. These isolates were all identified as *Salmonella* Brandenburg and so multiple vehicles were suspected in the outbreak. Ribotyping, IS200 probes and PFGE were compared for their ability to differentiate between *S.* Brandenburg strains. PFGE was the most discriminatory method, producing 10 profiles in the 32 strains. Strains from different geographical areas, outbreak-associated strains, and sporadic isolates from Switzerland could be distinguished clearly and reproducibly. PFGE profiles exhibited typeability and reproducibility comparable to IS200 profiling or ribotyping, but were more discriminatory for epidemiological purposes. [21]

2.5.4.3 *Enterococcus* spp.

Enterococcus was ranked the second most common agent of nosocomial infection from 1986 to 1989. Until recently, epidemiologic evaluations of enterococcal infections were hampered by lack of discriminatory typing systems. 41 isolates of *E. faecalis* were studied. They underwent ribotyping and PFGE to determine which method was better for subspecies differentiation. Using *Eco*RI as the restriction enzyme for both typing methods, seven different ribotypes were found, compared with 25 pulsed-field types. Closer examination to determine the similarities and differences between the types that each isolate was placed in revealed that ribotyping with *Eco*RI was inferior to PFGE for subspecies differentiation. The

ribotype patterns did not show sufficient polymorphism to distinguish between isolates that were epidemiologically unrelated. PFGE did show these isolates as having different patterns of DNA banding. [49]

In a study of 45 vancomycin resistant isolates of *E. faecium*, REA typing and PFGE were compared to the charts of patients in one hospital over a 15-month period. REA analysis is difficult to analyse when large collections of isolates are examined. REA typing was carried out with two different enzymes, *Hind*III and *Hae*III. The results were 20 patterns categorised into nine types and 21 patterns categorised into 19 types respectively. PFGE showed 27 distinct subtypes (patterns) that belonged to 21 types. Overall, many isolates were identified as clonal by PFGE and REA that had strong clinical data to support this result. [42]

2.5.4.4 Other organisms

2.5.4.4.1 Campylobacter jejuni

PFGE, ribotyping and phage typing were compared for their ability to differentiate between strains of *Campylobacter jejuni* serotype HS2. Seventy-seven strains were compared: 27 sporadic and 18 outbreak-associated from one area of England collected over a 1-month period, 30 epidemiologically-unrelated strains from other areas (including 13 animal strains) and 2 reference strains. Ribotyping using two enzymes did not differentiate epidemiologically unrelated strains of the same serotype. It would appear that the three copies of the 16s rRNA are insufficient to establish strain identity by this method. Typeability and reproducibility of phage typing was poor in this experiment. PFGE was the most discriminatory of the typing approaches and genetic heterogeneity was seen in epidemiologically-unrelated isolates that had the same ribotype. The PFGE results were reproducible and the technique was easier to perform, less labour intensive and more discriminatory than ribotyping. [50]

2.5.4.4.2 Streptococcus spp.

Group B *Streptococci* (GBS) are a major cause of mortality and morbidity in neonates. The modes of transmission and certain aspects of the infections in neonates have been defined through classical epidemiological studies. Markers such

as antibiotic susceptibilities, serotyping and phage typing were used in conjunction with these studies. PFGE was tested on some isolates to determine the potential utility of the technique as a tool for studying GBS infections. The system was tested against REA typing. PFGE was found to yield reproducible and easily readable patterns that were easier to analyse than the REA gel results. This was the main advantage seen with PFGE over REA typing and the researchers suggest that more comparative studies would be needed. [22]

2.5.4.4.3 Escherichia coli O157:H7

PFGE was compared to phage typing for 124 isolates of *Escherichia coli* O157:H7 (17 from animals) collected over a 17-month period in Scotland. PFGE profiles were compared to each other using the Dice coefficient, and a dendrogram (UPGAMA) showed the clustering of the isolates. Twelve phage types were detected in total. Seven point nine percent of the isolates were untypeable by this method. Distinctive PFGE profiles were shown for epidemiologically unrelated strains, and identical profiles were obtained for those isolates that were epidemiologically related. Comparison of the results showed that PFGE was more discriminatory. [13]

2.5.4.4.4 Mycobacterium tuberculosis

Mycobacterium tuberculosis was analysed with PFGE and compared with IS6110 and ribotyping. Eighty-four isolates of *M. tuberculosis* were examined and one *M. bovis* BCG isolate and one *M. africanum* isolate were included in the study as comparison organisms. The 84 isolates resulted in 66 profiles. One pattern was carried by nine isolates that were epidemiologically related. No strain predominated among the patterns. A high degree of polymorphism was found among isolates of both the Australian- and North European-born and the Asian-born groups of patients. PFGE was more discriminatory than RFLP with the pTBN12 probe. [11]

2.5.4.4.5 Listeria monocytogenes

A study was undertaken to evaluate a set of 51 *Listeria monocytogenes* strains by PFGE, ribotyping and AP-PCR to compare the results with those resulting from serotyping. There was a remarkable degree of uniformity in the DNA-based methods, but ribotyping was the least discriminatory technique, unable to

differentiate between strains from two separate outbreaks. PFGE gave clear and reproducible results that were easily interpreted although there was no difference in the ability of PFGE to differentiate between strains when compared with AP-PCR. [51]

2.5.4.4.6 Acinetobacter baumannii

Seventy-three isolates of the nosocomial pathogen *Acinetobacter baumannii* were analysed and compared using Ribotyping and PFGE. Twenty-six isolates were from 10 hospital outbreaks. Ribotyping with one enzyme showed 31 patterns, and a second enzyme added another 8 patterns. PFGE identified 49 distinct fingerprints. Nine epidemiologically-unrelated strains could not be differentiated from outbreak-associated strains by ribotyping, but they were by PFGE. PFGE was therefore more discriminatory than ribotyping. [52]

2.5.4.4.7 Vibrio cholarae O139

Forty-eight *V. cholarae* O139 strains were isolated over a 3-month period from patients in Thailand hospitals. These were compared to 4 strains of the same type from Bangladesh and India. Ribotyping and PFGE results were compared. PFGE was able to differentiate among epidemiologically-unrelated strains that were indistinguishable by ribotyping. However since PFGE did not demonstrate correlation between certain types or the origin of the isolates, the exact transmission routes were difficult to follow with PFGE alone. [37]

2.6 Potential Use of PFGE in Veterinary Epidemiology

Typing systems may be used in studies to identify sources of transmission of infecting organisms from general microflora. This understanding of the epidemiology of an infectious agent would assist with development of prevention and control strategies. [45]

As shown previously, PFGE has been used in several areas of human epidemiology. Most of the research to date has proved that PFGE is a more discriminatory method for strain typing than other typing methods. There is nothing to suggest that PFGE cannot be used equally in veterinary epidemiology as it has been in human.

Outbreak situations are studied in animals, and PFGE could provide laboratory confirmation of the presence of a single outbreak strain.

Typing data should supplement epidemiological investigations. The information provided could rule out an outbreak, hence prevent the cost of a study being incurred. On the other hand, it could demonstrate outbreaks involving more than one strain concurrently. Reliance on strain typing data without the underlying data from an epidemiologic investigation is to be discouraged, as results could be meaningless.[46] [33]

There is a great amount of diversity between the time scale seen in the studies mentioned previously. While most outbreak studies were of isolates collected over a few months [25], [38], there were also surveys conducted that observed isolates collected over the same time period [29], [34]. As well as the time scale, each of these studies compared strains from several geographic locations, between animals and between herds [29], between counties and states [38], within a ward [25] and within wards in 3 different countries [34]. It is clear to see that there are neither standard time frames nor standard spatial frames that studies have been designed to incorporate. It seems that investigators have used PFGE in the study regardless of what the study aimed to observe or prove.

The methods of the previous studies are compared to show the disparity between the use of PFGE in these situations. 175 isolates from an ICU ward in one hospital taken over 1 month resulted in nine strain types, one dominant. [25] Between 46 cows in 15 dairy herds, greater differences were observed between herds than between animals. [29] In typing isolates from three different countries, no isolate looked identical to that from another country, and considerable polymorphism was shown within each country. [34] Again in 265 isolates from nine counties (2 states), the outbreak strain from patients was indistinguishable from the food derived isolates that caused the outbreak. [38]

Studies that were conducted in under a year include those mentioned above as well as some others. One was of MRSA in a burn unit, nine isolates collected over a 6-month period showing no polymorphism between isolates, suggesting a resident

strain. [41] A study of another nosocomial bacterial infection spanning three hospitals led investigators to believe that dissemination of a plasmid was the cause behind the antibiotic resistance of *K. pneumoniae* to cephalosporins. This was determined by the presence of polymorphisms between 31 isolates collected from different wards of each hospital over eight months. [23] However, a particular strain of *S.* Brandenburg was isolated from several areas and several sources (human, animal feed,) in Switzerland, and this was compared to isolates from other countries (different patterns) and isolates from different years. [21] These studies were conducted over a "short" time frame whereas others span greater periods.

Several retrospective studies have incorporated outbreak-associated isolates to test the ability of PFGE to suggest clonally derived strains. One such study observed 45 isolates of *E. faecium* from 42 patients hospitalised over 15 months in one hospital. PFGE suggested epidemiologic association between 90-100% of isolates taken from one outbreak period, and in 38-75% of isolates taken during another. The sporadic cases, known to be unrelated, did not appear related to either the outbreak-related strains or each other. [42]

Some studies examined isolate differences over longer periods than two years. The study of *Salmonella enteritica* in poultry compared isolates from flocks that had been vaccinated with Strain Zoosaloral H against isolates from unvaccinated flocks in the previous nine years. The unvaccinated flocks exhibited more strain types (7) than were seen in the vaccinated flocks (3). [28] A two-year survey of isolates was conducted on MRSA and with the examination of 239 isolates, 26 patterns were seen, and one predominated despite the fact all the strains were epidemiologically unrelated. [24]

Two years collection of *Vibrio cholerae* El Tor isolates, including outbreak-associated isolates from three areas and sporadic isolates similar in time and geographic origin to the outbreaks, were examined by PFGE. Strains involved in the outbreaks were identical for each area, and isolates from sporadic cases were clearly different from each other and the outbreak strains. [19] This study showed that isolates were different over three geographical areas in Malaysia.

A study of *E.coli* O157:H7 broadened the geographical areas covered by isolates examined by PFGE, with outbreak-related isolates originating from Washington, Idaho, Nevada and California. Sporadic isolates from the outbreak period, the post-outbreak period and the pre-outbreak period were compared with isolates from the hamburger meat implicated in the outbreak and isolates from national herds of cattle. While a range of strain types was seen, the outbreak strain was seen in 100% of the outbreak, 48% of the sporadic (outbreak period), 27% of the post-outbreak, 1% of the pre-outbreak, 91% of the hamburger meat and 0% of the cattle isolates. This indicated the ability of PFGE to detect clonality in isolates from a broad geographical are if they were clonally derived. [18]

A study that observed strain types of *S. typhi* from three different countries in South East Asia examined isolates collected over a 7-year period. There were multiple patterns from each country, and some patterns were seen in two of the three countries. This suggested movement and mobility of the *S. typhi* strains via the moving human population. [15]

On a broader time scale still, some studies have incorporated several years of isolate collection into strain comparisons. *Salmonella* Enteritidis PT4 isolates were collected over 21 years and fell into 39 strain types. The origins of the isolates varied greatly, different sources and countries. [36] Increasing the scope of countries and host species examined, *Brucella* spp. were strain typed from the United States of America, the United Kingdom, Uganda, Africa and from different animal species bison, elk, cattle, humans, swine, sheep, dogs, goats and wood rats. Different brucellae showed different patterns, although *Brucella abortus* did not differ greatly between biovars. Bison and elk strains were similar to those seen in cattle. [27] In another study, *Campylobacter fetus* from Japan and the United States of America were compared. Although there were different strain types in each country, variation in the PFGE profiles did not appear to reflect the geographic origin of the bacteria. [16]

From the examples given it is clear to see that while there are many applications for PFGE in epidemiological studies, there are no guidelines for the time frame or spatial distribution of the isolates to which the technique can be applied.

2.7 Molecular Clock

The theory of the molecular clock is that the genome of bacterial strains changes constantly. However, it is not known how many asexual reproduction stages that bacteria go through before one of the random mutations causes a difference in the genome pattern as derived by PFGE. As this technique observes the fragments after the genome has been cut by rare-cutting restriction endonucleases, many nucleotides could be altered by mutation between the cleavage sites thereby potentially "hiding" the random mutations. Rare cutter sites are the most ancient landmarks available to typing systems, as they are the least frequent oligonucleotide sequences. [32]

To analyse epidemiological typing data, a measure of "genomic resolution" must be ascertained. This is the molecular clock of the markers according to the time and space scale of the investigation. The stability of the markers used must be sufficient to track variants through the chain of transmission, be it from animal-to-animal in under a week (outbreak situation) to country-to-country transmission over years (surveillance operations).

For genomic typing, this molecular clock can be measured by assessing the stability of strain types seen. Stability (S) is described as the ability of a typing system to recognise clonal ancestry of strains despite the phenotypic or genomic variation that may occur. (Suggested that 10 strains be studied after every fifth passage in an experiment of 50 passages/subcultures). [45]

Stabilities of bacteria in previous studies have been ascertained by various methods. Some studies followed the PFGE profiles of 40 generations as opposed to the suggested 50. [25] [24] Other studies examined stability using slightly different methods. RFLP data was used to estimate the molecular clock of the *E. coli* chromosome and it was determined that there would be one replacement per nucleotide per generation. [32] Stabilities of strain profiles were evidenced by finding the same profile in Swiss strains of *Salmonella* Brandenburg from 1983-4 and 1992. [21] *Yersinia pestis* was seen to be highly unstable *in vitro*, where five different profiles were seen among eight colonies of the same strain. In comparison, *Yersinia enterocolitica* displayed considerable *in vitro* stability when several colonies from

one strain were tested by PFGE, indicating that the genome is much more stable than that of *Y. pestis.* [20]

Genetic stability of the *Salmonella* Typhimurium vaccine strain Zoosaloral H was demonstrated when no differences in fragment patterns of seven vaccine strains reisolated during a 22-month period from the faecal samples of vaccinated chickens and the lyophilised vaccine. [28] Another vaccine strain (*Brucella abortus* RB51) was identified as being stable after passage through animals when no differences were seen between patterns from isolates from cattle lymph nodes 2-4 weeks after vaccination and the strain isolated from the lyophilised vaccine. [27]

The quantitative threshold of similarity used, as a working definition of a clone should be adjusted to the species studied, the typing system and the time-space frame of the epidemiologic investigation. Clonally-derived strains from a single host cluster at similarity levels above 80%. [45] Typing provides information on strain distribution within a host population over time and space. Surveillance programs (local, regional, national or country level) would benefit from such a library system where types can be compared over time and space between studies from different laboratories. [45]

3 SHEEP ABORTIONS

3.1 Ovine Abortions in New Zealand

Major infectious causes of ovine abortion include *Campylobacter* spp., *Chlamydia psittaci, Toxoplasma gondii* and various other bacterial and viral organisms. Clinical signs in the ewe and gross lesions in the foetus are often indistinguishable regardless of the aetiologic agent. [53]

A sheep abortion review was last carried out by MAF in 1984. Thirty-four percent of abortions were caused by *C. fetus fetus* and 27% by *Toxoplasma gondii*. [54] Four years later there were 57 outbreaks of sheep abortions, 40% due to *Campylobacter*, 7% of which involved flocks which had been vaccinated against this bacteria. Twenty-two percent of the sheep abortion outbreaks were caused by *Toxoplasma* and 3% were positive for both of these organisms. Neither *Chlamydia* spp. nor *Salmonella abortus ovis* were isolated from the abortions. [54]

A diagnosis was made in 60-80% of cases of abortions referred to labs in 1990. Generally, campylobacteriosis was the most common cause of sheep abortion. Other infections included several outbreaks of *Listeria ivanovii* abortion from the Lincoln region and outbreaks of both *Streptococcus agalactiae* and *Salmonella* Typhimurium abortion from the Invermay area. In the last of these, several ewes aborted and subsequently died. There was no evidence of *Chlamydia psittaci* or *Salmonella* Abortus ovis infection in any foetus or placenta. [55]

3.2 Salmonella spp.

Several serotypes of *Salmonella* have been associated with abortion in sheep. Two previous surveys (from 1958-1967 and 1968-1974) studied the incidence of ovine salmonellosis based on monthly reports from MAFF Labs in England. During the first survey, the majority of incidents were reported to be *S.* Abortus ovis followed by *S.* Dublin. The second survey showed the marked increase of *S.* Dublin infections, although the incidence of both *S.* Dublin and *S. abortus ovis* greatly decreased towards the end of the period. The number of incidents associated with other *Salmonella* serotypes increased. [56] Sporadic outbreaks of abortion involving

less common serotypes were reported, *S.* Derby, *S.* Agona and *S.* Montevideo were isolated from aborted materials in several flocks. [57] The decrease in incidence of *S.* Abortus ovis was of interest because of the economic importance of the organism in several countries. Reduced incidence suggested either that a carrier state does not develop or that once flocks have been infected and immunity develops, the organism has low pathogenicity. [56]

A changing pattern of the *Salmonella* serotypes causing animal disease in the UK was seen in the 1970's and early 1980's. *S.* Montevideo emerged as a significant cause of abortion in ewes [57] while *S.* Arizonae was implicated in abortions in a few flocks. [56] Abortion was the most common manifestation of *Salmonella* Dublin or *S.* Montevideo infections and the clinical picture was very similar for that described for *S.* Abortus ovis. Affected ewes generally did not scour and only showed a transient, mild illness before aborting. There was remarkably little evidence of scouring and ill health in lambs born at full term to ewes in the same group although they usually excreted the organism in their faeces for a time. The disease produced was much milder than that reported with other serotypes such as *typhimurium* where deaths from septicaemia were more frequent. [57]

4 BOVINE MASTITIS

Staphylococcus aureus is recognised worldwide as the most difficult mastitis pathogen to control. [58] It is ubiquitous, contagious and able to evade the hosts natural defences and responds poorly to antimicrobial treatment. Infections tend to be subclinical, producing mild to moderate elevations in herd Somatic Cell Counts. [59] Transmission of *Staphylococcus aureus* is from infected milk (primary reservoir) to an uninfected, susceptible quarter. This usually occurs during the milking process. [60] [61]

There are few estimates of the exact prevalence of *Staphylococcus aureus* in the literature, as there are difficulties in obtaining, updating and maintaining Bulk Tank Somatic Cell Counts on both a regional and national level. Large surveys conducted up to a decade ago estimated that more than 20% of cows had intramammary infections caused by *Staphylococcus aureus* [62] although these surveys were undertaken in Canada and Britain not in New Zealand. [63]

Widely recommended control plans advise a number of methods be used to reduce the rate of new infections. These include: proper milking management, adequately functioning milking equipment, teat cup disinfection, post milking teat disinfection, segregation of infected cows, dry cow therapy, improved cow resistance factors, and screening herd replacements[64-73]. [74] [75] [76] [77] [78] [60] [79] [80] [81] [82] [61]

Despite these control methods, herds still experience ongoing problems with new intramammary infections caused by *Staphylococcus aureus*[68,83-87]. [88] [89] [59] [90] [91] [60] [92]
It has been discovered that even when the general udder health improves on the farm, the relative importance of *Staphylococcus aureus* does not decrease. This is unexpected in light of the decrease of importance of Streptococcus agalactiae as health conditions improved. [93]

Dry cow therapy is an integral part of *Staphylococcus aureus* control on dairy farms. It has been shown to be up to 75% effective [77], although this result may be misleading as efficacy was calculated in quarters. An infected quarter remains a reservoir for other cows and therefore ‘cows’ should be the practical measure of

prevalence, not quarters. However there is conflicting evidence on the efficacy of dry cow therapy.

Some cows tested, cultured negative for *Staphylococcus aureus* within the first thirty days after calving, but were positive after 60 days. [77] This may be due to the cyclic nature of *Staphylococcus aureus* shedding. [94] Whether or not the infection was due to the same strain of *Staphylococcus aureus* was not determined.

It has also been discovered that a cow with 3 or 4 infected quarters is more likely to remain infected following dry cow therapy than a cow with only 1 or 2 infected quarters. [76] It was not shown whether the cow was as susceptible to new strains of *Staphylococcus aureus* as the old.

Researchers have been working on the development of vaccinations for *Staphylococcus aureus* for a decade with variable success. The successes are few and far between because of the highly strain specific nature of the vaccines produced[95]. [96] [90] [97] It has been thought that there are only a few strains of *Staphylococcus aureus* that predominate in mastitis, and they have a genetic composition ideal for evading host defences and antimicrobial treatments. [98] However, there may be more virulent strains emerging.

Eradication of *Staphylococcus aureus* from dairy herds is not yet a practical option. This means that the prevention of spread of infection and enhancement of the immune response are the most important pathways to reducing mastitis caused by *Staphylococcus aureus.* [99]

5 MATERIALS AND METHODS

5.1 Salmonella Brandenburg

5.1.1 LABORATORY STUDY

PFGE has become a very popular method of strain typing for *Salmonella* spp. among other bacteria. [2] [14] [15] [28] [21] [36] [48] It is highly reproducible, provided all the protocols are copied and the conditions under which the gel is run are identical.

PFGE has been used to determine the relatedness between strains of bacteria to support epidemiological studies. The reliability of this technique is dependent on choosing the correct enzyme. At least 10 bands must be produced, so that the genetic fingerprint of the bacteria can be compared. Random genetic changes (insertions, deletions, point mutations) must be taken into account when comparing fingerprints, as they give rise to slightly different patterns for two isolates of the same strain. [2]

The *Salmonella* Brandenburg outbreak in 1998 in Southland, Otago and Canterbury New Zealand was an ideal opportunity to use PFGE in the context of an epidemiological study. It allowed investigators to determine the strain type of the bacteria that caused the disease. This confirmed there was a definite outbreak strain. Subsequently, theories of the transmission and the origin of the strain were made based on this information. Strains were compared to those recovered from sheep in previous years and from humans.

PFGE was used to determine whether the sheep abortions were occurring due to infection by the same organism. This would indicate either a common source of infection or a common time of infection. A description of how the epidemic spread so quickly, why it only spread to certain farms, and whether it was the same strain of *Salmonella* Brandenburg, that caused all of the abortions, was desired.

5.1.1.1 Transport

Salmonella Brandenburg was isolated from ewes and their aborted materials by veterinary laboratories close to the area the animals were in. Isolates were delivered in airtight containers on nutrient agar slopes. When containers were received at

Massey University, they were kept in a refrigerator at 4oC until a sub culture was made.

5.1.1.2 Culture

A loopful of the growth on the nutrient agar slopes was streaked on a plate of Sheep Blood Agar (Columbia Blood Agar Base [Difco Laboratories] and 5% defibrinated sheep blood [GibcoBRL (Life Technologies)]) to obtain single colonies. The plate was incubated at 36^{o}C for 12-24hours. A colony from the plate was removed with a wire loop and added to Brain Heart Infusion Broth BHIB (Difco Laboratories). Following incubation for 24hours at 37^{o}C, 200µl of the broth was removed and used to prepare plugs for Pulsed-Field Gel Electrophoresis.

5.1.1.3 Plug Preparation

The broth was added to eppendorf tubes and centrifuged at 13000rpm for 5 minutes. The supernatant was removed (using a pipette) and the pellet resuspended in 150µl PETT IV Buffer (1M NaCl [BDH], 10mM Tris [BDH], and 10mM Na+EDTA [BDH]). This step was repeated and 50µl PETT IV Buffer used to resuspend the pellet the second time.

Bio-Rad Pulsed Field Certified Agarose was melted at a concentration of 1% w/v in PETT IV Buffer in a water bath. 75µl of molten cooled agarose was added to the re-suspended cells in the eppendorf tubes. This mixture was immediately transferred to a plug mould. The moulds were placed on ice to aid solidification.

Plugs were removed from the mould and added to new eppendorf tubes. 1ml of Lysis buffer (1M Urea [BDH], 50mM Tris [BDH], 50mM Na+EDTA [BDH], 1% N-lauryl sarcosine [SIGMA], 0.2% Na+deoxycholate [SIGMA] and 0.5mg/ml Proteinase K [Boehringer Mannheim]) was added to each tube and the plugs incubated in this buffer for 24 hours at 56^{o}C. Plugs were then removed from the eppendorf tubes and added to glass universal tubes. 10ml of TE Buffer (10mM Tris [BDH] and 1mM Na+EDTA [BDH]) was added to the tubes which were then plunged in ice and rocked for 1hour. The plugs were removed from the cleaning

buffer and placed in new eppendorf tubes with 1ml of TE Buffer. The plugs were then kept at 4^{o}C until needed.

5.1.1.4 Enzymatic Cleavage

A third of a plug was needed for the electrophoresis. The plugs were cut with a scalpel, the remaining two thirds placed back in the TE Buffer and returned to storage at 4^{o}C. The small pluglet was added to 100µl of restriction buffer (12% SureCut Buffer H [Boehringer Mannheim] and 1% 100X BSA (BioLabs) as suggested by the manufacturer of the enzyme. After equilibrating on ice for 45 minutes, the restriction buffer was removed and 80µl of cutting buffer (8% SureCut Buffer H [Boehringer Mannheim], 0.8% 100X BSA (BioLabs) and 15 Units *Xba1* [Boehringer Mannheim]) were added. After equilibrating for a further 45 minutes on ice, the tubes were added to a waterbath and incubated for 24 hours at 37^{o}C.

5.1.1.5 Pulsed-Field Gel Electrophoresis

A 1% w/v Pulsed-Field Gel Electrophoresis agarose gel containing 10% TBE Buffer (1M Tris [BDH], 1M Orthoboric Acid [BDH] and 2mM Na+EDTA [BDH]) was poured into a mould. The 20-well 1.5mm Bio-Rad comb was added after the gel was poured to allow correct well formation. The gel was left aside to set at RT for 1 hour. After this time, it was placed in the electrophoresis chamber and covered with 0.5% TBE Buffer. The gel equilibrated in the chamber for 1 hour as the buffer was cooled to 14^{o}C and pumped at a rate of 1L/min. After equilibration, the gel was removed and pluglets were added to the wells. Forceps were used to push the pluglet to the front wall of each well. Bio-Rad lambda ladders for Pulsed Field were added to the first, last and middle (if more than 10 isolates) lanes and the loaded gel was replaced in the chamber.

The parameters for Pulsed-Field Gel Electrophoresis used in this experiment were as follows. A voltage of 6V/cm was applied across the gel. Pulse times began at 3 seconds and were ramped in a linear manner to end at 25 seconds after 23 hours. The angle between pulses was 120^{o}. These settings were used for all the *Salmonella* Brandenburg isolates.

5.1.1.6 Imaging

Following electrophoresis, the gel was removed and added to a staining solution of 0.1% w/v Ethidium Bromide [BDH] in MQ water. The gel was stained for 10 minutes and the Ethidium Bromide solution poured into a storage bottle for recycling. The gel was then illuminated with UV light and a Polaroid (Polaplan 667) picture taken. Following this manual procedure, a Gel-Doc System (Bio-Rad and AlphaImager) was used to store the image in a digital manner for further analysis.

5.1.2 *SALMONELLA* BRANDENBURG QUESTIONNAIRE

A questionnaire was designed to quickly identify the risk factors of *Salmonella* Brandenburg abortions by a group of people in AgriQuality New Zealand. It was then sent to the veterinarians that were reporting the abortions amongst their clientele. The veterinarians then sent the questionnaire to the farmers of the affected farms and the completed document was sent back to them. Some veterinarians took this opportunity to add some questions that they would have liked answered themselves. Unfortunately, these questions did not go out to all farmers, hence some of these later questions could not be analysed. The completed questionnaires were gathered and sent to Massey University for analysis.

The questionnaire pertained to: lambing practices, movements onto and off the farm, vaccination history and feed management, previous abortion history, losses incurred in the 1998 season, and the outbreak itself. Appendix A contains a sample questionnaire.

The farmers were rapid in filling in the questionnaires as the abortions were obviously of some concern. Many farmers lost a great deal of revenue with the abortions – both in lambs lost as well as ewes that died. There was a good response rate from the farmers with abortions, but very little response from ‘control’ farmers. Consequently only limited information was available for identification of risk factors that were present on case farms and not on control farms.

Information obtained was in enough detail to give a reasonably accurate case description of both the abortions and the farms upon which they occurred.

5.2 Staphylococcus aureus

Staphylococcus aureus isolates were collected from 7 farms in 1996 from subclinical mastitis cases. Some were sequentially collected from the same animal (but often a different quarter) on more than one occasion. Some bacteriology needed to be carried out to ensure the bacteria isolated are *Staphylococcus aureus*.

Pulsed-Field Gel Electrophoresis is a highly discriminatory method of strain typing human *Staphylococcus aureus* infections[100,101], and has also been used for *Streptococcus* species isolated from bovine mastitis. All isolates were typed using this method, to elucidate the genotypes of the most virulent strains, thereby identifying the appropriate ones to be included in the search for a vaccine/bacterin.

5.2.1 CULTURE & IDENTIFICATION

Fifty-one *Staphylococcus aureus* isolates were collected from seven farms between May 1996 and January 1998. The animals all had sub-clinical mastitis and the milk samples taken from each quarter were checked for bacteria. These were stored at –70^{o}C for two years in Glycerol Broth (GB). 48 samples that had been taken from one animal on two different occasions were studied. A swab of the GB was spread onto Sheep Blood Agar (SBA) and incubated for 24 hours at 37^{o}C. A representative colony was then streaked onto another SBA plate and added to BHIB to obtain a pure growth. The plate and broth were incubated as above.

Colonies that exhibited typical *S. aureus* morphology were tested biochemically for catalase, hyaluronidase and DNAse activity and Gram stained. Colony morphology of *S.aureus* on SBA is flat and opaque yellowish colonies with a or b hydrolysis zones.

DNAse activity was measured by streaking a section of a colony onto a DNAse agar plate (Difco Laboratories). The plate was incubated as before and the growth covered with a 1N solution of Hydrochloric Acid (BDH) after 24 hours. An Oxford *S. aureus* positive control and a *Streptococcus* spp negative control were also grown on the same plate for comparison. Clear zones around the growth under the HCl indicated a positive result.

Catalase activity was measured on a slide. A small section of a colony was smeared onto a glass slide, and a drop of 3% Hydrogen Peroxide solution (BDH Analar 30%w/v) added to the smear. A positive result was recorded following immediate bubbling.

When testing for the presence of hyaluronidase, *Streptococcus equi* was smeared onto half of a SBA plate. A swab of the *S. aureus* suspension in BHIB was dragged over the plate in a line, until the swab just touched the line of *S. equi* inoculum. Positive and negative controls (*Staphylococcus aureus* and *Staphylococcus intermedius* respectively) were also swabbed onto the plate. An area of no *S. equi* growth close to the end of the streak of *S. aureus* indicated a positive result.

A colony of *S. aureus* from the purity plate was then added to BHIB and incubated at 37^{o}C for 24 hours to be used for making plugs as above.

5.2.2 PLUG PREPARATION

The method for plug preparation follows the method for *Salmonella* very closely. However, there is a pre-lysis step necessary for *Staphylococcus spp* due to the unusually thick cell wall of these bacteria. The plugs were removed from the mould and added to eppendorf tubes containing 1ml of Pre-Lysis buffer (1M NaCl [BDH], 10mM Tris [BDH], 100mM Na+EDTA [BDH], 0.5% N-lauryl sarcosine [SIGMA], 0.2% Na+Deoxycholate [SIGMA], 1mg/ml Lysozyme [Boehringer Mannheim] and 50µg/ml Lysostaphin [SIGMA]). The plugs were incubated in this buffer at 37^{o}C for 24 hours.

Then the plugs were removed from the pre-lysis buffer and added to the Lysis buffer as for the *Salmonella* technique.

5.2.3 ENZYMATIC CLEAVAGE

The same technique was used for both bacteria, although *Sma1* (Boehringer Mannheim) and SureCut Buffer A (Boehringer Mannheim) were substituted for *Xba1* and SureCut Buffer H.

5.2.4 PULSED-FIELD GEL ELECTROPHORESIS

Again, the same parameters were used, although the run time was reduced from 23hours in the *Salmonella* method to 21 hours for the *Staphylococcus* method to prevent DNA from running off the end of the gel.

5.2.5 IMAGING

There was no change in the method for this stage.

6 RESULTS

6.1 Salmonella Brandenburg

6.1.1 THE OUTBREAK STRAIN

The outbreak strain has a particular fingerprint or pattern. This consists of the following bands (in order of highest to lowest base pair size). The size of the DNA strands (bands) is measured in base pairs.

BAND NUMBER	SIZE (BP)
Band 1:	436
Band 2:	366
Band 3:	311
Band 4:	289
Band 5:	275
Band 6:	247
Band 7:	238
Band 8:	224
Band 9:	200
Band 10:	164
Band 11:	148
Band 12:	136
Band 13:	127

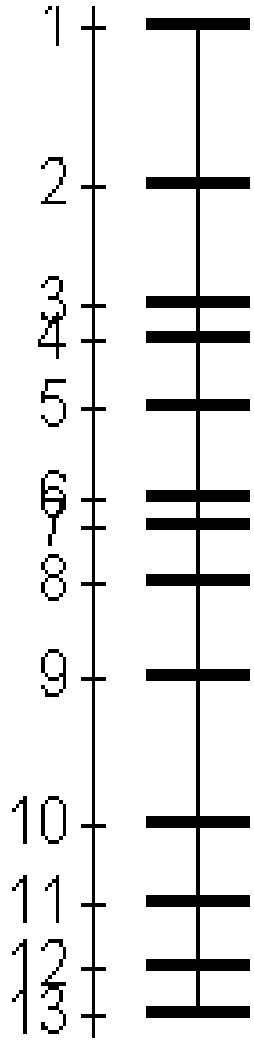


Figure 2: The outbreak strain

This strain differs from the *S.* Brandenburg reference strain held by ESR (Environmental Science and Research) in Porirua, Wellington. The similarity

between the outbreak strain and the reference strain was only 57.1%. This indicates that approximately half of the bands were present in both strains. The difference between the Outbreak strain and the *S*. Hindmarsh reference (also held by ESR) was even greater, with a similarity index of only 33.3%.

6.1.2 QUESTIONNAIRE ANALYSIS

Seventy-four percent of the 97 farms that had abortions on a large scale were laboratory diagnosed as having *Salmonella* Brandenburg. The remainder of the farms was diagnosed by the symptoms of the abortions, and often the fact that neighbouring farm had been diagnosed with *Salmonella* Brandenburg.

On 60% of the farms with *Salmonella* Brandenburg infections lambing was due to begin between the 1st and 13th of September, with mating occurring 150 days earlier. Rams were brought in on only 10% of the farms. However, veterinarians in the area believe the average to be closer to 80%. Twenty-five percent of the farms stated that there had been stock movement of any description on to the farm within the last year. This question was optional and some farmers may have chosen not to answer. Some farmers may not have thought of rams being brought in as stock movement and hence not reported it. Whether these figures are abnormal for sheep farming in these areas cannot be ascertained from the current data, as no data for the unaffected control group is available yet. The mean size of the affected mobs was 827 sheep, with a maximum size of 4000 on one farm. The median (787) may be a better representation of the average mob size as it is unaffected by outliers such as this 4000 head mob or those farmers that did not respond to this question.

Most farmers (76%) stated that the abortions occurred in stock that were either two-tooth age, mixed age, or both, although 14% stated that losses occurred mainly in the four-tooth's. Seventy-five percent of the farmers noted that the abortions occurred primarily in twinning or multiple lamb ewes. This may be due to the opportunistic nature of *Salmonella* in that these ewes would be under more stress than those carrying single foetuses and therefore more susceptible to infection and subsequent abortion.

The average (both mean and median) duration of the abortion outbreak on farms was 29 days, although one farm reported only a 7-day period of abortion. The maximum duration was recorded as being 107 days although this may have been an anomaly as the next smaller value was 75 days. This information was calculated by subtracting the date of the first observed abortion from the date of the last observed abortion; so those farmers that did not answer one or both of these questions (16%) could not be included in the analysis. The information above included only those farms where laboratory confirmation had occurred., and it should be taken into consideration that only a limited number of the abortions would have been confirmed by laboratory diagnosis

The primary affected areas were Winton and Milton in South Otago, each accounting for 27 and 18% of the affected farms respectively.

Most affected farms reported a lambing loss of 17%. In farms where scanning was carried out prior to lambing (60% of the farms included in the questionnaire), the loss was determined by the formula [% at lambing/% scanned x 100].

In terms of how many lambs were lost, the average number was 132 lambs, which represents a financial loss of approximately NZ$5280 ($40 per lamb) for that farm. However, only 34% of the respondents answered the question. The loss of lambs ranged from 23 to 500, which meant financial losses were in some cases up to $20,000. These results must be viewed carefully though, as not all of the losses would have been due to *Salmonella* Brandenburg.

Losses of ewes were also very important, with a range of 15 to 350 ewes being lost on farms. Slightly more farmers answered this question than that regarding the lambs (48%). The average loss was 128, which would result in a financial loss of $6400 for that farm ($50 per ewe) not to mention subsequent lambing potential which must also be considered.

Most farmers either shifted the mobs daily (32%) or break fed (29%) prior to the onset of the abortions. In the face of the outbreak, they altered the management to either setting the stock (30%) or spreading it out (21%). Feeding before and during

the outbreak changed very little, with the majority of farmers allowing the sheep pasture with no supplements (30% before and 44% during outbreak).

Several farmers had other species of animals on the property. Sixty-four percent did not respond to this question, although this is not an indication that they did NOT farm any other species. Other enterprises were primarily cattle (30% of farms), with other species occurring less frequently (deer 10%, pigs 1% and goats 1%).

The epidemic curve showed a peak around week 35 of the year (beginning on the 31st August 1998) and a sharp decline towards the end of week 39 (20th September 1998) of the epidemic. This is likely to be due to the nature of the problem; sheep can only abort while they are carrying a lamb - if there is no pregnancy, there can be no abortion.

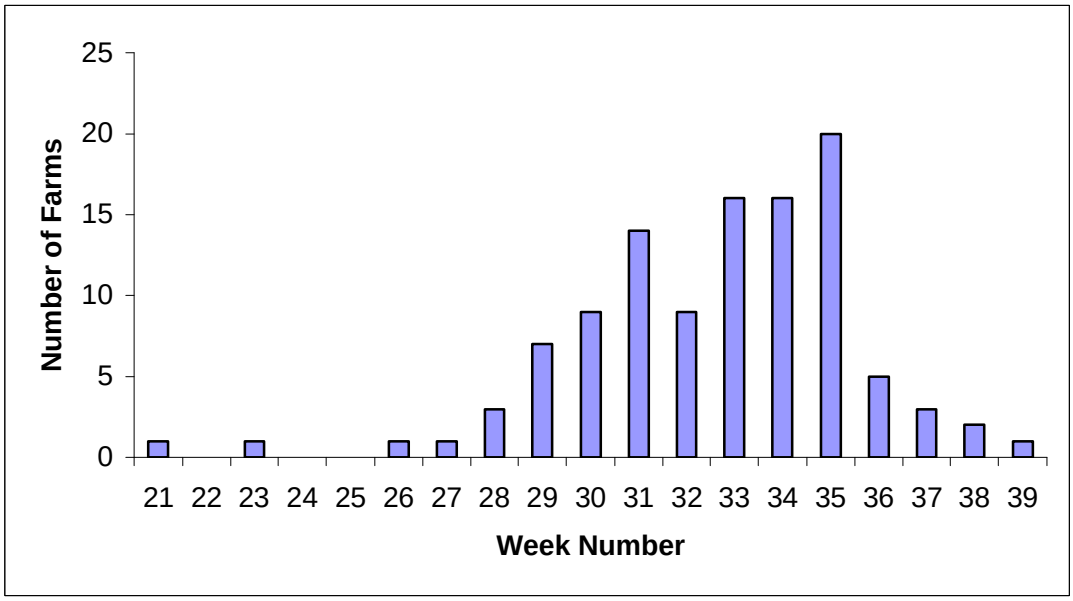


Figure 3: Epidemic Curve of *Salmonella* Brandenburg abortions in 1998

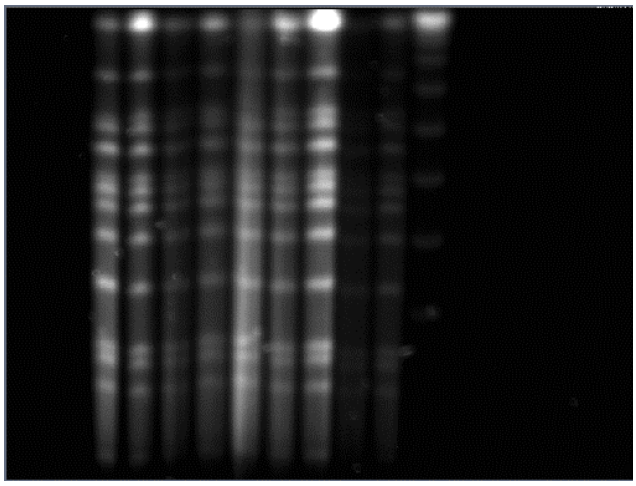
All the farms exhibited infection by the same strain of *Salmonella* Brandenburg. While some strains appeared slightly different, (smallest similarity index of ovine isolates was 81.8%) the genetic differences between them could be explained by the deletion of two restriction sites (which could be due to a simple point mutation in each). The amount of genetic difference between these few strains does not lead us to believe them to be different, but one may be a substrain of the other.

This indicates that while all the strains were the same (or at least, similar enough to be considered ‘the same’) there must be something on the farm that linked the strain to certain farms. Due to the lack of information on the control farms, it is impossible to determine why some farms were affected with *Salmonella* Brandenburg and others in the same area remained uninfected.

6.1.3 IMAGES OF *SALMONELLA* BRANDENBURG

Details of the photographs show the laboratory numbers of the isolate sent for Pulsed-Field Gel Electrophoresis. Where known, the area and the species have also been included. The lanes read left to right as the column of details reads downwards. Lambda ladders are at both ends of the gel and on occasion in the middle as well. Descriptions of each gel are situated beneath the data.

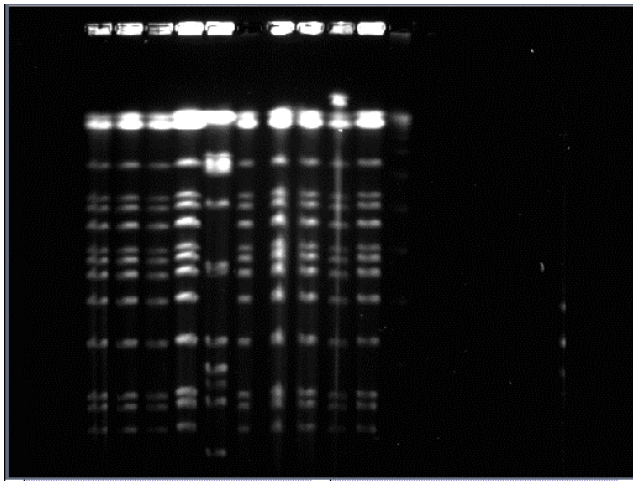
GEL 1



ISOLATE NUMBER	SPECIES	AREA
9058	Ovine	Dipton
9062	Ovine	Oreti
8093	Ovine	Lochiel
7881	Ovine	South Hillend
9008	Ovine	Winton
9108	Ovine	Heddon Bush
9079	Ovine	Milton
8160	Ovine	Moneymore
8684	Ovine	Winton
8946	Ovine	Otautau
LL		Lambda Ladder

These isolates were amongst the first to be sent to Massey University for strain typing by PFGE. They had been collected and isolated by the laboratory to which aborted material was sent by local veterinarians in the areas. As well as being the first isolates typed, the PFGE method was tested on these isolates and it was found to work well.

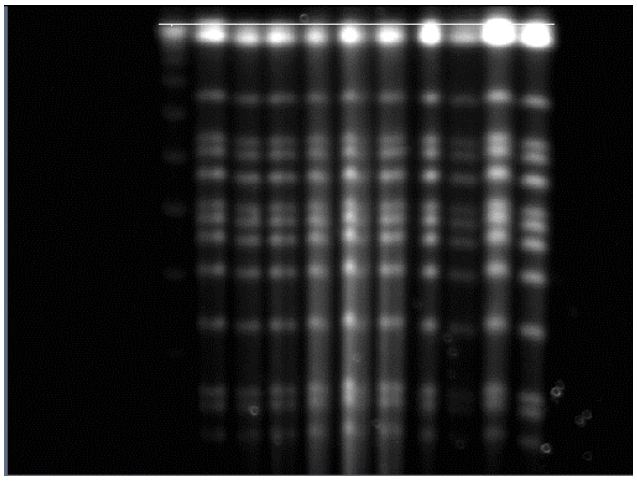
GEL 2



ISOLATE NUMBER	SPECIES	AREA
8736	Ovine	Grove Bush
8500	Ovine	Invercargill
9023	Ovine	Milton
8908	Ovine	Spar Bush
9027	Ovine	Waipahi
8414	Ovine	Milton
8524	Ovine	Invercargill
8568	Ovine	Winton
8289	Ovine	Milton
8884	Ovine	Milton
LL		Lambda Ladder

These isolates had been sent in one batch with those in Gel 1. With the isolates was a list of the areas that the veterinarians had found the aborted materials. Some of these isolates may have been found in ewes rather than the foetal materials but Massey University was not informed if this was the case.

GEL 3



ISOLATE NUMBER	SPECIES	AREA
LL		Lambda Ladder
8663	Ovine	
8332	Ovine	
8848	Ovine	
8686	Ovine	
8471	Ovine	
8646	Ovine	
8642	Ovine	
8882	Ovine	
8709	Ovine	
8659	Ovine	

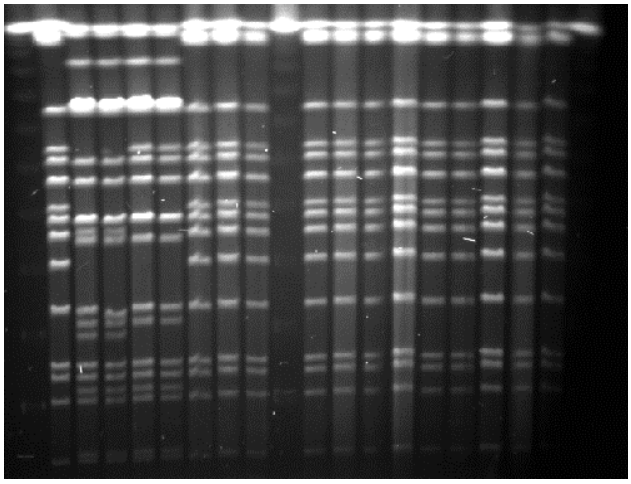
The second batch of isolates sent to Massey University did not contain any information about the origins of the bacteria. Oral communication determined that

they were from sheep in the affected areas, but no further details could be elucidated.

GEL 4	ISOLATE NUMBER	SPECIES	AREA
	LL		Lambda Ladder
	3844	Human	
	4200	Human	
	4337	Human	
	4393	Human	
	4472	Human	South Canterbury
	4476	Human	Otago
	4579	Human	Southland
	4692	Human	
	LL		Lambda Ladder
	4740	Human	
	4866	Human	
	4881	Human	
	5013	Human	
	4942	Human	Sth Auckland
	4219	Human	Sth Canterbury
	4814	Human	Sth Canterbury
	4839	Human	Sth Canterbury
	LL		Lambda Ladder

This gel contains isolates sent from ESR to Massey University. They are all from humans and are from the same period as the outbreak occurred in the sheep. Similarities are clear although there seems to be more smearing at the higher molecular weight bands. All but one isolate shows the same pattern of bands as the sheep isolates show and that is the isolate from South Auckland (4942). One isolate from South Canterbury (4219) failed to run and resulted in a smear that could not be evaluated.

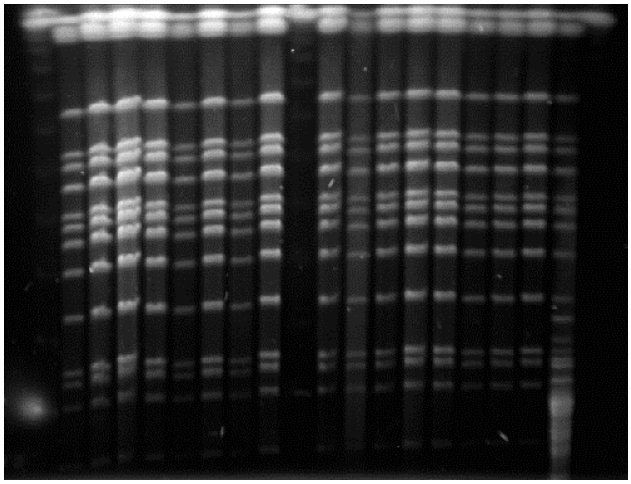
GEL 5



ISOLATE NUMBER	SPECIES	AREA
LL		Lambda Ladder
8052	Ovine	Oreti Plains
9113	Ovine	Otautau
8051	Ovine	Winton
9180	Ovine	Winton
8272	Ovine	Milton
9181	Ovine	Centre Bush
8145	Ovine	Riverton
8233	Ovine	Hokonui
LL		Lambda Ladder
7860	Ovine	Milton Area
8098	Ovine	Winton
7551	Ovine	Milton
7858	Ovine	Milton
8146	Ovine	Riverton
97211	Human	Christchurch
97303	Human	Palmerston North
97442	Canine	Palmerston North
971520	Poultry	North Island
LL		Lambda Ladder

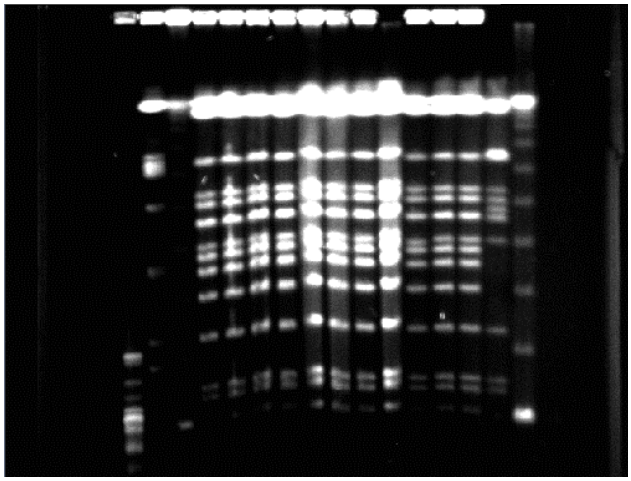
This gel completes the isolates from the first batch that were sent to Massey University. Here one can see that there are some discrepancies in the strains. Isolates 9113, 8051, 9180 and 8272 all from sheep within the area affected by the outbreak were unrelated from the strain type assigned "outbreak strain". [46] The similarity index between these strain patterns and the outbreak strain patterns were 87% and 91% for the two pairs respectively and as this falls above the 80% threshold suggested for outbreak studies, they can be assumed to have been part of the outbreak. [45] The gel also shows that humans, avian species and dogs from other parts of the country are able to shed the bacterial strain of S. Brandenburg that has been involved in the outbreak. It was not known (and nor can it without epidemiologic study) whether these animals were infected by the same source as infected the sheep

in the South Island or whether they were infected by contact with sheep that were infected. Further details are unlikely to be forthcoming as the isolates from any other than sheep were sent from ESR in Kenepuru and there are confidentiality issues to consider.



GEL 6	ISOLATE NUMBER	SPECIES	AREA
	LL		Lambda Ladder
	8146	Ovine	Riverton
	8172	Ovine	Milton
	9006	Ovine	Winton
	9063	Ovine	Tuturau
	9090	Ovine	Spar Bush
	9110	Ovine	Otautau
	9111	Ovine	Birchwood
	9114	Ovine	Otautau
	LL		Lambda Ladder
	299263	Seagull	
	299261	Seagull	
	299262	Seagull	
	9436	Bovine	Milton
	9461	Ovine	Winton
	9468	Ovine	Gore
	9504	Bovine	Clinton
	9523	Canine	Winton
	9526	Ovine	Southland
	LL		Lambda Ladder

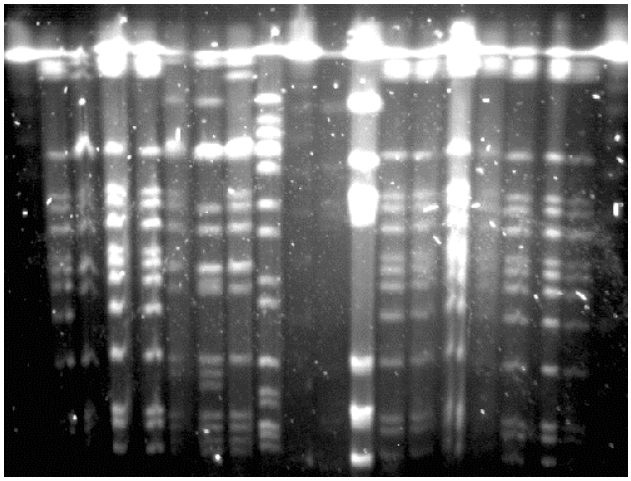
The isolates in the gel above were a mixture of those sent from the laboratory related to the outbreak investigation, and those that were from a case control investigation into the likely role of black backed gulls in the transmission of the S. Brandenburg strain. Black-backed gulls were sacrificed for the study, and intestinal bacterial counts were carried out. [102] The dog and cow had diarrhoea symptoms and isolates were sent with the sheep strains for strain typing. The very last lane on this gel shows considerably more bands in the lower molecular weight range than have been seen previously. Other than these bands (that are unexplainable) there were no differences. Further analysis with Diversity Database (BioRad) shows that the similarity index between this isolate and the "outbreak strain" was greater than 80% so it can be considered associated with the outbreak [45]



GEL 7	ISOLATE NUMBER	SPECIES	AREA
			Pasteurella
			Hindmarsh
	LL		Lambda Ladder
	99745	Seagull	
	95933	Ovine	
	95935	Ovine	
	95937	Ovine	
	98395	Ovine	
	983911	Ovine	
	983918	Ovine	
	983919	Ovine	
	9885	Seagull	
	9966	Ovine	
	0006	Seagulls	
			Brandenburg
	LL		Lambda Ladder

Most of these strains were part of the case control study mentioned earlier hence the lack of details in regards to area of isolation. The first lane is a *Pasteurella* spp that was being tested with this PFGE program to see if the enzymes were able to cleave the bacteria etc. This gel also included a few standard strains that were sent from ESR in Kenepuru. These references included one Brandenburg, and one Hindmarsh strain to determine the differences between them. The Brandenburg reference shares a lot of DNA fragments with the outbreak strain, whereas the Hindmarsh reference is considerably different. Similarity indices are 57.1 and 33.3% respectively.

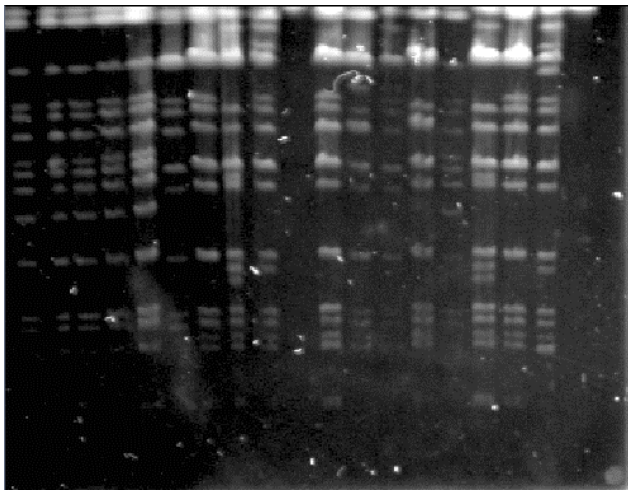
GEL 8



ISOLATE NUMBER	SPECIES	AREA
LL		Lambda Ladder
983914	Ovine	
8471	Ovine	
8848	Ovine	
7136	Ovine	
971520		
973146	Poultry	North Island
973309	Human	Hutt Valley
973385	Human	Waikato
LL		Lambda Ladder
6909	Ovine	
6925	Ovine	
6926	Ovine	
6927	Ovine	
7037	Ovine	
7039	Ovine	
7126	Ovine	
7136	Ovine	
9570	Ovine	
LL		Lambda Ladder

This gel was not particularly well captured. However it is possible to see that there are some differences in isolates 971520, 973146, 973309 and 973385. These isolates were from a different year (1997) and from different areas of the country, therefore some difference is expected. It is also interesting to note that isolate 6909 is more similar to the Hindmarsh reference in Gel 7 than to the outbreak strain. The sheep isolates in this gel are from Invermay in the South Island, while the human isolates are once again from ESR.

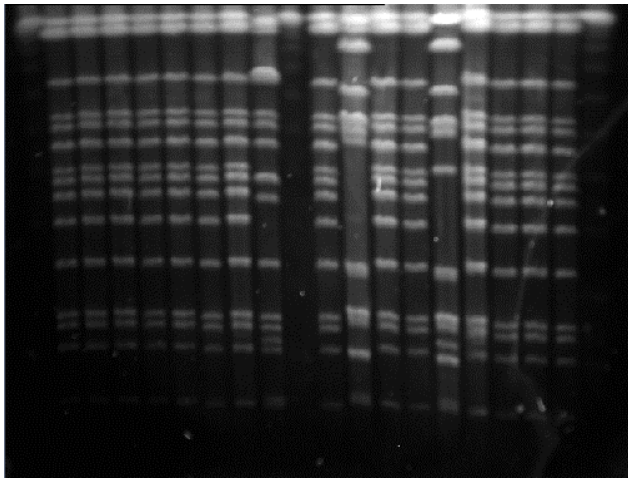
GEL 9



ISOLATE NUMBER	SPECIES	AREA
LL		Lambda Ladder
702/543	Ovine	
702/541	Ovine	
702/540	Ovine	
702/385	Ovine	
702/383	Ovine	
963635	Human	Auckland
963557	Human	Overseas travel
963197	Human	Christchurch
963187	Human	
LL		Lambda Ladder
961845	Human	Auckland
961159	Human	Hutt Valley
96660	Human	Christchurch
96539	Human	Hutt Valley
95684	Human	Pattern 5
943976	Human	Pattern 2
94484	Human	Pattern 4
91182	Human	Pattern 1
LL		Lambda Ladder

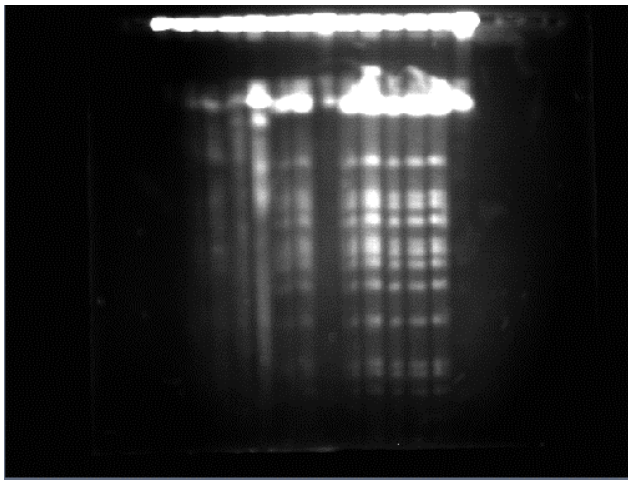
Again, all the human isolates in this gel are from ESR. The laboratory in Lincoln in the South Island sent the first 5 ovine isolates to Massey University. They show no difference to the outbreak strain. The rest are different from the outbreak strain, albeit in different ways. Three human isolates appear to be similar to each other (963557, 963187, and 961845) but the remaining human isolates differ from each other by at least one band.

GEL 10



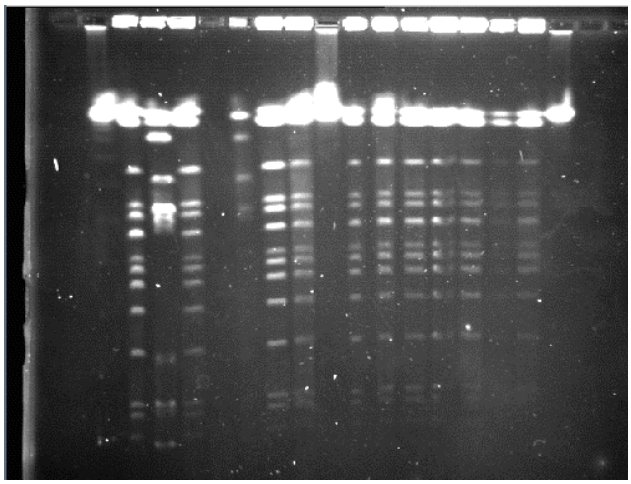
ISOLATE NUMBER	SPECIES	AREA
LL		Lambda Ladder
7070	Ovine	
7113	Ovine	
7126	Ovine	
7136	Ovine	
7339	Ovine	
7126A	Ovine	
702545	Ovine	
7489	Ovine	
LL		Lambda Ladder
7575	Ovine	
7636	Ovine	
7722	Ovine	
29702A	Ovine	
8113	Ovine	
29702AD	Ovine	
29702AC	Ovine	
702265	Ovine	
8126	Ovine	
LL		Lambda Ladder

These isolates are mainly from Lincoln laboratory as mentioned above. Although they were all isolated from sheep samples, there were differences seen in the PFGE patterns as evidenced above. Isolates 7636 and 8113 were similar to each other but different from the outbreak strain (similarity indices 87% and 81% respectively). This categorises them in the same type as the outbreak strains according to criteria mentioned in other studies [45]. The remaining isolates show the typical banding pattern of the outbreak strain.



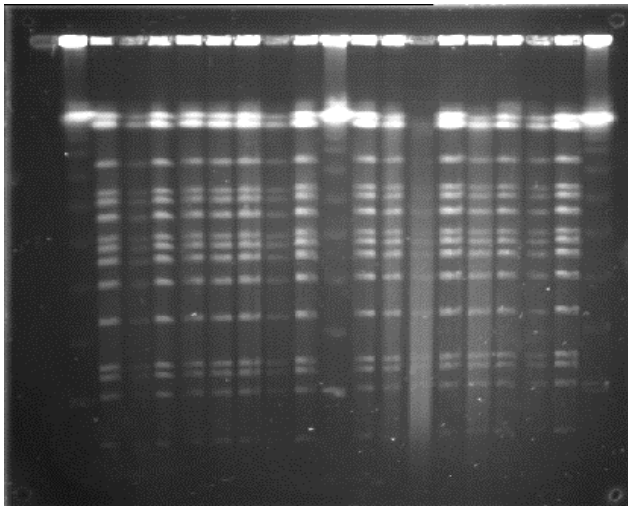
GEL 11	ISOLATE NUMBER	SPECIES	AREA
	LL		Lambda Ladder
	7070	Ovine	
	7113	Ovine	
	7126	Ovine	
	7136	Ovine	
	7339	Ovine	
	7126	Ovine	
	LL		Lambda Ladder
	702/545	Ovine	
	7489	Ovine	
	7575	Ovine	
	7636	Ovine	
	7722	Ovine	
	LL		Lambda Ladder

Though faint, the banding patterns that can be seen in this gel are clearly indistinguishable from the outbreak strain. It is not known why this gel should have been so faint, although at about this time a small crack was noticed in the electrophoresis chamber close to the drain manifold. Leakage was quite great and there may not have been sufficient buffer in the chamber during the run to prevent the degradation of DNA.

GEL 12	ISOLATE NUMBER	SPECIES	AREA
	LL		Lambda Ladder
	702/545	Ovine	
	7636	Ovine	
	7722	Ovine	
	29702	Ovine	
	8113	Ovine	
	29702	Ovine	
	29702	Ovine	
	LL		Lambda Ladder
	702/265	Ovine	
	702/383	Ovine	
	702/385	Ovine	
	702/540	Ovine	
	702/541	Ovine	
	702/543	Ovine	
	702/544	Ovine	
	LL		Lambda Ladder

This gel shows some differences between strain types again. Isolate 7636 and 8113 are different from the outbreak strain. Both isolates were typed in a previous gel (10) and the similarity between the lanes in gel 10 and the lanes in gel 12 show that PFGE was reproducible in duplicate strains.

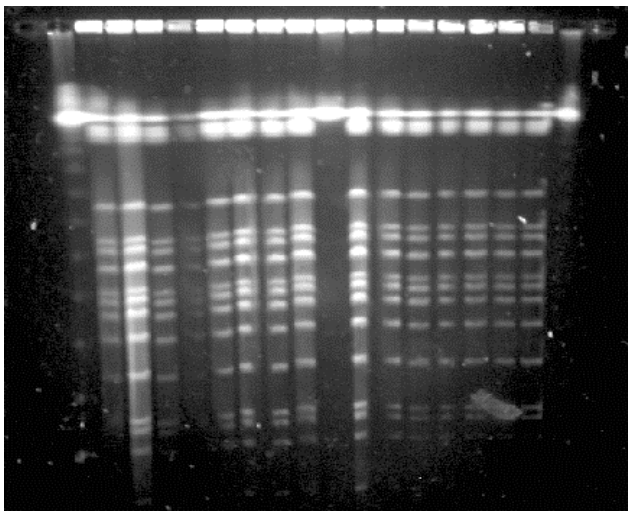
GEL 13



ISOLATE NUMBER	SPECIES	AREA
LL		Lambda Ladder
8371	Ovine	
8385	Ovine	
8460	Ovine	
8531	Ovine	
8713	Ovine	
8842	Ovine	
8921	Ovine	
9007	Ovine	
LL		Lambda Ladder
98090552	Ovine	Winton
98010568	Bovine	Balclutha
95011146	Bovine	Balclutha
98011676	Bovine	Shirling
9805223/5	Ovine	Winton
98015223/17	Ovine	Winton
98062840	Bovine	Balclutha
98061997/10	Ovine	Winton
LL		Lambda Ladder

All except from one isolate was typed on this gel. Isolate 95011146 from a cow resulted in a smear. Upon careful inspection, some bands at the lower molecular weight can be seen through the smear that suggest that there were nucleases present in the preparation stages that were not present in those for the other isolates. From this gel, cows and sheep can be colonised by the same strain of *Salmonella* Brandenburg.

GEL 14



ISOLATE NUMBER	SPECIES	AREA
LL		Lambda Ladder
9083	Ovine	
9171	Ovine	
9173	Ovine	
9306	Ovine	
9359	Ovine	
9359	Ovine	
9358	Ovine	
7881	Ovine	South Hillend
LL		Lambda Ladder
7375	Ovine	Centre Bush
9181	Ovine	Riverton
8145	Ovine	
8233	Ovine	Hokonui
7551	Ovine	Milton
9063	Ovine	Tuturau
29926	Seagull	
LL		Lambda Ladder

This penultimate gel included some strains from different laboratory batches that had been sent to Massey University. Those before the Lambda Ladder arrived in the second Invermay batch (without details as to the area the sheep were found in) and those after were from the first. The seagull isolate was from the case control study as mentioned previously. There were several isolates from seagulls with the same number and this one was not typed earlier. All isolates appear indistinguishable from the outbreak strain.

GEL 15	ISOLATE NUMBER	SPECIES	AREA
	LL		Lambda Ladder
	963202	Ovine	Ashburton
	963453	Canine	
	972798	Porcine	
	972895	Human	Canterbury
	973030	Human	South Canterbury
	973407	Ovine	
	98358	Human	Christchurch
	LL		Lambda Ladder
	98458	Human	Christchurch
	984019	Cockles	
	984113	Cockles	
	984114	Cockles	
	984171	Human	Southland
	984232	Human	Southland
	LL		Lambda Ladder

The final gel was very faint indeed. As the gel was not visualised with the Gel Doc system the isolates were not incorporated into analysis, similarity indices could not be calculated as the gel could not be incorporated into the Diversity Database (BioRad). The single porcine isolate included in the whole study resulted in a smear. No bands could be seen through the smear. The faint bands that could be seen in the remainder of the gel appeared to be similar to the outbreak strain including those isolates from raw cockles.

6.2 Dendrogram of Salmonella Brandenburg strains

It was important to quantify the similarity between the strains profiled by PFGE. Once this was done, UPGAMA (previously described in Chapter 2.2) was used to determine which isolates were clonally descended.

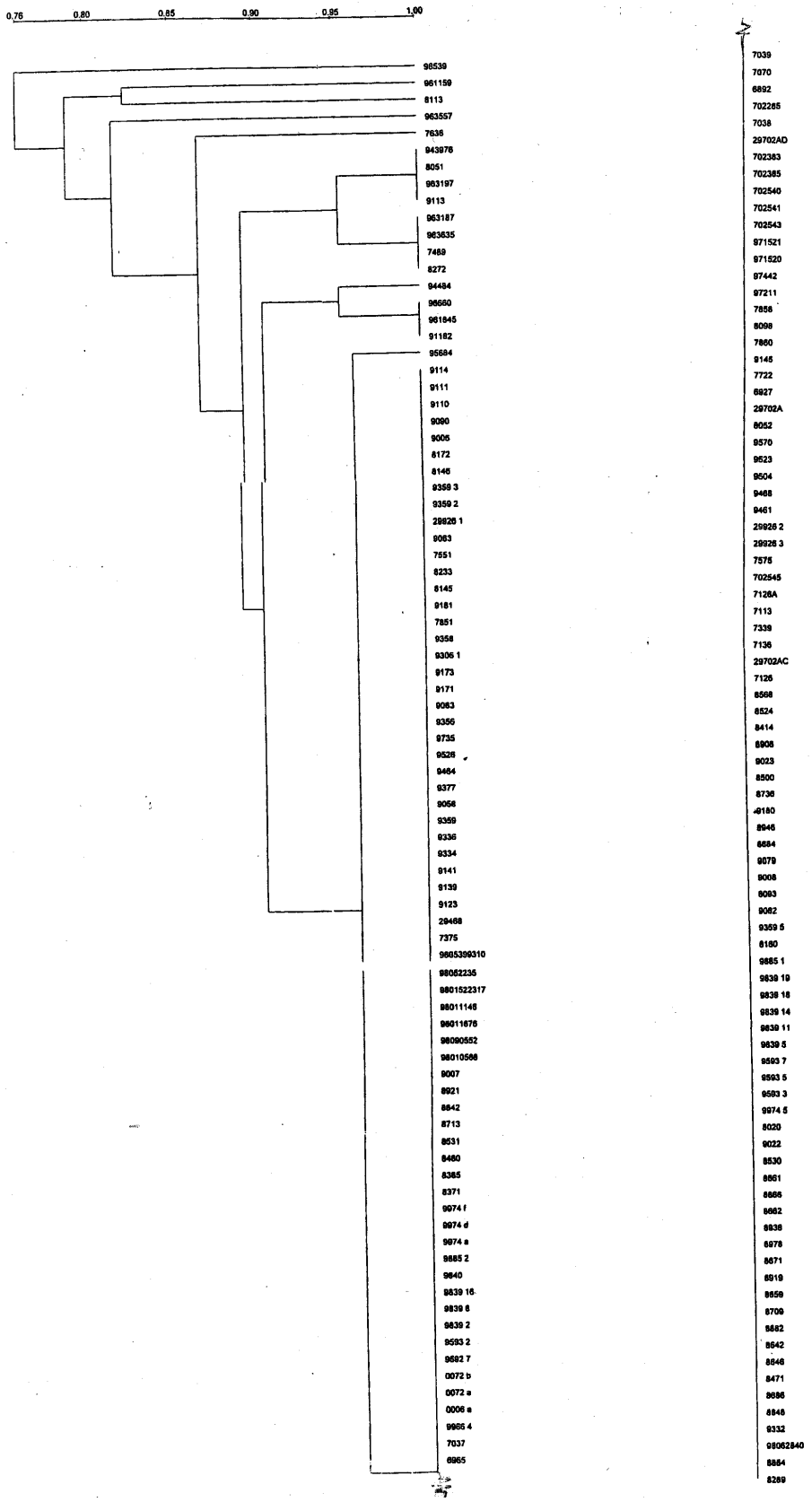


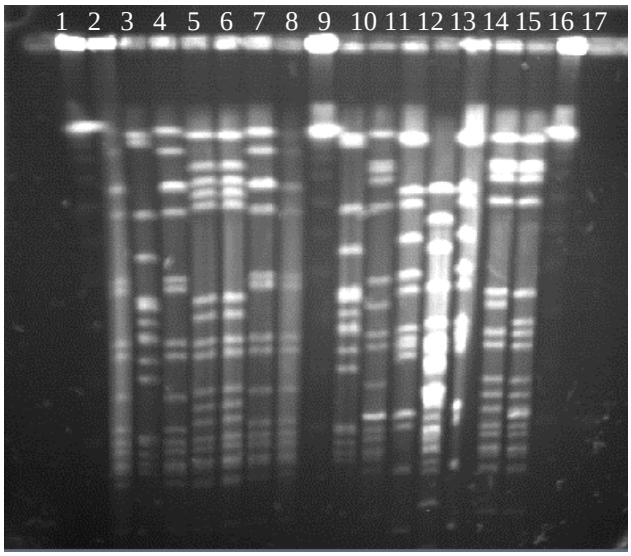
Figure 4: UPGAMA phylogenetic tree developed using the Dice coefficient of *Salmonella* Brandenburg isolates sent to Massey University for typing by PFGE

6.3 Staphylococcus aureus

6.3.1 IMAGES OF *STAPHYLOCOCCUS* AUREUS

The following photographs show restriction patterns following Pulsed-Field Gel Electrophoresis of bacterial isolates. Each sample was taken from a cow that was considered 'subclinical' for mastitis. The area and farm number, date, cow identity tag and quarter have also been included. The mammary gland of the cow is divided into four quarters. There are two at the front of the udder, and two at the rear. Hence the codes RR (Right Rear), LR (Left Rear), RF (Right Front) and LF (Left Front) refer to the quarters as they would appear to the cow, not to the veterinarian standing in front of the cow. The lanes read left to right as the column of details reads downwards. Lambda ladders are at both ends of the gel and in the middle as well. Descriptions of each gel are situated beneath the data.

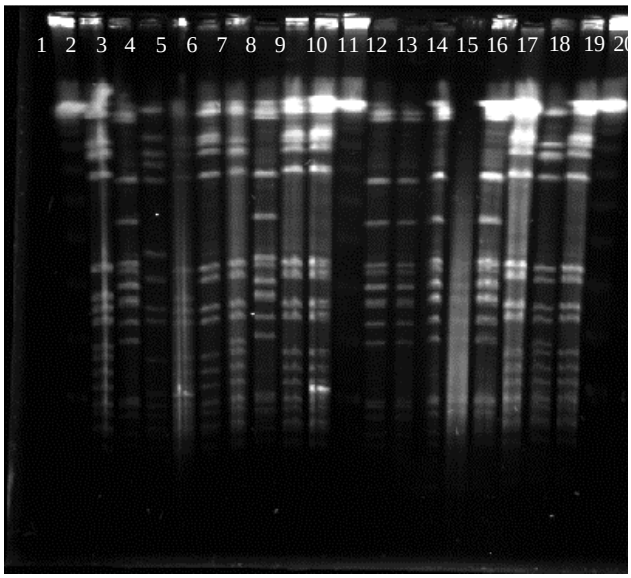
GEL 1



LAB #	COW ID	DATE	FARM
LL			Lambda Ladder
231	170 LR	12/10/96	Hawera 18
239	142 RR	12/10/96	Hawera 18
441	170 LR	26/10/96	Hawera 18
442	142 RR	26/10/96	Hawera 18
316	155 RR	29/10/96	Palmerston Nth 6
317	155 LF	29/10/96	Palmerston Nth 6
240	205 RF	12/10/96	Hawera 7
LL			Lambda Ladder
241	82 LF	12/10/96	Hawera 7
402	233 LR	12/10/96	Hawera 7
404	205 LF	12/10/96	Hawera 7
405	205 LR	12/10/96	Hawera 7
459	233 LR	26/10/96	Hawera 7
460	205 LF	26/10/96	Hawera 7
461	205 LF	26/10/96	Hawera 7
462	82 LF	26/10/96	Hawera 7
LL			Lambda Ladder

Lanes 2, 4, 7 and 8 appear to be clonally derived strains although isolated from three different farms and four different animals. Lanes 5 and 6 again similar patterns, and isolated from different animals on different farms. This pattern appears again in Gel 2 and in Gel 3. Lanes 3 & 10 seem to be the same strain, from the same region but different animals on different farms. Lanes 11, 15 & 16 show the same strain, which would be expected as they were taken from the same farm. The strains are different from those found in the same quarter on a different sampling. (Cf. lanes 14, 12 and 13 respectively) Lanes 12 & 14 are the same fragment pattern and they were isolated at different sampling times from different animals on the same farm. Lane 13 appears to be a different strain type to those seen otherwise on the farms in this gel.

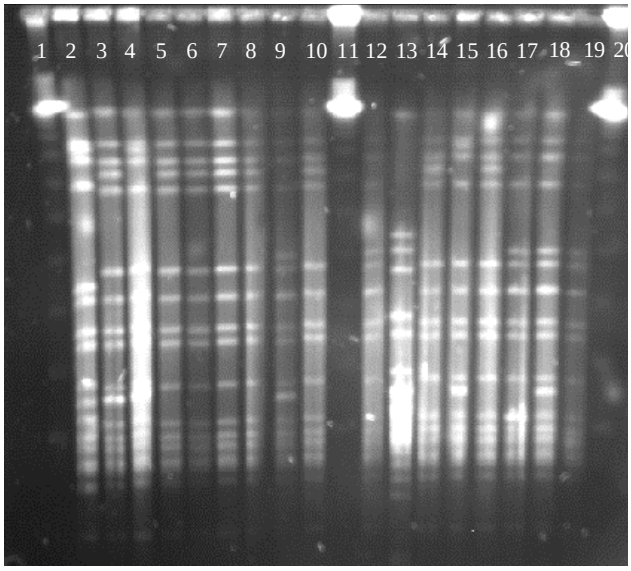
GEL 2



LAB #	COW ID	DATE	FARM
LL			Lambda Ladder
322	377 LR	29/10/96	Palmerston Nth 6
355	387 RF	29/10/96	Palmerston Nth 6
245	66 LF	15/10/96	Rangiotu 2
413	155 LF	13/11/96	Palmerston Nth 6
414	182 RR	13/11/96	Palmerston Nth 6
415	182 LR	13/11/96	Palmerston Nth 6
416	240 LR	13/11/96	Palmerston Nth 6
419	314 RF	13/11/96	Palmerston Nth 6
420	377 RF	13/11/96	Palmerston Nth 6
LL			Lambda Ladder
446	387 RF	29/10/96	Palmerston Nth 6
447	387 RR	29/10/96	Palmerston Nth 6
448	240 RF	29/10/96	Palmerston Nth 6
449	240 RR	29/10/96	Palmerston Nth 6
450	240 LR	29/10/96	Palmerston Nth 6
451	182 RF	29/10/96	Palmerston Nth 6
452	182 LR	29/10/96	Palmerston Nth 6
453	314 LF	29/10/96	Palmerston Nth 6
LL			Lambda Ladder

Lanes 2, 4, 5, 6, 7, 9, 10, 17, 18 and 19 show the same strain. In several cases these isolates were taken from the second sampling of the same cow different quarters, although in one case, the same strain was isolated from the same cow in the same quarter (Lanes 7 & 18). All but one strain was from the same farm (Lane 4). Lanes 3, 8, 12, 13, 14 and 16 were also from the same farm as the other pattern. In these lanes, the same strain was seen in repeated samples of the same quarter (Lane 3 & 12) and the same strain appeared to have spread to another quarter in the same cow (Lane 13). The same situation can be seen with another cow on the same farm (Lanes 8, 14 and 16)

GEL 3 **LAB** **COW** **DATE** **FARM**



#	ID		
LL			Lambda Ladder
454	314 LR	29/10/96	Palmerston Nth 6
243	148 LF	15/10/96	Rangiotu 2
249	66 LF	15/10/96	Rangiotu 2
250	228 LR	14/10/96	Rangiotu 2
252	228 RR	14/10/96	Rangiotu 2
253	128 LR	14/10/96	Rangiotu 2
254	128 RF	14/10/96	Rangiotu 2
261	100 LF	15/10/96	Rangiotu 2
262	85 RF	15/10/96	Rangiotu 2
LL			Lambda Ladder
376	228 RR	30/10/96	Rangiotu 2
377	204 LR	30/10/96	Rangiotu 2
387	128 LR	30/10/96	Rangiotu 2
383	148 LF	30/10/96	Rangiotu 2
388	128 LF	30/10/96	Rangiotu 2
389	66 RF	30/10/96	Rangiotu 2
391	85 RR	30/10/96	Rangiotu 2
392	66 LF	30/10/96	Rangiotu 2
LL			Lambda Ladder

All of these lanes show a remarkable likeness to each other. This is to be expected on one farm. However, the isolate taken from a different farm (Lane 2) could also be clonally related to those from this farm. The isolates from subsequent sampling of the same cows (3 & 16; 4, 17 & 19; 10 & 18; 5, 6 & 12; and 7, 8 & 14, 16) differ very slightly from one another, showing in some cases that one strain has infected more than one quarter. The strain types in evidence here appear similar to the strain type seen in the previous gel for the Rangiotu (and some Palmerston North isolates) farm as well, indicating that one strain may be predominant in both areas.

7 DISCUSSION

Pulsed-Field Gel Electrophoresis was the second strain-typing method used on the *Salmonella* Brandenburg isolates. Initially, each isolate identified as *Salmonella* spp. was sent to ESR for serotyping. The amount of anti-sera required for serotyping decreases the usefulness of the technique for laboratories other than national reference laboratories. After the isolates were identified as the Brandenburg serotype, they were sent from the original laboratory to Massey University for strain typing by PFGE. This technique was used because of the availability of the equipment, the expertise and because it had previously been used by ESR on *Salmonella* Brandenburg in New Zealand. [103]

In the previous study conducted on Salmonella Brandenburg in New Zealand, strain types of 115 recent and historical isolates from humans and non-human sources were observed. Thirteen different patterns were found. This laboratory used slightly different parameters for running the PFGE gels upon which the strains were seen, the patterns would have been comparable to those resulting from an "in house" method (Massey University) previously used on other organisms e.g. *Streptococcus uberis, Yersinia enterocolitica*.

Several studies document PFGE typing of *Staphylococcus aureus*, in particular MRSA (as shown previously in Chapter 7). While the organism that causes bovine mastitis was not identified as MRSA, there was no reason to presume that PFGE could not be used to observe *S. aureus* strains that caused mastitis in the North Island. The technique had previously been used to examine the strain differences between *Streptococcus uberis* strains that caused bovine mastitis in the same area (Douglas, V., personal communication).

Few problems were encountered using this system of strain typing. The main downfall of PFGE strain typing was the time consuming nature of the technique. While not labour intensive, it required up to 10 days for a result to be seen. If any intermediate steps in the method failed, the 10 days resulted in nothing more than a genomic smear. When the technique worked however, bands were clear and sharp and easy for the computer to detect. Pulsed-Field Gel Electrophoresis gave clear

indications as to the strain types that were seen in the outbreak of sheep abortions in 1998.

Analysis of the gels was complex without the use of a computer software program designed for this purpose. Between-gel uniformity was difficult to ensure as running conditions were always marginally different. It was noted that the gels run during the winter months of the year were clearer to read than those run when the ambient temperature in the laboratory was higher. This was despite the cooling unit designed to maintain constant buffer temperature during the run. It could, however, be a by product of the time taken to prepare the plugs, and the activity of nucleases may have increased at warmer temperatures.

Approximately halfway through the laboratory investigation, the molecular weight marker (lambda ladder) appeared to have changed. The company and the supplier remained the same, but the bands of smaller base pairs were less distinct. DNA was present in the pre-prepared agarose plugs, shown by a thick, bright band in the high molecular weight region of the lanes that contained the marker. It was speculated that the company might have decreased the reaction time for the enzymatic cleavage of the lambda concatemers, as there was less DNA in the lower molecular weight region than seen in previous gels.

The two organisms examined in this study required slight differences in both the parameters under which the gel was run, and the preparation of the DNA. On occasion, the incorrect temperature was used to incubate the enzyme for the cleavage of *S. aureus* DNA and new plugs had to be made. Despite the slight differences in the technique it would have been possible to run both organisms on the same gel. This was not carried out, as confusion may have occurred in the analysis stage. The equipment used in this study was straightforward to program for both organisms.

The results of the laboratory investigation of the *S. Brandenburg* isolates were clear in most aspects. One strain type was observed in all but four isolates. These isolates were run in Gel 5 (9113, 8051, 9180 and 8272) and were considered epidemiologically related, as they were cultured during the course of the outbreak

from sheep on farms in a well-defined affected area. These four isolates were grouped together into pairs. Both 9113 and 8051 exhibited 10 differences (4 band losses, and 6 band gains) when compared to the "outbreak strain". The similarity index given to the strains by the computer program was however, 87%. The other two isolates (9180 and 8272) showed 7 distinct fragment differences from the outbreak pattern. The similarity index in this case was 91%. Following the guidelines mentioned above, these strains would fall into the "different" category. That is, they would be considered genetically unrelated to the outbreak. [46] [104] Other guidelines suggest that they be considered related, based entirely on the similarity indices. [45]

The origin of the two unusual strain types is uncertain. They may have been the first and only isolates of a different *Salmonella* Brandenburg strain carried by sheep that aborted. It is possible that the sheep may have carried two strains consecutively and although one was predominantly isolated, the other strains were rarely recovered. It is also possible that the *Salmonella* were misidentified as Brandenburg serotype and may be another. (A *Salmonella* Hindmarsh strain was sent to Massey University for PFGE by mistake during this study) This may mean that another serotype of *Salmonella* caused the abortions, or perhaps the sheep aborted for other reasons. Further epidemiological investigation into the different isolates is clearly needed. The farms from which these four isolates were obtained should be compared with other affected farms in the area to determine whether there are any differences in regard to risk factors and exposures between them.

The strains seen on all of the gels were compared with those seen in previous years in New Zealand [103] and also with strains isolated from human salmonellosis in Scotland [105]. While both of these studies used different parameters (as mentioned previously) if the strains were similar a similar banding pattern would have been seen. The outbreak strain from the sheep abortions in New Zealand in 1998 showed no similarities to strains seen in either of these other studies. However, some of the patterns seen previously in New Zealand were not particularly clear, and bands may not have been seen due to poor imaging.

The original outbreak strain was first isolated from Ashburton in the middle of 1996 where one merino ewe aborted [106]. *Salmonella* Brandenburg had not previously been isolated from sheep before and there was no reason to investigate the appearance of the unusual serotype. Prior to 1998, the *Salmonella* serotype Brandenburg had never been associated with abortions in sheep although several others had been. [56] [57] In 1997, a further outbreak of abortions involving 18 farms in Southland and Otago occurred. Once again, though the serotype was unusual, it was deemed a small, localised outbreak of no great importance. The widespread outbreak of abortion storms studied here was seen the following year (1998). Over 120 farms were affected in the same areas as the previous year, though the areas had extended. Because *Salmonella* Brandenburg was cultured from the placenta and/or foetal material, it was considered the aetiologic agent.

It is possible that the animals infected with the outbreak strain of *Salmonella* Brandenburg were infected from the same source. This study was a laboratory study only, there was no epidemiological investigation to pinpoint a common source or exposure on the affected farms, or to compare affected and unaffected farms throughout the outbreak. This study indicated that the strain type found in all the animals that aborted was clonally derived (stemmed from a single ancestral type). [30]

It was hypothesised that people could have introduced the strain to sheep. It is known that humans are capable of carrying the same strain as a number of human cases have occurred associated with sheep infections. The strain type had not previously been seen in New Zealand, but it may have been hidden by the low notification rate of gastroenteritis caused by *Salmonella* spp. There is also a chance that the strain was imported via the tourist industry in Christchurch, an area known for the arrival of many visitors. It is possible a visitor to the area worked on a farm in Ashburton, where the initial ovine case was seen. It is also possible that sewage sludge containing the strain was used as a fertiliser on a paddock subsequently used for grazing sheep. [107] It is known that some serotypes of *Salmonella* can multiply under laboratory conditions in sterile sewage sludge and this could occur under natural conditions. [108] In addition gulls have been known to feed at sewage outfalls and have previously been implicated in the transmission of *Salmonella* serotypes to animals, [109] [110] [111] including the serotype Brandenburg [112]. The evidence to suggest

that the black backed gulls were involved with the transmission of *Salmonella* Brandenburg to farms in this study is circumstantial, but cannot be ignored.

A case-control study was conducted to identify the black-backed gulls as the vehicle of transmission. Carriers of *Salmonella* Brandenburg were found more often on the control farms (where *S.* Brandenburg had yet to be isolated) than they were on the case farms (where *S.* Brandenburg had already been isolated in relation to sheep abortions). This may suggest that the gulls were not as important in the transmission of *Salmonella* Brandenburg as first believed, or that some sheep were able to resist the infection or that they reacted less violently to infection. In all birds that carried the organism, it was found in higher numbers than would be expected in healthy individuals. [102]

A study of *Salmonella* Brandenburg in humans in Switzerland suggested several methods of dissemination, multiple food products enabled the widespread transmission of the same bacterial strain. This was hypothesised when food and sporadic isolates from the pre-outbreak period and isolates from other countries were tested alongside those from outbreak-associated patients. The food isolates were examined after the patients had reported eating the food types prior to becoming ill although no "formal" epidemiologic investigation occurred during the outbreak. [21] Nevertheless the source could only be postulated and not proved, as with this study. The necessity of a thorough epidemiological investigation to confirm hypotheses based on laboratory findings is reiterated.

Gel 9 shows pre-outbreak human isolates. Seven or more differences were discernible between them and the outbreak strain. This was sufficient to suggest that they were "different" from the outbreak strain. [46] [104] One isolate was "possibly" related to the outbreak strain with 5 band differences (Isolate 94484), and one was indistinguishable from the outbreak strain with a 96% similarity index. (Isolate 95684). [104] [46] Massey University received all isolates "blind", and the similarity was discovered during analysis. While this certainly appeared promising, the circuitous route in which isolates arrived at Massey University caused concern. Further investigation of the origin of this isolate must be conducted and is currently underway.

It was demonstrated the outbreak strain was not host specific as dogs, cattle, humans and gulls in the area of the outbreak shed the organism. It appeared that the outbreak had one source and several vehicles of transmission. The organism should be easier to control by vaccination than if several strain types were observed as it suggests vehicles of transmission rather than ubiquitous presence of *Salmonella* Brandenburg in the environment. This indicates that once the mode of transmission is elucidated, control measures can be designed and implicated.

A vaccine against *Salmonella* (Salvexin™) produced by Schering Plough is available in New Zealand and Australia. It protects sheep against *Salmonella* Typhimurium. It was suggested the vaccine be altered slightly to protect the sheep from a wider range of *Salmonella* serotypes. A study is to be conducted to examine the molecular basis of infection with *Salmonella* Brandenburg, in the hope of designing a novel vaccine that may be used to protect against other abortifacients.

Control measures may have prevented further transmission. Dogs scavenged carcasses and were likely to become infected with *Salmonella* Brandenburg through licking and eating foetal or placental material after abortions had occurred, particularly given the high numbers of organisms that were recovered from such tissue (Orr, M. Personal communication). Burning and burying animal remains occurred on some farms, as soon as possible after the abortion occurred to prevent scavengers (including wild cats, ferrets, and stoats, scavenging birds and farm dogs, etc) becoming carriers of the organism. Additional measures to disinfect the area where the aborted material lay may have reduced the number of other animals that become infected or carriers.

It is hypothesised that cattle were infected in a similar manner to the sheep. Again, gulls (including the black backed gulls indicated in this study) have been implicated in the transmission of *Salmonella* spp. to this ruminant overseas. The reaction in the cattle was limited to scouring not abortions during the sheep outbreak in 1998 in New Zealand, and the reasons for this were not clear. The sheep were under considerable stress, carrying multiple foetuses, having had slight drought conditions during the summer and a very wet winter in the south of the South Island. The cattle may have been under less stress (generally single foetuses) and that may have had an

impact on the severity of the infection. Further investigation into the reaction of different animals to the bacteria would be beneficial.

Recent observations show *S.* Brandenburg has become endemic in the sheep of the South Island as apart from abortions. It has been isolated from hoggets in 1999 (Clark, G. Personal communication). It was the same strain type as that implicated in the abortion storms, and the effect it may have on sheep in the future is uncertain.

Bovine mastitis has been examined microbiologically for years. Recently a study was completed at Massey University that examined strain types of *Streptococcus uberis* in bovine mastitis cases in the North Island of New Zealand (Douglas, V. personal communication). Isolates of *S. aureus* were recovered from cows during that study and were not strain typed prior to this study.

Visual examination of the *S. aureus* gels showed strains that could be grouped into 5 strain types, within which there were 6 or fewer subtypes. One type dominated in this study (Gel 1, isolate 404, Lane 11). [104] Considerable homogeneity was observed among the *S. aureus* strains, for instance, indistinguishable patterns were isolated from four different animals on three different farms. The movement of the dairy cattle was unknown; the animals may have been part of one herd at one time. This clearly demands further investigation.

Different strains were isolated from the same quarter on subsequent sampling. This has implications for diagnosis and treatment where different antibiotic susceptibility patterns may be exhibited by different strains. It was not clear why only a few clones were seen on all 4 farms in this study. It is possible that only certain strains are virulent for cattle. Examination as to whether this was true was beyond the extent of this study and may be pursued in future.

There appeared to be spread of the strains both between and within farms and the spatial distribution of the strains is wide. The same strain seen in Palmerston North was also evident on two farms in Hawera and in Rangiotu. Those three areas were the only areas involved in the study so further investigations are required to determine whether these strains are more widespread. There may be strains

particular to each island in New Zealand due to the physical barrier of the Cook Strait, with the ability to "quarantine" the islands from one another if animal movements are prevented.

A comparison of the ages at which the animals become colonised with each strain of *S. aureus* would extend the information achieved by this study. Cows may be free of this pathogen until they first enter the milking shed. The ages of the animals in this study were unknown, so whether strains are particular to the age of the host could not be determined. As there was no period of dry cow therapy (DCT) between the two sampling periods it is not known whether one strain can be displaced and another become resident in the udder.

This study indicated the direction for further work required to understand the colonisation of bovine udders by *S. aureus*. The investigation showed few clones; therefore control by vaccination might no longer be implausible for mastitis caused by this pathogen. As more knowledge of the ecology of *S. aureus* is acquired, more control options will be available to farmers in the future.

The methods used in this study are not standardised. To date, there are few standardised methods in place for PFGE other than for *Salmonella* Typhimurium, *E. coli* O157:H7 and *Listeria monocytogenes* in the United States of America. PulseNet, a network of databases that store information and strain types of these organisms, shows a great advantage over several operators using different "in house" methods on the same organism. It is thus important for researchers working on similar organisms to communicate freely.

Addition of epidemiological data to suggest where an outbreak began, which strains were present in which areas, whether there were endemic strains or not, would have been of particular value at the analysis stage. The use of PFGE would provide confirmation to support such data. In all future veterinary investigations of this genre, an epidemiologic study MUST be conducted alongside the laboratory study. It is not enough to know that all the strains are identical. Once this was done and the laboratory confirmed the strain type to be clonal, then spatial analysis could be attempted following standard methods. Where strains diverged and became slightly

different, the origin farm may be on the outskirts of the outbreak. This would depend on the similarity threshold determined for the study being conducted. Spatial analysis techniques could be used to investigate associations between the occurrence of cases caused by the same and different strains. This would have been attempted with the *Salmonella* Brandenburg isolates, however, the underlying maps for the farms in the South Island were not available during the time frame of this investigation.

8 CONCLUSION

In both an outbreak situation and an endemic disease investigation, PFGE has proved a useful tool for laboratory studies. However, without the thorough epidemiologic investigation of either situation, PFGE can only allude to patterns through space and time.

Salmonella Brandenburg has become an endemic organism in sheep in the South Island. One strain type was seen throughout the laboratory-based investigation of the outbreak, and the origins of this strain have been hypothesised. Further investigation of the possible human origin is underway at Massey University. Many abortions were again observed in 1999. There is no evidence to suggest the strain has moved to sheep in the North Island.

Staphylococcus aureus was found to exhibit clonality amongst cows on several farms over a wide area of the North Island. This has positive implications for the control of the organism, although more in depth study is needed on a wider scale before control measures making use of this feature can be advised. Research is underway worldwide for a vaccine to *S. aureus* mastitis, so the notion that only a few clones need be targeted may be of assistance, however a more extensive New Zealand-wide investigation needs to be performed to confirm the findings from this study.

9 REFERENCES

1. Townsend KM. & Dawkins HJ *Journal of Chromatography. 618(1-2):223-49, 1993 Aug 25* .

2. Maule J *Molecular Biotechnology. 9(2):107-26, 1998 Apr* .

3. Carter GL., Wilson LJ. & Dunn-Coleman NS *Progress in Industrial Microbiology. 29:667-83, 1994* .

4. Bustamante C., Gurrieri S. & Smith SB *Trends In Biotechnology. 11(1):23-30, 1993 Jan* .

5. Romling, U., Grothues, D., Heuer, T. & Tummler, B. *Electrophoresis* **13**, 626-631 (1992).

6. Vogt, W. & Nagel, D. *Clinical Chemistry* **38**, 182-198 (1992).

7. Dice, L. R. *Ecology* **26**, 297-302 (1945).

8. Sourdis, J. & Krimbas, C. *Molecular and Biological Evolution* **4**, 159-166 (1987).

9. Struelens, M. J., Gheldre, Y. D. & Deplano, A. *Infection Control and Hospital Epidemiology* **19**, 565-569 (1998).

10. Hanninen ML., Pajarre S., Klossner ML. & Rautelin H *Journal of Clinical Microbiology. 36(6):1787-9, 1998 Jun* .

11. Feizabadi MM., Robertson ID., Edwards R., Cousins DV. & Hampson DJ *Journal of Medical Microbiology. 46(6):501-5, 1997 Jun* .

12. Liu PY., Ke SC. & Chen SL *Journal of Clinical Microbiology. 35(6):1533-5, 1997 Jun* .

13. Krause U., Thomson-Carter FM. & Pennington TH *Journal of Clinical Microbiology. 34(4):959-61, 1996 Apr* .

14. Murase T., Okitsu T., Suzuki R., Morozumi H., Matsushima A. & Nakamura A. Yamai S *Microbiology & Immunology. 39(9):673-6, 1995* .

15. Thong KL. *et al. Journal of Clinical Microbiology. 33(7):1938-41, 1995 Jul* .

16. Fujita M., Fujimoto S., Morooka T. & Amako K *Journal of Clinical Microbiology. 33(6):1676-8, 1995 Jun* .

17. Bannerman TL., Hancock GA., Tenover FC. & Miller JM *Journal of Clinical Microbiology. 33(3):551-5, 1995 Mar* .

18. Barrett TJ. *et al. Journal of Clinical Microbiology. 32(12):3013-7, 1994 Dec* .

19. Mahalingam S., Cheong YM., Kan S., Yassin RM., Vadivelu J. & Pang T *Journal of Clinical Microbiology. 32(12):2975-9, 1994 Dec* .

20. Najdenski H., Iteman I. & Carniel E *Journal of Clinical Microbiology. 32(12):2913-20, 1994 Dec* .

21. Baquar N., Burnens A. & Stanley J *Journal of Clinical Microbiology. 32(8):1876-80, 1994 Aug* .

22. Gordillo ME., Singh KV., Baker CJ. & Murray BE *Journal of Clinical Microbiology. 31(6):1430-4, 1993 Jun* .

23. Liu, P. Y., Tung, J., Ke, S. & Chen, S. *Journal of Clinical Microbiology* **36**, 2759-2762 (1998).

24. Prevost, G., Jaulhac, B. & Piemont, Y. *Journal of Clinical Microbiology* **30**, 967-973 (1992).

25. Prevost, G., Pottecher, B., Dahlet, M., Bientz, M., Mantz, J. M. & Piemont, Y. *Journal of Hospital Infection* **17**, 255-269 (1997).

26. Feizabadi MM., Robertson ID., Hope A., Cousins DV. & Hampson DJ *Australian Veterinary Journal. 75(12):887-9, 1997 Dec* .

27. Jensen AE., Cheville NF., Ewalt DR., Payeur JB. & Thoen CO *American Journal of Veterinary Research. 56(3):308-12, 1995 Mar* .

28. Schwarz S. & Liebisch B *Letters in Applied Microbiology. 19(6):469-72, 1994 Dec* .

29. Baseggio, N., Mansell, P. D., Browning, J. W. & Browning, G. F. *Molecular and Cellular Probes* **11**, 349-354 (1997).

30. Tenover, F. C. *et al. Journal of Clinical Microbiology.* **32**, 407-15 (1994).

31. Mendez-Alvarez S., Pavon V., Esteve I., Guerrero R. & Gaju N *Microbiologia. 11(3):323-36, 1995 Sep* .

32. Grothues D. & Tummler B *Molecular Microbiology. 5(11):2763-76, 1991 Nov* .

33. Maslow, J. & Mulligan, M. E. *Infection Control and Hospital Epidemiology* **17**, 595-604 (1996).

34. Murray, B. E., Singh, K. V., Heath, J. D., Sharma, B. R. & Weinstock, G. M. *Journal of Clinical Microbiology* **28**, 2059-2063 (1990).

35. Jarvis, W. R. *Infection Control and Hospital Epidemiology* **15**, 500-503 (1991).

36. Powell, N. G., Threlfall, E. J., Chart, H. & Rowe, B. *FEMS Microbiology Letters* **119**, 193-198 (1994).

37. Dalsgaard A., Skov MN., Serichantalergs O. & Echeverria P *Epidemiology & Infection. 117(1):51-8, 1996 Aug* .

38. Khambaty FM., Bennett RW. & Shah DB *Epidemiology & Infection. 113(1):75-81, 1994 Aug* .

39. Cardinali G., Pellegrini L. & Martini A *Yeast. 11(11):1027-9, 1995 Sep 15* .

40. Dib JC., Dube M., Kelly C., Rinaldi MG. & Patterson JE *Journal of Clinical Microbiology. 34(6):1494-6, 1996 Jun* .

41. Winters, M. A., Goering, R. V., Boon, S. E., Morin, R., Sorensen, M. & Snyder, L. *Medical Microbiological Letters* **2**, 33-41 (1993).

42. Savor, C., Pfaller, M. A., Kruszynski, J. A., Hollis, R. J., Noskin, G. A. & Peterson, L. R. *Journal of Clinical Microbiology* **36**, 3327-3331 (1998).

43. van Belkum A. *et al. Journal of Clinical Microbiology. 36(6):1653-9, 1998 Jun* .

44. Tamplin ML., Jackson JK., Buchrieser C., Murphree RL., Portier KM., Gangar V. Miller LG. & Kaspar CW *Applied & Environmental Microbiology. 62(10):3572-80, 1996 Oct* .

45. Struelens, M. J. *Clinical Microbiology and Infection* **2**, 2-11 (1996).

46. Tenover, F. C., Arbeit, R. D. & Goering, R. V. *Infection Control and Hospital Epidemiology* **18**, 426-439 (1997).

47. Saulnier, P., Bourneix, C., Prevost, G. & Andremont, A. *Journal of Clinical Microbiology* **31**, 982-985 (1993).

48. Olsen, J. E., Skov M. N. , Threlfall, E. J. & Brown, D. J. *Journal Od Medical Microbiology* **40**, 15-22 (1994).

49. Gordillo ME., Singh KV. & Murray BE *Journal of Clinical Microbiology. 31(6):1570-4, 1993 Jun* .

50. Gibson JR., Fitzgerald C. & Owen RJ *Epidemiology & Infection. 115(2):215-25, 1995 Oct* .

51. Louie M., Jayaratne P., Luchsinger I., Devenish J., Yao J., Schlech W. & Simor A *Journal of Clinical Microbiology. 34(1):15-9, 1996 Jan* .

52. Seifert H. & Gerner-Smidt P *Journal of Clinical Microbiology. 33(5):1402-7, 1995 May* .

53. Hansen, D. E., Hedstrom, O. R., Sonn, R. J. & Snyder, S. P. *Journal of the American Veterinary Medical Association* **196**, 731-734. 14 ref (1990).

54. Orr, M. B. *Surveillance (Wellington)* **16**, 24-25. 1 ref (1989).

55. Orr, M. *Surveillance (Wellington)* **18**, 27-28 (1991).

56. Sojka, W. J., Wray, C., Shreeve, J. E. & Bell, J. C. *British Veterinary Journal* **139**, 386-392. 15 ref (1983).

57. Linklater, K. A. *Veterinary Record* **112**, 372-374. 7 ref (1983).

58. Owens, W. E. *et al. National Mastitis Council Annual Meeting Proceedings* 144-145 (1995).

59. Erskine, R. J., Eberhart, R. J., Hutchinson, L. J. & Spencer, S. B. *Journal of the American Veterinary Medical Association* **190**, 1411-6 (1987).

60. Leslie, K. E. & Schukken, Y. H. *Proc. Nat. Mastitis Council Mtg.* 63-72 (1993).

61. Sol, J., Harink, J. & van Umm, A. *Proc. Intl. Mast. Symp, Indinapolis, Indiana* 118-123 (1990).

62. Wilson, C. D. & Richards, M. S. *Veterinary Research* **106**, 431-436 (1980).

63. Brooks, B. W., Barnum, D. A. & Meek, A. H. *Canadian Veterinary Journal* **23**, 156-159 (1982).

64. Boddie, R. L. & Nickerson, S. C. *Journal of Dairy Science* **69**, (1986).

65. Buddle, B. M., Herceg, M., Ralston, M. J. & Pulford, H. D. *Veterinary Microbiology* **15**, 191-9 (1987).

66. Erskine, R. J. *National Mastitis Council Annual Meeting Proceedings* 20-25 (1994).

67. Goodger, W. J. & Ferguson, G. *JAVMA* **190**, 1284-1289 (1987).

68. Leslie, K. E. & Schukken, Y. H. *Proc. Nat. Mastitis Council Mtg.* 63-72 (1993).

69. Mellenberger, R. W. & Troyer, B. *National Mastitis Council Annual Meeting Proceedings* 364-365 (1994).

70. Nickerson, S. C. *National Mastitis Council Annual Meeting Proceedings* 67-73 (1998).

71. Nickerson, S. C., Owens, W. E. & Boddie, R. L. *Journal of Dairy Science* **78**, 1607-18 (1995).

72. Owens, W. E., Watts, J. L. & Boddie, R. L. et. a. *Journal of Dairy Science* **71**, 3143-3147 (1988).

73. Sol, J., Harink, J. & van Umm, A. *Proc. Intl. Mast. Symp, Indinapolis, Indiana* 118-123 (1990).

74. Baldelli, R., Prosperi, S., Bonicelli, F. & Restani, R. *Obiettivi e Documenti Veterinari* **6**, 115-117. 15 ref (1985).

75. Boddie, R. L. & Nickerson, S. C. *Journal of Dairy Science* **69**, (1986).

76. Buddle, B. M., Herceg, M., Ralston, M. J. & Pulford, H. D. *Veterinary Microbiology* **15**, 191-9 (1987).

77. Erskine, R. J. *National Mastitis Council Annual Meeting Proceedings* 20-25 (1994).

78. Goodger, W. J. & Ferguson, G. *JAVMA* **190**, 1284-1289 (1987).

79. Mellenberger, R. W. & Troyer, B. *National Mastitis Council Annual Meeting Proceedings* 364-365 (1994).

80. Nickerson, S. C. *National Mastitis Council Annual Meeting Proceedings* 67-73 (1998).

81. Nickerson, S. C., Owens, W. E. & Boddie, R. L. *Journal of Dairy Science* **78**, 1607-18 (1995).

82. Owens, W. E., Watts, J. L. & Boddie, R. L. et. a. *Journal of Dairy Science* **71**, 3143-3147 (1988).

83. Bartlett, P. C., Miller, G. Y., Lance, S. E. & Heider, L. E. *Preventative Veterinary Medecine* **12**, 59-71 (1992).

84. Erskine, R. J., Eberhart, R. J., Hutchinson, L. J. & Spencer, S. B. *Journal of the American Veterinary Medical Association* **190**, 1411-6 (1987).

85. Fox, L. K. & Gay, J. M. *Veterinary Clinics of North America - Food Animal Practice* **9**, 475-87 (1993).

86. Lacy-Hulbert, J. *National Mastitis Council Annual Meeting Proceedings* 28-34 (1998).

87. Sears, P. M. *National Mastitis Council Annual Meeting Proceedings* 4-11 (1993).

88. Hoblet, K. H., Bailey, J. S. & Pritchard, D. E. *Journal of the American Veterinary Medecine Association* **192**, 777-779 (1988).

89. Bartlett, P. C., Miller, G. Y., Lance, S. E. & Heider, L. E. *Preventative Veterinary Medecine* **12**, 59-71 (1992).

90. Fox, L. K. & Gay, J. M. *Veterinary Clinics of North America - Food Animal Practice* **9**, 475-87 (1993).

91. Lacy-Hulbert, J. *National Mastitis Council Annual Meeting Proceedings* 28-34 (1998).

92. Quai, V. & Turdean, I. *Revista De Cresterea Animalelor* **26**, 30-36. 10 ref (1976).

93. VanDevanter DR., Yirdaw G., Do C., Tysseling KA., Drescher CA., Forseth BJ. Von Hoff DD. & McNutt MA *Biotechniques. 13(6):884-7, 1992 Dec* .

94. Sears, P. M., Smith, B. S., English, P. B., Herer, P. S. & Gonzalez, R. N. *Journal of Dairy Science* **73**, 2785-9 (1990).

95. Tyler, J. W., Cullor, J. S. & Ruffin, D. C. *Veterinary Clinics of North America - Food Animal Practice* **9**, 537-49 (1993).

96. Nickerson, S. C., Ray, C. H., Tomita, G. M., Owens, W. E., Boddie, R. L. & Boddie, N. T. *National Mastitis Council Annual Meeting Proceedings* 295-296 (1998).

97. Tyler, J. W., Cullor, J. S. & Ruffin, D. C. *Veterinary Clinics of North America - Food Animal Practice* **9**, 537-49 (1993).

98. Sordillo, L. M., Scott, N. L. & Aarestrup, F. M. *National Mastitis Council Annual Meeting Proceedings* 148-149_ (1995).

99. Schukken, Y. H., Leslie, K. E., Lam, T. G. M. & Sol, J. *National Mastitis Council Annual Meeting Proceedings* 19-26 (1993).

100. Bannerman, T. L., Hancock, G. A., Tenover, F. C. & Miller, J. M. *Journal of Clinical Microbiology* **33**, 551-5 (1995).

101. Prevost, G., Jaulhac, B. & Piemont, Y. *Journal of Clinical Microbiology* **30**, 967-73 (1992).

102. Clark, G., Fenwick, S., Boxall, N. S. & Nicol, C. *Proceedings of The Sheep and Beef Cattle Conference* (Continuing Education, Massey University.

103. Wright, J., Brett, M. & Bennett, J. *Epidemiology and Infection* **121**, 49-55 (1998).

104. Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelson, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. *Journal of Clinical Microbiology* **33**, 2233-2239 (1995).

105. Mather, H. henry@ssrl.demon.co.uk *Brandenburg strains* E-mail to Stan Fenwick S.G.Fenwick@massey.ac.nz (July 1999).

106. Bailey, K. M. *Surveillance (Wellington).* **24**, 10-11 (1997).

107. Findlay, C. R. *The Veterinary Record* **93**, 100-101 (1973).

108. Findlay, C. R. *The Veterinary Record* **93**, 102-103 (1973).

109. Johnston, W. S., MacLachlan, G. K. & Hopkins, G. F. *Veterinary Record* **105**, 526-527 (1979).

110. Butterfield, J., Coulson, J. C., Kearsey, S. V., Monaghan, P., McCoy, J. H. & Spain, G. E. *Journal of Hygiene* **91**, 429-436 (1983).

111. Coulson, J. C., Butterfield, J. & Thomas, C. *Journal of Hygiene* **91**, 437-443 (1983).

112. Fenlon, D. R. *Journal of Hygiene* **86**, 195-202 (1981).

10 APPENDIX A

A copy of the questionnaire that was completed by farmers of affected farms during the *Salmonella* Brandenburg abortion outbreak in 1998.

SALMONELLA QUESTIONNAIRE

Farmer: .....

Address: .....

.....

How was this diagnosed - Lab ☐ Other.....

Any previous problems with Ewe deaths from Salmonella over the previous twelve months

Vaccinations (camp/toxo/salmonella)

Mating date:

Lambing date:

Movements of stock on to farm in previous 8 months: (i.e. 1998)

Details of Abortion Problem:

- **Date first noticed:**
- **Age and breed of ewes affected**
- **Breeds of sheep on farm**
- **Daily abortion rate**
- **Date when last abortion/dead lamb seen**
- **No. of mobs affected and mob size of each**

- Mostly - singles affected
- twins affected

Details of management of affected mob (i.e. crop, baleage, grass, hay)

- Over the previous two weeks before abortions started

- During abortion outbreak

Any changes to management during abortion outbreak and effects of these changes?

Expected lambing % from scanning

Final lambing %

Assessment of financial loss.

Other stock on the property?

Any meal supplements fed to sheep?

Any further comments/observations:

Details of any abortions in last few seasons: eg Salmonella Brandenburg