To the Graduate Council:

I am submitting herewith a thesis written by Debra Arnett entitled “Evaluation of Methods for Detecting the Apramycin Resistance Gene in *E.Coli* Isolated from Cold-Stressed Swine.” I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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(Original signatures are on file in the Graduate Student Services office)

EVALUATION OF METHODS FOR DETECTING THE APRAMYCIN RESISTANCE GENE IN *E. COLI* ISOLATED FROM COLD-STRESSED SWINE

A Thesis
Presented for the
Master of Science
Degree
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Debra Beth Arnett
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ABSTRACT

This study was an attempt to locate the apramycin resistance gene in *E. coli* isolated from cold-stressed swine. Six pigs were administered apramycin for 14 days during a 150-day study and were exposed to cold stress in the form of an 8°C reduction in recommended daily temperature. Fecal swabs were taken on eight different days for the recovery of *Escherichia coli,* and isolates were tested for sensitivity to apramycin. Plasmids were isolated from selected *E. coli* from each day of sampling and were electroporated into receptive bacteria to see whether those bacteria in turn would become receptive so that a resistant plasmid could be identified. Electroporated plasmids could not be recovered so AP PCR was examined instead to identify resistant plasmids. Fingerprints were made of all plasmids using two different primers. PFGE of DNA, using two different restriction endonucleases, was also performed. Profiles from AP PCR and PFGE were compared separately for similarity by eye and also by using a molecular analyst software program that creates dendrograms. No resistant plasmids were identified, the source of resistance could not be linked to a plasmid or plasmids, and the apramycin resistance gene was not discovered.

TABLE OF CONTENTS

1. Review of the Literature ----------1
    - I. Subtherapeutic Antibiotic Use in Swine Feeds----------1
    - II. Antibiotic Resistance and Stress----------2
    - III. Mechanisms of Resistance----------3
    - IV. Plasmids and Conjugation----------4
    - V. Plasmid Incompatibility----------7
    - VI. Transposons and Insertion Sequences----------8
    - VII. *Escherichia coli* in Pigs----------9
    - VIII. Use of Apramycin----------10
    - IX. Protocols for Characterization of Plasmids----------12
        - IXA. Alkali Lysis Method of Plasmid Separation----------12
        - IXB. Electroporation of Bacteria----------13
        - IXC. Arbitrarily Primed PCR----------13
        - IXD. Agarose Gel Electrophoresis----------16
    - X. Protocol for Chromosomal DNA: Pulsed-field Gel Electrophoresis-17
    - XI. Justification for Research----------18
2. Materials and Methods----------19
    - I. Previous Study----------19
    - II. Current Study----------20
        - IIA. Electroporation----------22
        - IIB. AP PCR----------24
        - IIC. Pulsed-Field Gel Electrophoresis----------26
        - IID. Analysis of AP PCR and PFGE Profiles----------28
3. Results----------30
    - I. Previous Study----------30
    - II. Current Study----------30
        - IIA. Electroporation----------30
        - IIB. AP PCR----------33
        - IIC. Pulsed-Field Gel Electrophoresis----------45
        - IID. Analysis of AP PCR and PFGE Fingerprints----------45
            - IIDa. Analysis by Eye----------45
            - IIDb. Analysis by Computer----------45
4. Discussion----------55
    - I. Electroporation----------55
    - II. AP PCR----------57
        - IIA. Analysis by Eye----------58
        - IIB. Analysis by Computer----------59
    - III. Pulsed-Field Gel Electrophoresis----------61
        - IIA. Analysis by Eye----------61
        - IIB. Analysis by Computer----------61
    - IV. Overall Results----------62
    - V. What Could Have Been Done Differently?----------62

Literature Cited----------65

TABLE OF CONTENTS, CONT.

Vita----------73

LIST OF TABLES

Table #	Table Description	Page
1	Day, pig number, isolate number, and MIC reading for all isolates on which PCR was performed	25
2	PCR cycles used on *E. coli* plasmids	26
3	MIC readings (μg/ml) for *E. coli* resistance to apramycin in cold stressed pigs on each sampling day of previous study	31
4	Bands found for each plasmid and both primers for AP PCR, as analyzed by eye by two different people	48
5	Groups of plasmids that were deemed “related” by eye for primer OPB-17, primer 23L and both primers used in AP PCR	49
6	Bands identified by eye for PFGE MRPs for each isolate digested witih enzyme Xba I	50
7	Bands identified by eye for PFGE MRPs for each isolate digested with enzyme Spe I	51

LIST OF FIGURES

Fig #	Figure Description	Page
1	Schematic representation of conjugation between donor and recipient bacterial cells	6
2	Schematic representation of PCR amplification of DNA, demonstrating the three stages of denaturation, primer annealing, and primer extension	14
3	Plasmid profile (lanes 2-4) of day 5, pig 20, isolate 2.	32
4	Plasmid profile (lanes 2-4) of day 5, pig 23, isolate 2.	32
5	Plasmid profile of day 3, pig 23, isolate 1.	34
6	Results of electroporation	34
7	Plasmid profile of day 5, pig 21, isolate 4 (lanes 2-4) and day 3, pig 23, isolate 1 (lanes 5-7)	35
8	Plasmids extracted from electroporated *E. coli*	36
9	Plasmid profile of day 1, pig 23, isolate 1 plasmids (sensitive strain)	37
10	Day 1, pig 23, isolate 1 plasmid fingerprints	37
11	Day 1, pig 23, isolate 1, plasmid #3 PCR fingerprint	38
12	Day 1, pig 19, isolate 2 (sensitive strain) plasmid profile	38
13	PCR fingerprint of the only plasmid from day 1, pig 19, isolate 2	38
14	Day 2, pig 23, isolate 3 (sensitive strain) plasmid profile	38
15	Day 2, pig 23, isolate 3, plasmid 1 and plasmid 2 PCR fingerprints	39
16	Day 2, pig 23, isolate 3, plasmid 3 and plasmid 4 PCR fingerprints	39
17	Day 2, pig 23, isolate 3, plasmid 5 and plasmid 6 PCR fingerprints	39

Fig #	Figure Description, cont.	Page
18	Day 3, pig 22, isolate 1 (sensitive strain) plasmid profile	39
19	PCR profile of the only plasmid from day 3, pig 22, isolate 1	40
20	Plasmid profile of day 3, pig 23, isolate 1 (resistant strain)	40
21	PCR fingerprints of day 3, pig 23, isolate 1, plasmid 1 and plasmid 2	40
22	PCR fingerprints of day 3, pig 23, isolate 1, plasmid 3 and plasmid 4	40
23	PCR fingerprints of day 3, pig 23, isolate 1, plasmid 5 and plasmid 6	41
24	Day 4, pig 19, isolate 2 (resistant isolate) plasmid profile	41
25	PCR fingerprint of the only plasmid from day 4, pig 19, isolate 2	41
26	Plasmid profile of day 5, pig 20, isolate 3	41
27	PCR fingerprint of the only plasmid from day 5, pig 20, isolate 3	42
28	Plasmid profile from day 6, pig 24, isolate 1 (sensitive isolate) in lanes 2-4, and from day 6, pig 24, isolate 2 (resistant isolate) in lanes 5-7	42
29	PCR fingerprint of the only plasmid from day 6, pig 24, isolate 1	42
30	PCR fingerprint of the only plasmid from day 6, pig 24, isolate 2	42
31	Plasmid profile from day 7, pig 20, isolate 2 (sensitive isolate)	43
32	PCR fingerprints of day 7, pig 20, isolate 2 plasmids	43
Fig #	Figure Description	Page
33	Plasmid profile of day 8, pig 22, isolate 4 (sensitive isolate)	44
34	PCR fingerprints of plasmids from day 8, pig 22, isolate 4	44
35	PCR fingerprints of plasmids from day 8, pig 22, isolate 4, continued	44
36	Chromosomal DNA MRPs generated by PFGE using restriction enzyme Xba I	46
37	Chromosomal DNA MRPs generated by PFGE using restriction enzyme Spe I	47
38	Dendrogram generated by Molecular Analyst software for AP PCR on all plasmids using primer OPB-17	52
39	Dendrogram generated by Molecular Analyst software for AP PCR on all plasmids using primer 23L	53
40	Dendrogram generated by Molecular Analyst software for PFGE on all chromosomal DNA using restriction enzyme Xba I	54
41	Dendrogram generated by Molecular Analyst software for PFGE on all chromosomal DNA using restriction enzyme Spe I	54

1. REVIEW OF THE LITERATURE

I. SUBTHERAPEUTIC ANTIBIOTIC USE IN SWINE FEEDS

Antibiotics can be administered to swine in one of three different methods; therapeutic (for the treatment of bacterial disease), subtherapeutic (the daily use in feeds to improve growth rates and animal performance), and prophylactic (to help prevent disease from occurring) (Maiden, 1998).

It was discovered in the 1940's that low (subtherapeutic) levels of antibiotics, when added to the diets of chicks, could help improve growth rates and performance (Moore et al., 1946). Other studies in the early 1950's proved that antibiotics also had similar effects on other livestock such as swine and cattle (Bartley et al., 1950; Cunha, 1950; and McGinnis et al., 1950). Since then, antibiotics have commonly been used at subtherapeutic levels to help prevent disease and improve growth rates and performance. In swine units, antibiotics have been used to improve farrowing rate, litter size, birth weight, and the number of pigs weaned; and reduce mastitis, metritis, and agalactia (NRC, 1999).

Some believe that the above benefits are most likely due to the action of the antibiotics on some component of normal bacteria (Novick, 1981). Others have postulated that antibiotics lead to a reduction of immunologic stress by lowering circulating levels of IL-1 (which mediate responses to infection), thus allowing for greater growth and performance (Roura et al., 1992). However, the

exact mechanisms by which antibiotics help improve performance are still largely unknown (Gustafson and Bowen, 1997).

A drawback to the use of antibiotics in swine is that they can contribute to the emergence of antibiotic-resistant bacteria (Dawson et al., 1984). The use of antibiotics exerts a strong selective pressure, inducing resistance to antimicrobial agents among bacteria (Hinton et al., 1986). This is of concern because such bacteria arising from agricultural practices can not only cause difficulty in treatment of animal diseases, but also can be transferred to human environments, therefore causing the treatment of bacterial infections in humans to become difficult or impossible (Khachatourians, 1998). An additional problem is that resistance often involves drugs to which the bacteria and animals have never previously been exposed (Novick, 1981). This can occur because of transfer via plasmids, which will be discussed later in this review.

II. ANTIBIOTIC RESISTANCE AND STRESS

It has been shown that when animals are exposed to various types of stress, an increase in bacterial resistance to antibiotics is often seen. An example of such a stress is transportation of pigs. A study by Langlois and Dawson (1999) showed that antimicrobial resistance of some bacteria in pigs increased after transporting them for thirty minutes. This occurred even though the pigs had never before been exposed to antibiotics.

Exposure of pigs to excessive cold is also a source of stress. Newborn piglets require an air temperature in housing units of about 35°C (Curtis, 1992).

Their bodies, before developing, lack subcutaneous fat and thus require such warmer temperatures (Widdowson, 1950). Exposing younger pigs to excessively cool temperatures (cold stress) has been shown to cause an increase antibiotic resistance (Moro et al., 1998).

It is difficult to explain why stressors cause increases in resistance, as a number of different factors may be contributing at the same time (Moro et al., 1998). Stressors may affect heat exchange and rate of feed intake in animals' bodies, thus altering bacteria in the digestive tracts of animals. In turn, the increase of certain bacteria and not others could change pH levels or production of hydrogen peroxide, so that growth of some species would be favored over others (Moro et al., 1998). It is unlikely that an increase in resistance can be explained by sudden and selective increases in resistant microbes, because the resistance occurs very quickly; one study found an increase in less than ten minutes (Langlois and Dawson, 1999). Rather, the increase appears to be due to the selective excretion of resistant bacteria by animals during the times of stress (Langlois and Dawson, 1999). This could be due to adhesion factors possessed by the resistant microbes, or perhaps rapid motility of the intestine due to the stress.

III. MECHANISMS OF RESISTANCE

Bacterial resistance to antibiotics can either be intrinsic or acquired (Murray and Hodel-Christian, 1991). If the resistance is acquired, it occurs in one of two ways; either mutations develop in existing DNA of the bacteria, or new

DNA conferring resistance is acquired. Acquired resistance implies that an organism which was previously sensitive to an antibiotic develops resistance to it. Some mutations that can develop in existing DNA to cause resistance include deletions, insertions, or substitutions of nucleotide/s within the sequence of the DNA (Murray and Hodel-Christian, 1991).

Most common, however, is resistance that is collected through the acquisition of new, foreign DNA. This acquisition can occur by transformation, transduction, or conjugation. Transformation involves cells taking up free DNA directly from their environments, transduction involves transfer of DNA by a bacterial virus from one cell to another, and conjugation involves transfer of DNA from one cell to another by way of plasmids and direct cell-to-cell contact (Snyder and Champness, 1997). The method examined in this study is conjugation of plasmids.

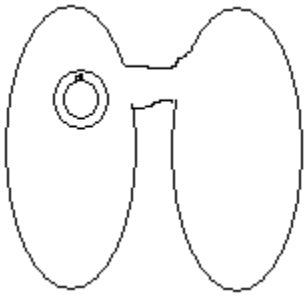
IV. PLASMIDS AND CONJUGATION

Plasmids are autonomously-replicating, extrachromosomal, mostly closed, circular elements of DNA that can encode for many different traits, including antibiotic resistance, carbohydrate fermentation, toxins, and adhesion factors. (Murray and Hodel-Christian, 1991). They vary largely in size, ranging from less than one to greater than 300 x 10^6 daltons (or, from a few thousand to hundreds of thousands of base pairs). Both gram-positive and gram-negative bacteria contain plasmids, however plasmids from gram-positive bacteria do not appear to

encode for a sex pilus, which allows the gram-negative bacteria to transfer plasmids from cell to cell (Murray and Hodel-Christian, 1991).

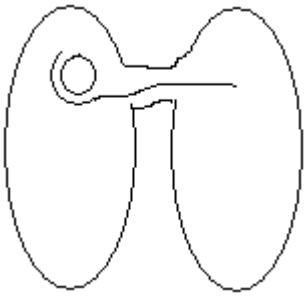
During transfer of plasmids (conjugation), only one strand of the double-stranded plasmid is transferred. First a pilus is formed, which is a means of connection between the donor and recipient cells. A single-stranded nick is then formed on the plasmid in the donor cell, and the circular plasmid relaxes. One of the strands of DNA travels through the pilus from the donor to the recipient. As the strand leaves the donor cell, a complementary one is simultaneously synthesized in the donor strand. Once inside the recipient cell, the DNA strand becomes circular again. Replication of complementary strands in both donor and recipient cells is not essential for transfer, so often single-stranded plasmids are found in cells (Snyder and Champness, 1997). It is in this way that genes encoding for antibiotic resistance can be transferred from one cell to the next (Figure 1). DNA information encoded on plasmids is often a determinant of antibiotic resistance (Son et al., 1997).

Plasmids can be transferred to bacteria of the same or different species. For example, conjugal transfer of plasmids and antibiotic resistance has been shown to occur among *E. coli* (Son et al., 1997), and between bacteria of diverse origins (Kruse and Sorum, 1994). Such genetic variation, especially involving resistance to antibiotics, is known to develop rapidly (Saunders 1984).



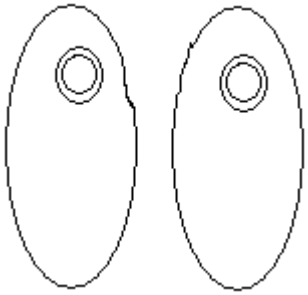
donor recipient

A. Plasmid is nicked at a specific site; cells joined by pilus



donor recipient

B. A single strand is transferred to recipient



donor recipient

C. Both cells now have copy of plasmid

Figure 1. Schematic representation of conjugation between donor and recipient bacterial cells. Adapted from Snyder and Champness, 1997.

V. PLASMID INCOMPATIBILITY

Plasmids may be classified according to antibiotic resistance pattern (genes), incompatibility groups, phage susceptibility, or molecular characterization (such as a characteristic set of fragments created by restriction enzymes) (Dale, 1994). Bacteria can contain more than one type of plasmid (Snyder and Champness, 1997). However, not all types of plasmids can coexist in a cell at the same time; as sometimes one will interfere with another's replication or functioning. In this case, one or both will be lost from the cell more readily than is normal. Two plasmids that cannot exist together are members of the same incompatibility (Inc) group, and this phenomenon is called plasmid incompatibility (Snyder and Champness, 1997).

Reasons for possible plasmid incompatibility include: a) two plasmids share the same mechanism of replication control, and/or b) they share the same partitioning functions (Projan and Novick, 1986). Under normal circumstances, plasmids have different replication control systems, so each daughter cell is not likely to receive the same number of each plasmid (Snyder and Champness, 1997). But in subsequent divisions, cells will eventually copy the plasmids and contain the same numbers of each of the plasmids, so very few cells will lose either one. In a case of two incompatible plasmids, daughter cells will still not receive the same number of the two plasmids. However, the two plasmids will replicate until the total number of plasmids in each cell is equal to the copy number (average number of a particular plasmid per cell), because they will regulate each other's replication. This will result in an uneven distribution of both

plasmids. In subsequent generations there is a great chance that one or both of the plasmids will be lost (Snyder and Champness, 1997).

Partitioning systems in plasmids are methods by which plasmids can avoid being lost from dividing cells (Snyder and Champness, 1997). They ensure that at least one copy of the plasmid is passed onto each new cell at the time of division. The units regulating these systems are known as par functions. When two plasmids share the same par function, one or the other will always be passed on to the next generation of cells. However, sometimes one cell will receive one plasmid, and the second cell will receive another plasmid, resulting in cells that will not contain both of the original plasmids (Snyder and Champness, 1997).

VI. TRANSPOSONS AND INSERTION SEQUENCES

Transposons are mobile genetic elements that can move from one place to another in DNA and carry with them their own transposases, which are enzymes that promote transposition (Snyder and Champness, 1997). All transposons contain inverted repeats at each end. They may enter bacteria via plasmid transfer or introduction by phages and can be incorporated into the chromosome. The smallest transposons (750 to 2000 base pairs) are known as insertion sequences and usually encode for only enzymes that promote their transposition (Snyder and Champness, 1997).

Two of the same type of insertion sequences can form a composite transposon, in which the insertion sequences bracket other genes (such as

genes that encode for antibiotic resistance). The flanking insertion sequences form “cassettes” of genes that can be moved from one piece of DNA to another. Many plasmids are thought to be formed of such cassettes, especially plasmids that carry genes for resistance to several different antibiotics (Snyder and Champness, 1997).

Integrons are sites on plasmids or transposons where antimicrobial resistance genes have been integrated. They are places were antibiotic resistance genes accumulate, and are a powerful mechanism by which plasmids can develop resistance to multiple antibiotics (Rice and Bonomo, 1996).

VII. *Escherichia coli* IN PIGS

Escherichia coli (*E. coli*) belongs to the family Enterobacteriaceae, which includes gram-negative, facultatively anaerobic rods (Bertschinger et al., 1992). Some of these organisms cause a large variety of intestinal and extraintestinal diseases in swine; other varieties are normal gastrointestinal inhabitants. Disease-causing *E. coli* can lead to enteric colibacillosis, edema disease, systemic infections, coliform mastitis, and urinary tract infections (Bertschinger et al., 1992). The disease of special concern is colibacillosis, which involves *E. coli* attaching to epithelial cells of the intestine and causing a secretory diarrhea that can lead to dehydration and sometimes results in death (Mortensen et al., 1996). Such diseases are often of concern for swine producing units, therefore it is important that antibiotics work against such pathogens.

However, *E. coli* in pigs have often been found to be resistant to antibiotics, especially tetracycline (Linton et al., 1975). Administering antibiotics facilitates transfer of resistance from bacterium to bacterium (Anderson et al., 1973). Also, resistant *E. coli* may persist in the majority of fecal flora in pigs even in the absence of any antibiotic selection (Hartley and Richmond, 1975; Linton et al., 1978; and Petrocheilou et al., 1976 and 1977).

VIII. USE OF APRAMYCIN

Antibiotics are divided into about twenty-five different "classes," according to chemical structure (Murray and Hodel-Christian, 1991). Three of the larger classes are quinolones, aminoglycosides, and beta-lactams. Apramycin is an aminoglycoside antibiotic that has a structure of an amino sugar or sugars linked to a hexose or aminocyclitol nucleus via a glycoside bond (Mortensen et al., 1996).

Apramycin has been used in veterinary practice (including use in swine) since 1980, but is not commonly used in humans (Wray et al., 1986). Administration of apramycin to pigs is either through the drinking water (as a soluble powder given at 12.5 mg/kg for seven days) or in the feed (as a premix given after weaning for 14 days at a concentration of 150 g/ton of feed) (Mortenson et al., 1996). It is one of several different antibiotics used in swine-producing facilities.

The drug is produced by *Streptomyces tenebrarius* and is an inhibitor of protein synthesis in bacteria (Vasiljevic et al., 1993). Irreversible binding of the

antimicrobial to the ribosome disrupts normal protein synthesis by causing tRNA misreading, thus killing the cell (Davies, 1991). The drug also induces errors in translation, causing cell death (Davies, 1991).

Bacteria, specifically *E. coli*, have been shown to become resistant to apramycin. This was first documented in animals in 1982 in England (Mortensen et al., 1996). Today, resistance to apramycin remains prevalent in animals and is observed on swine farms, especially in bacteria from younger pigs (Mathew et al., 1998).

The resistance of bacteria to aminoglycosides often occurs in one of three ways; a) prevention of transport of the drug across the membrane so that the bacterial ribosomes are not available for modification, b) modification of the ribosomes so that the drug cannot bind to them, and c) use of aminoglycoside-modifying enzymes by the bacteria (Mortensen et al., 1996). The enzymes are encoded by transferable plasmids and transposons (Mortensen et al., 1996).

These enzymes include acetyltransferases, nucleotidyltransferases, and phosphotransferases (Shaw et al., 1993). Specifically, the enzyme 3-N-aminoglycoside acetyl-transferase type IV (AAC(3)IV) is the only enzyme produced by resistant organisms that acetylates apramycin; this same enzyme also acetylates gentamicin and tobramycin (other aminoglycosides) (Johnson et al., 1995). Plasmids have been found in *E. coli* that carry the genes that encode for these enzymes (Davies and O'Connor, 1978). Many of the genes are associated with transposons, which allow for the rapid dissemination of the

genes and thus drug resistance in many different species of bacteria (Shaw et al., 1993).

Resistance to aminoglycosides is thought to have come about in one of two ways; either resistance genes were derived from the organisms that produce the aminoglycosides, or the genes are derived from bacterial genes that encode enzymes involved in normal cellular metabolism (Shaw et al., 1993).

IX. PROTOCOLS FOR CHARACTERIZATION OF PLASMIDS

IXA. Alkali Lysis Method of Plasmid Separation

It is the relatively small size of plasmids, compared to chromosomal DNA, that allows for plasmid separation in the alkali lysis method (Snyder and Champness, 1997). In this treatment, cells are first lysed with lysozyme to allow separation of DNA components from other cell parts such as proteins and RNA. DNA strands are separated with addition of alkali, which changes the pH from neutral to about 12.0-12.6. At that pH range, chromosomal DNA will denature completely but covalently closed circular DNA (plasmids) will not because they are physically interlinked (Dale, 1994). The physical interlinking of the plasmids allows for easy renaturing. Then, a high concentration of salt is added, which neutralizes the high pH, causes the strands of chromosomal DNA to reassociate at numerous random places, and leads to the formation of an insoluble DNA complex (Birnboim, 1983). The plasmids reassociate and are left intact in the supernatant.

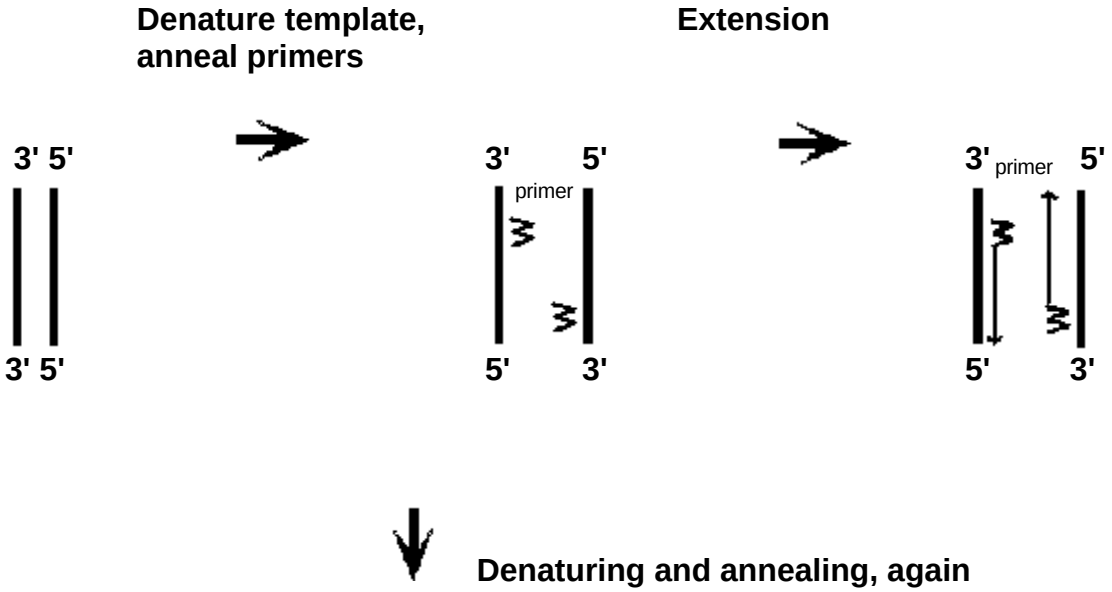
IXB. Electroporation of Bacteria

Electroporation involves the application of brief, high-voltage shocks to a sample of cells (bacterial, plant, or animal) and DNA (including plasmids) so that the DNA may be introduced into the bacteria (Miller, 1994). It is thought that these shocks result in a change in membrane structure so that electropores (holes) are formed in the membrane, and the magnitude and frequency of the shocks determine whether these electropores will remain open or will close again (Ho and Mittal, 1996). A transmembrane potential is created where negative and positive charges accumulate in the cell at areas closest to the cathode and anode, which causes the electropores to form (Clementz et al., 1988). The efficiency of electroporation decreases as the size of the DNA increases (Atherly et al., 1999). Electroporation is a method that can be used to introduce plasmids into bacteria.

IXC. Arbitrarily Primed PCR

Polymerase Chain Reaction, or PCR, is a method by which specific segments of DNA are amplified (Erlich, 1989). The uses for PCR are many and include gene amplification and site directed mutagenesis (Sharrocks, 1994). PCR takes advantage of the fact that DNA polymerases (thermostable and thermoactive) require a primer, a template, buffer and free dNTPs to polymerize DNA and make a complementary copy of the template (Snyder and Champness, 1997).

Cycle One



Cycle Two

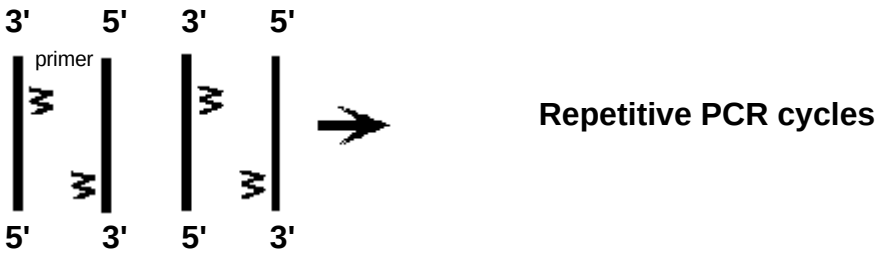


Figure 2. Schematic representation of PCR amplification of DNA, demonstrating the three stages of denaturation, primer annealing, and primer extension. Adapted from Snyder and Champness, 1997.

A PCR reaction basically consists of repetitive cycles of DNA denaturation (achieved with high temperatures), annealing of primers to template DNA (cooler temperatures), and extension of the DNA by nucleotide addition (Atlas and Bej, 1994) (Figure 2). Temperatures are very critical at each stage. By repeating these stages, a nearly exponential increase in the amount of desired DNA is achieved, with the amount of DNA doubling with each cycle. Specialized types of PCR have been developed for specific applications, and the number of cycles used depends on the degree of amplification required and the need to amplify selectively the target DNA sequence (Atlas and Bei, 1994).

Arbitrarily primed PCR is a versatile method that can be used to create a fingerprint of DNA (McClelland and Welsh, 1995). Fingerprints are created by using arbitrarily selected primers that amplify the DNA, sometimes on opposite strands, even when the matching of primer to template is not perfect (McClelland and Welsh, 1995). The most efficient of pairs of priming events will “compete” with each other to produce a fingerprint, the products of which are referred to random amplified polymorphic DNAs (RAPDs) (Williams et al., 1990).

Experimentation must be used to acquire the desired number of fragments; too many products can make a pattern too complex for interpretation, and too few may not be enough to generate a fingerprint. Either one primer may be used (as in the case of this study), or two may be used in what is known as “pairwise combination” (McClelland and Welsh, 1995). Resulting fingerprints may be analyzed by eye or with the use of molecular analyst software.

IXD. Agarose Gel Electrophoresis

Gel electrophoresis is used to estimate the size of DNA fragments, whether plasmid or chromosomal, or whether generated by PCR, restriction enzymes, or plasmid isolation techniques (Atherly et al., 1999). A gel is cast which has small wells molded into it and is composed of agarose, TBE buffer and a trace amount (5 μl) of ethidium bromide. DNA samples, premixed with a sucrose-containing loading buffer to weigh down the samples and keep them from floating into the TBE buffer, are loaded into the wells. The gel is immersed in TBE buffer that contains a trace amount of ethidium bromide and an electric current is passed through everything. The ethidium bromide intercalates between bases in the DNA sequence and allows the DNA to later be visualized under fluorescent light (Atherly et al., 1999).

Since DNA is negatively charged and is loaded near the negative electrode, it will migrate through the gel away from the negative charge and towards the positive electrode. Larger fragments travel more slowly than smaller ones. A molecular ladder is simultaneously run with the DNA so that the DNA can be compared to fragments of known size and accurate size can be determined. The DNA is visualized as bands, and each band represents a collection of fragments of a particular size (Atherly et al., 1999).

When plasmids are separated with gel electrophoresis, the conformation of the plasmid will affect its mobility (Dale, 1994). Plasmids can be in a supercoiled form, a nicked open circular form, or a linear form. Those in the supercoiled form migrate well ahead of those in the open circular form. In this

way, a single plasmid can give rise to multiple bands; thus it is often difficult to visually determine the number of plasmids present in a strain of bacteria without running further tests such as PCR or restriction enzyme analysis (Dale, 1994).

X. PROTOCOL FOR CHROMOSOMAL DNA: PULSED-FIELD GEL ELECTROPHORESIS

Pulsed-field gel electrophoresis (PFGE) is a technique that is used to separate larger DNA fragments (greater than 50 kilobase pairs, usually using chromosomal DNA) based on size with the use of restriction endonucleases and application of a static electric field (Thomson-Carter et al., 1993). The electric field is changed periodically so that the DNA migrates in different directions (Atherly et al., 1999). Since molecules reorient themselves every time there is a shift in the field, and larger molecules take more time to reorient, the larger DNA will thus move more slowly than the smaller DNA.

PFGE machines have one of several different types of electric field configurations (Maslow et al., 1993). One type of electric field is field inversion gel electrophoresis (FIGE), which involves two parallel electrodes whose polarity is inverted. Another is the contour-clamped homogenous electric field (CHEF) system, which involves a hexagonal array of electrodes where the electric field is alternated at a constant angle to the direction of migration. In this study, the CHEF system is used because several researchers have found that it gives better results than other electric field methods, especially for *E. coli* isolates (Allardet-Servent et al., 1989; Harsono et al., 1993; On et al., 1997).

The end result is restriction patterns of the isolates, which are then visually compared with one another to determine relatedness (Tenover et al., 1995). However, patterns may also be evaluated with the use of molecular analyst software. In this study, chromosomal macrorestriction profiles (MRPs) generated by PFGE can be compared to plasmid profiles generated by AP PCR to help determine the genetic source of antibiotic resistance.

XI. JUSTIFICATION FOR RESEARCH

Since plasmids are a means by which bacteria can acquire resistance, and since development of *E. coli* resistance to apramycin in cold-stressed swine is of concern, it would be helpful to know whether development of resistance can be linked to one, several, or any plasmids. This study is an attempt to determine whether or not levels of antibiotic resistance of bacteria can be related to plasmid profiles, so that a genetic location of resistance could possibly be identified. By doing so, the development of bacterial resistance in swine could be better understood, then perhaps measures could be taken to help producers stop or slow resistance development.

2. MATERIALS AND METHODS

I. PREVIOUS STUDY

This project utilized bacterial isolates collected from a previous study that is outlined elsewhere in detail. Briefly, fifty-eight weaned pigs (18 days old) with no history of antibiotic exposure were challenged intranasally with 10^{11} CFU of *Salmonella enterica* serovar Typhimurium (Salmonella Typhimurium) containing a nalidixic acid resistance marker (National Animal Disease Center, Ames, Iowa). Pigs were randomly assigned to one of eight separate treatments; control without apramycin, control with apramycin, cold stress, heat stress, oxytetracycline, poor sanitation, overcrowding, and intermingling. Each group was administered sub-therapeutic levels of the feed-based antibiotic, apramycin sulfate (150 g/ton for 14 d) two days post inoculation (day 2), with the exception of the first control group.

The only treatment group utilized by the current study was the cold stress group. In this group, pigs were exposed to cold stress in the form of an 8°C reduction in recommended daily temperature. Cold stress treatment was started seven days after intranasal challenge so that acclimation could occur. On the 149th day of the study (150 days total), all pigs were transported to a new facility.

Fecal swabs were taken from each pig prior to inoculation (day 0) and again on days 2, 7, 14, 28, 64, 148 (prior to shipping), and 149 (post-shipping) (isolates designated as days 1-8, respectively for current study) for the recovery of *Escherichia coli*. The *Salmonella typhimurium* were recovered and used in the original study. Swabs were streaked onto lactose MacConkey agar (Difco,

Detroit, MI) and incubated for 24 h at 37^{o}C for the isolation of *E. coli*. Isolates were transferred to Trypticase Soy Agar plates containing 5% defibrinated sheep blood and incubated for another 24 h at 37^{o}C to select for non-hemolytic colonies.

Once confirmed as *E. coli*, bacterial isolates were tested for sensitivity to apramycin sulfate via a broth dilution minimum inhibitory concentration (MIC) method according to the National Committee for Clinical Laboratory Standards (NCCLS). The antibiotic dilution range was 2-128 ug/mL. For the purpose of the previous study, those isolates with a breakpoint of <2 μg/mL were considered sensitive to apramycin, whereas those exhibiting a breakpoint of >32μg/mL were considered to be resistant.

II. CURRENT STUDY

The current study utilized one apramycin resistant and one apramycin sensitive isolate (if both were present) from each of the eight days of sampling (12 isolates total) from the cold stress group. For purposes of the current study, resistant isolates exhibited an MIC of $\geq$128 μg/ml, and sensitive isolates expressed an MIC of <2 μg/ml.

For plasmid isolation, isolates were grown overnight in media (2YT broth) containing an appropriate antibiotic (depending on their MIC readings). A Bio-Rad Quantum® Prep plasmid maxiprep kit (Hercules, CA) was used on each isolate to separate plasmids from all other cell components. The kit utilizes an alkaline lysis separation method and generates 1-3 mg of plasmid DNA.

Plasmids were separated by gel electrophoresis using 1% agarose gels at 105 volts for approximately 30 minutes, and were visualized on an Electrophoresis Systems 312nm Transilluminator (Fisher Biotech, Pittsburgh, PA). Photographs of gels were taken with Polaroid's MP 4+ System (Cambridge, MA) and Polaroid instant sheet film type 55. Plasmids were numbered from top to bottom, with number one representing the largest, "highest" plasmid on the gel, and higher numbers representing the smaller, "lower" plasmids.

Individual plasmids were cut out of gels with a razor blade and separated from the agarose using the Wizard® PCR Preps DNA Purification System (Promega, Madison, WI). The kit involves melting the gel at 70°C, combining it with a resin that binds to plasmids in the gel, vacuuming the resin mixture through a filter that retains the plasmids, then rinsing the filter with 80% isopropanol to leave the plasmids, but not the resin, in the filter. TE buffer was added to the filter and the filters were centrifuged in microcentrifuge tubes (FisherScientific, Pittsburgh, PA), resulting in a TE-plasmid mixture. At that point, plasmids were purified for use in PCR reactions, restriction enzyme analysis, or electroporation experiments. Spectrophotometer readings were taken on all plasmid mixtures to determine the quantity of acquired DNA (Spectronic 1201, Milton Roy, Ivyland, PA). The quantity of plasmid DNA acquired was usually between 12 and 30 ng/μL (where optical density A_{260} 1.0 = 50 μg DNA/mL).

IIA. Electroporation

Electroporation was attempted first. A receptive strain of *E. coli* that is reportedly sensitive to many antibiotics (strain 25922, ATCC) was tested beforehand for non-resistance to apramycin by growing on nutrient agar plates containing 0, 16, 32, 48, 96, 112, and 128 μg/ml apramycin. No growth was observed on the plates (except at 0 μg/ml apramycin), so electrocompetent cells (those prepared to accept plasmids through electroporation) were made from the *E. coli* 25922 strain according to manufacturer's instructions for the Electroporator II (Invitrogen, San Diego, CA). Electrocompetent cells were then grown on 0, 16, 32, 48, 96, 112, and 128 μg/ml apramycin on both 2YT and nutrient agar to determine which medium would be optimal for testing growth, and to determine which apramycin concentration would best determine whether or not electroporated bacteria had developed resistance.

Electroporation was performed as follows. First, 39μl of electrocompetent cells and 1μl of plasmids with a concentration of 10pg – 10ng were combined and chilled on ice, then transferred to a chilled electroporation cuvette (Invitrogen, San Diego, CA). Cuvettes were pulsed in the Electroporator II at 1500V, 25mA, 25 watts, 50 μF capacitance and 150Ω resistance. Immediately after pulsing, 2 ml of SOC media (made according to the manufacturer's instructions) was added to the cuvette and mixed with the plasmid-cell mixture. The mixtures were transferred to sterile tubes and shaken in an incubator at 37°C for one hour.

Plasmids were first extracted and individually isolated from the previous study's day 5, pig 20, isolate 2 *E. coli* (resistant) and day 5, pig 23, isolate 2 (resistant) *E. coli*. The resulting plasmids were electroporated into the electrocompetent *E. coli*. Mixtures were plated on nutrient agar plates containing 128 μg/ml apramycin and 0 μg/ml apramycin. Also plated, as a control, was nonelectroporated, electrocompetent cells. In a second trial using the same isolates, mixtures were plated on nutrient agar plates containing 0, 16, 48, 80, 96, and 128 μg/ml apramycin.

Day 3, pig 23, isolate 1 (resistant) plasmids were then electroporated into the same electrocompetent bacteria (the ATCC strain), plated on 0, 16, 48, 80, 96, and 128 μg/ml apramycin plates, and also inoculated into 5 different flasks containing 30ml of nutrient broth and 0, 16, 32, 80, and 128 μg/ml apramycin. A plasmid prep was performed on the flask with 0 μg/ml apramycin to test for the electroporated plasmid.

Next, new electrocompetent cells were created using day 3, pig 22, isolate 1 *E. coli* (which is a sensitive strain from the previous study), and the method recommended by the manufacturer. Day 3, pig 23, isolate 1 plasmids were electroporated into the cells, and the resulting mixture was grown overnight in 2YT media with 0, 64, and 128 μg/ml apramycin and on nutrient agar plates containing 0, 96, and 128 μg/ml apramycin. Plasmids were extracted from bacteria in the flask with 0 μg/ml apramycin to test for the electroporated plasmid.

Two new solutions of SOC were created; one with 4 μg/ml apramycin and the other with 8 μg/ml apramycin. Previous SOC did not contain apramycin. Plasmids were extracted from day 3, pig 23, isolate 1 and day 5, pig 21, isolate 4 and were electroporated into the day 3, pig 22, isolate 1 electrocompetent *E. coli*. Each plasmid recipient, after electroporation, was grown in each of the two different solutions of SOC. Resulting bacteria were used to inoculate four vials of 0, 16, 64, and 128 μg/ml apramycin in nutrient broth.

SOC that contained 2 μg/ml apramycin was used next, and compared to SOC with no apramycin. Plasmids from day 3, pig 23, isolate 1 were used again, and electroporated into day 3, pig 22, isolate 1 electrocompetent *E. coli*. This time the resulting bacteria were used to inoculate 4 vials of 0, 16, 64, and 128 μg/ml apramycin in nutrient broth, and were spread on nutrient agar plates containing 0, 16, 64, and 128 μg/ml apramycin. Plasmid preparations were performed on bacteria that grew in the vials.

IIB. AP PCR

AP PCR was performed on plasmids from both apramycin-resistant and apramycin-sensitive isolates from each day of the previous study. Before "final" PCRs were run, many "trials" were performed to determine the proper amount of DNA, Taq polymerase, buffer, primer, etc. to add to each reaction. Two primers, 23L (sequence CCGAAGCTGC) and OPB-17 (sequence AGGGAACGAG) (both Operon Technologies, Alameda, CA), were used on all isolates so that two fingerprints were generated for each isolate. The primers were chosen because

they have been found by other researchers to generate acceptable fingerprints in plasmids exposed to random amplified PCR (Lin et al., 1996).

Table 1 shows all of the isolates on which PCR was performed, along with their corresponding MIC readings. If both were present, one sensitive and one resistant isolate were arbitrarily chosen from each day of the previous study.

Each reaction was composed of the following: 2.5 units of Taq polymerase (Promega), 5 μl of dNTPs (Invitrogen), 10 μl of Invitrogen's buffer C, 1 μl of 100 μmol primer (Operon Technologies), 32 μl of water, and 10-20 ng of plasmid DNA. After reactions were mixed together in 2ml thin walled PCR tubes (Eppendorf, Westbury, NY), they were placed in Eppendorf's Mastercycler® gradient machine (Hamburg, Germany) and cycled according to the protocol in Table 2. Cycles were determined empirically.

Table 1. Day, pig number, isolate number, and MIC reading for all isolates on which PCR was performed

Day	Pig #	Isolate #	MIC reading
1	23	1	<2
1	19	2	>128
2	23	3	<2
3	22	1	<2
3	23	1	>128
4	19	2	>128
5	20	3	<2
5	23	2	>128
6	24	1	<2
6	24	2	>128
7	20	2	<2
8	22	4	<2

Table 2. PCR Cycles used on *E. coli* plasmid DNA

NUMBER OF CYCLES	TEMPERATURE	TIME
8 cycles	94°C	4 minutes
	30°C	4 minutes
	72°C	4 minutes
40 cycles	94°C	30 seconds
	30°C	1 minute
	72°C	2 minutes
1 cycles	72°C	5 minutes

Resulting fragments were placed in wells with loading buffer (adjacent to molecular standards for comparison) in 1% agarose gels containing 10 μg/mL ethidium bromide (Mallinckrodt, Paris, KY) and were run at 105 volts for approximately 30 minutes. Gels were then visualized on FisherBiotech's Electrophoresis Systems 312nm Transilluminator. Photographs of gels were taken with the MP 4+ System and instant sheet film type 55 (Polaroid, Cambridge, MA).

IIC. Pulsed-Field Gel Electrophoresis (PFGE)

Fingerprint profiles of chromosomal DNA were generated by CHEF PFGE using the method described by Gautom (1997). Bacterial colonies were grown overnight on 2YT agar plates at 37°C with appropriate antibiotic (Barrett et al., 1994). Colonies were suspended in 2-3 mL of TE buffer (100mM Tris and 100mM EDTA) until 20% transmittance was attained as measured by a bioMerieux Vitek colorimeter (Hazelwood, MO). Bacteria (200 mL) were transferred to a 1.5 mL microcentrifuge tube (Eppendorf) and treated with 10 μL

of proteinase K (20 mg/mL) (Roche Diagnostics, Indianapolis, IN). Two hundred microliters of 1.6% InCert agarose/SAS (BMA, Rockland, ME) were added and the resulting suspension was placed into plug molds (Bio-Rad Laboratories, Hercules, CA). Plugs were placed in microcentrifuge tubes containing 1.5 mL of ES buffer (0.5 M EDTA, 1% sodium-lauroyl-sarcosine), were treated with 40 μL of proteinase K (20 mg/mL) and were incubated for 1 hour at 55°C. Plugs were then placed into individual screen caps (Bio-Rad) and sealed in a 1.5 in. PVC washing tube for four washings; once in sterile water for 15 minutes at 50°C, and three times in plug wash TE (100mM Tris, 100mM EDTA, pH 5). Plugs were stored in 2 mL of plug wash TE at 4°C.

A sterile razor blade was used to cut 1 mm slices from each plug. These slices were digested individually in 86 μL sterile water, 10 μL 10X enzyme buffer, 1 μL BSA and 3 μL of 30 U of restriction enzyme (Roche Diagnostics) at 37°C for 1.5 hours. The enzyme mix was then removed and replaced with 500 μL plug wash TE buffer.

Plugs were electrophoresed along with two copies of a reference strain (*E. coli* O157:H7 from CDC, Washington State) and a Megabase lambda DNA standard (BCM). This took place in 1.0% agarose (SeaKem Gold, Roche Diagnostics) in 0.5X TBE using the CHEF-mapper system (Bio-Rad) with a run time of 14 hours, initial switch time of 2.16 seconds, final switch time of 35.07 seconds, angle of 120°, gradient of 6.0V/cm with a linear ramping factor at 14°.

Gels were stained with one drop of 10 mg/mL ethidium bromide (Sigma Alldrich, St. Louis, MO) in 500 μL of water.

Two cycles of PFGE on the same isolates were performed, one for restriction enzyme *Spe* 1 and another for enzyme *Xb*a 1 (both Roche Diagnostics). These two enzymes have been found previously to generate sufficient banding with *E. coli* strains using PFGE (Mitsuda et al., 1998).

IID. Analysis of AP PCR and PFGE Profiles

AP PCR fingerprints were compared by eye, and were determined to be related if patterns were identical (by number and size of bands). PFGE patterns were compared by eye according to the method of Tenover et al. (1995). Using this method, isolates are considered to be indistinguishable if restriction patterns have the same numbers of bands (or differ by just one band), and if corresponding bands are the same apparent size. If two isolates differ by two or three bands, then they are considered to be closely related. Isolates that differ by four to five bands are considered to be possibly related, and finally, those that differ by six or more bands are considered to be unrelated (Tenover et al., 1995). Two different people performed all “by eye” analyses to ensure identification of location for all bands.

Photos of resulting AP PCR (plasmid) and PFGE (chromosomal) fingerprints were also analyzed by computer. Photographs were converted to digital form using a ScanJet 3300C scanner (Hewllett Packard, Palo Alto, CA) and analyzed with Molecular Analyst software, v. 1.6, 1992-98 (Bio-Rad). That

software has an automatic band-searching feature; however, manual scoring of bands was necessary because often the computer would score wells and random background markings as being part of the fingerprint. Thus, bands were scored visually and manually, based on a presence-absence basis. Two different people scored the bands.

The software compares lanes of profiles by creating dendrograms that show a hierarchic representation of linkage levels between pairs of individuals or groups (Software Instruction Manual, 1992). Dendrograms were created using the Dice coefficient, which is used to estimate the proportion of restriction fragments shared by two populations with the formula:

$$\frac{2n_{AB}}{n_A + n_B}$$

where n_{AB} is the number of bands common for A and B, n_A is the total number of bands in A, and n_B is the total number of bands in B (Molecular Analyst Software Manual, 1992). The dendrograms were also created using the unweighted pair group method using arithmetic averages (UPGMA) clustering, where the level of the branch which links two “Operational Taxonomic Units” (OTUs) determines the correlation between the OTUs; along with a 1% tolerance in band position differences (Molecular Analyst Software Manual, 1992).

For AP PCR, one dendrogram was made for each primer (and all plasmids) used in the reactions. For PFGE, one dendrogram was made for each restriction enzyme used in the reactions.

3. RESULTS

I. PREVIOUS STUDY

Results from the previous study can be seen in Table 3. In general, resistance of *E. coli* to apramycin increased until day 14 and day 28, then decreased after that, with a total absence of resistance seen on days 148 and 149.

II. CURRENT STUDY

IIA. Electroporation

It was determined that nutrient agar would be better for growth of electroporated bacteria, since electrocompetent cells grew at all concentrations of apramycin on the 2YT plates. On nutrient agar, *E. coli* only grew on 0 and 16 μg/ml apramycin, so that medium was the more effective determinant for growth on antibiotics. It was also determined that if electroporated *E. coli* grew at or greater than 80 μg/ml apramycin, it would be considered to have developed resistance to the apramycin, so that concentration was determined to be the "cutoff" point for resistance testing for the electroporated cells.

Photographs of plasmids isolated from day 5, pig 20, isolate 2 and day 5, pig 23, isolate 2 *E. coli* can be seen in Figure 3 and Figure 4. Seven plasmids were found in each isolate. No growth was observed on any of the antibiotic plates when day 5, pig 20, isolate 2 plasmids were electroporated, nor when day 5, pig 23, isolate 2 plasmids were electroporated.

Table 3. MIC readings (ug/ml) for *E. coli* resistance to apramycin in cold stressed pigs on each sampling day of initial study

Pig #	Isolate	Day 0	Day 2	Day 7	Day 14	Day 28	Day 64	Day 148	Day 149
19	1	>128	-	-	>128	128	<2	<2	<2
19	2	>128	-	-	>128	128	4	<2	<2
19	3	>128	-	-	-	>128	<2	<2	<2
19	4	>128	-	-	-	64	4	<2	<2
20	1	<2	-	-	-	128	>128	4	-
20	2	<2	-	-	-	128	128	<2	-
20	3	<2	-	-	-	<2	>128	<2	-
20	4	4	-	-	-	128	128	<2	-
21	1	<2	<2	-	>128	>128	8	<2	4
21	2	<2	<2	-	>128	>128	8	<2	<2
21	3	<2	4	-	>128	>128	4	4	4
21	4	<2	<2	-	>128	128	16	<2	<2
22	1	4	4	<2	>128	>128	-	-	8
22	2	-	<2	-	>128	>128	-	-	4
22	3	4	4	-	>128	128	-	-	<2
22	4	4	<2	-	128	128	-	-	<2
23	1	<2	4	>128	>128	8	4	<2	<2
23	2	<2	4	>128	128	>128	4	<2	<2
23	3	4	<2	<2	128	<2	8	-	<2
23	4	<2	<2	-	>128	<2	4	-	<2
24	1	<2	<2	128	-	128	<2	<2	<2
24	2	<2	<2	-	-	>128	>128	<2	<2
24	3	>128	-	-	-	128	>128	<2	<2
24	4	4	-	-	-	-	>128	<2	<2

- = no sample available

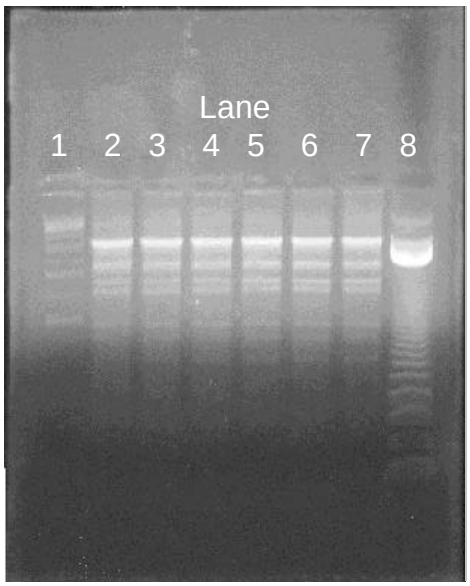


Figure 3. Plasmid profile (lanes 2-4) of day 5, pig 20, isolate 2. Lanes 5-7 are pig 23, isolate 2 plasmids. Lanes 1 and 8 are molecular standards. Seven plasmids can be seen.

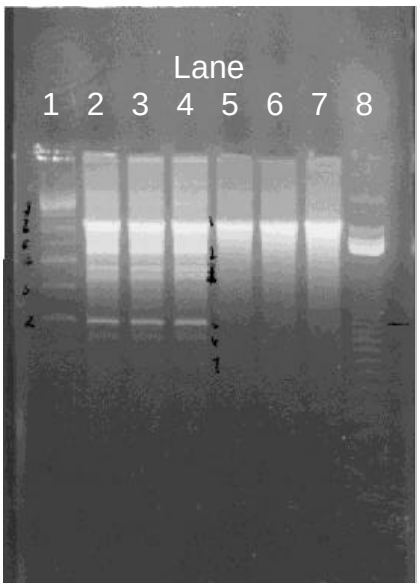


Figure 4. Plasmid profile (lanes 2-4) of day 5, pig 23, isolate 2. Lanes 1 and 8 are molecular standards. Seven plasmids are present.

Plasmids isolated from day 3, pig 23, isolate 1 can be seen in Figure 5. Four plasmids are present. Cells electroporated with day 3, pig #23, isolate 1 plasmids only grew in non-antibiotic broth and on non-antibiotic agar plates. The plasmid prep on the resulting electroporated cells did not reveal the electroporated plasmid.

When plasmids from day 3, pig 23, isolate 1 *E. coli* were electroporated into sensitive day 3, pig 22, isolate 1 cells, growth was seen only on non-antibiotic plates and in non-antibiotic broth. Again, the plasmid prep on the resulting electroporated cells did not reveal the electroporated plasmid. See Figure 6.

The plasmids used in the 2 μg/ml, 4 μg/ml and 8 μg/ml apramycin SOC experiments can be seen in Figure 7. No growth was observed in inoculated antibiotic nutrient broth for the 4 μg/ml and 8 μg/ml trials. No growth was observed in inoculated antibiotic broth for the 2 μg/ml trial, or on any of the antibiotic plates. The plasmid prep was done only on vials containing 0 apramycin (because that was the only place that growth was observed), and that did not reveal electroporated plasmids either.

See Figure 8 for the resulting plasmid prep; in that photograph, it can be seen that the original plasmids were not found in the electroporated bacteria.

IIB. AP PCR

See Figures 9-35 for each isolate's plasmid profile followed by the PCR fingerprint for each plasmid, in order of days.

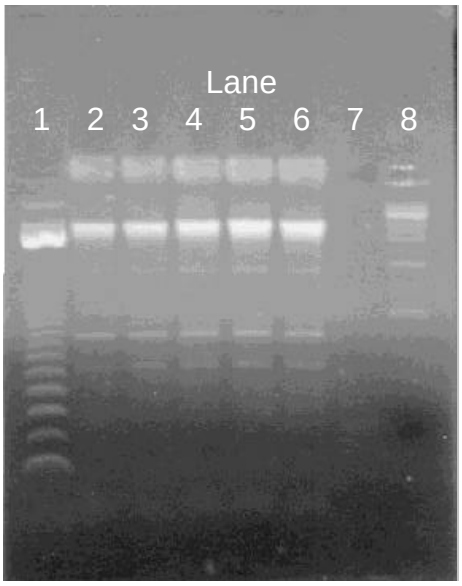


Figure 5. Plasmid profile of day 3, pig 23, isolate 1. Lanes 1 and 8 are molecular standards. Four plasmids can be seen.



Figure 6. Results of electroporation.

Lanes 1,2,3: day 3, pig 23, isolate 1 plasmid #2 electroporated into day 3, pig 22, isolate 1 *E. coli*.
Lane 4: only plasmid #2 from day 3, pig 23, isolate 1.
Lanes 5, 6, 7: day 3, pig 23, isolate 1, plasmid #3 electroporated into day 3, pig 22, isolate 1 *E. coli*.
Lane 8: only plasmid #3 from day 3, pig 23, isolate 1.

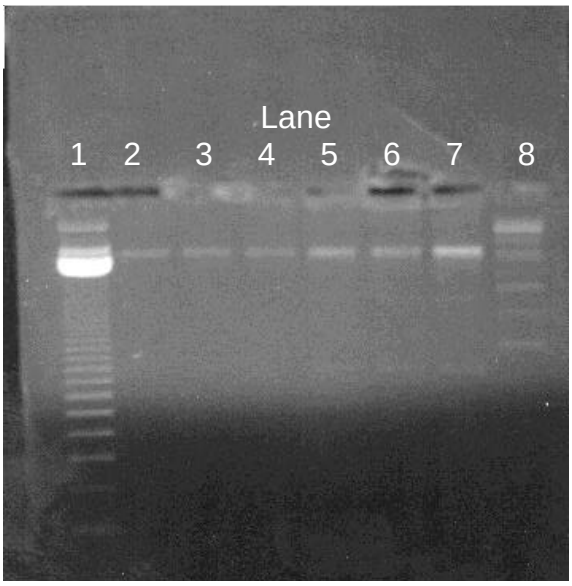


Figure 7. Plasmid profile of day 5, pig 21, isolate 4 (lanes 2-4) and day 3, pig 23, isolate 1 (lanes 5-7). Lanes1 and 8 are molecular standards

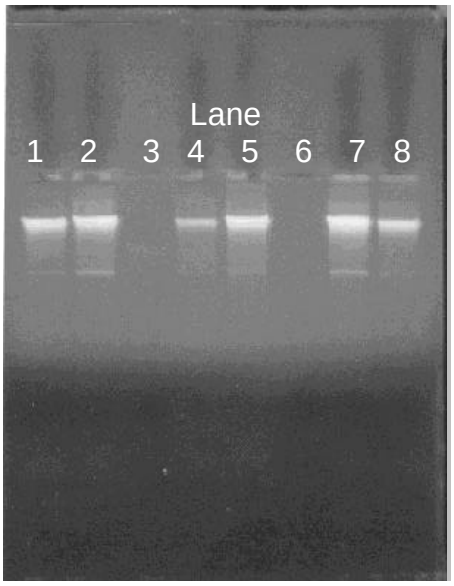


Figure 8. Plasmids extracted from electroporated *E. coli*. Only the original plasmids are seen in each of the electroporation lanes.

<u>Lane 1</u>: plasmid 1 from day 3, pig 23, isolate 1 electroporated into day 3, pig 22, isolate 1 in 0 apramycin SOC

<u>Lane 2</u>: plasmid 1 from day 3, pig 23, isolate 1 electroporated into day 3, pig 22, isolate 1 in 2 μg/ml apramycin-containing SOC

<u>Lane 3</u>: Only plasmid 1 from day 3, pig 23, isolate 1

<u>Lane 4</u>: plasmid 2 from day 3, pig 23, isolate 1 electroporated into day 3, pig 22, isolate 1 in 0 apramycin SOC

<u>Lane 5</u>: plasmid 2 from day 3, pig 23, isolate 1 electroporated into day 3, pig 22, isolate 1 in 2 apramycin SOC

<u>Lane 6</u>: Only plasmid 3 from day 3, pig 23, isolate 1

<u>Lane 7</u>: plasmid 3 from day 3, pig 23, isolate 1 electroporated into day 3, pig 22, isolate 1 in 0 apramycin SOC

<u>Lane 8</u>: plasmid 3 from day 3, pig 23, isolate 1 electroporated into day 3, pig 22, isolate 1 in 2 apramycin SOC

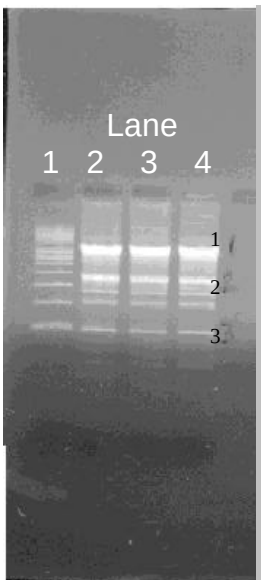


Figure 9. Plasmid profile of day 1, pig 23, isolate 1 plasmids (sensitive strain). The three numbered plasmids are the ones used to generate the next three fingerprints. Lane 1 is a molecular standard.

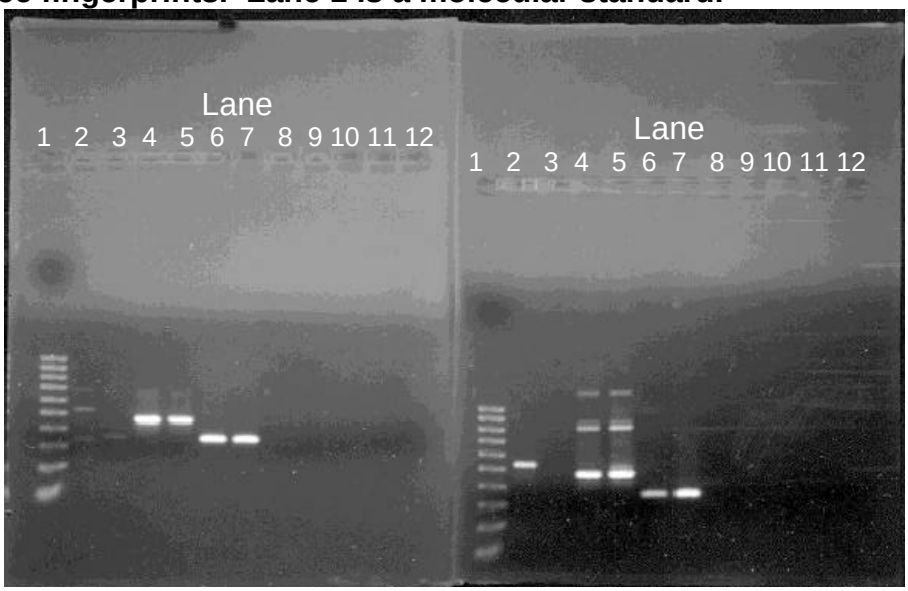


Figure 10. Day 1, pig 23, isolate 1 plasmid fingerprints. The gel on the right is plasmid #1, on the left is plasmid #2. For each gel, lanes 4 and 5 are primer 23L, lanes 6 and 7 are primer OPB-17, and lane 1 is a molecular standard.



Figure 11. Day 1, pig 23, isolate 1, plasmid #3 PCR fingerprint. Lanes 4 and 5 are primer 23L, lanes 6 and 7 are primer OPB-17, and lane 1 is the molecular standard.

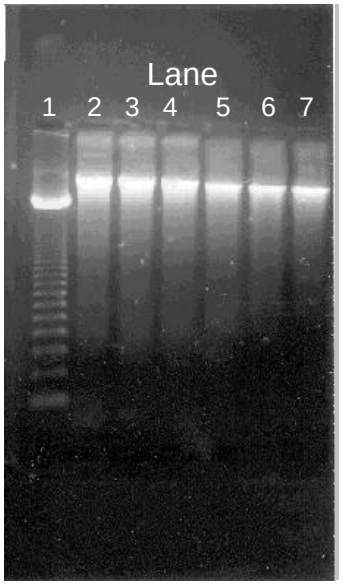


Figure 12. Day 1, pig 19, isolate 2 (sensitive strain) plasmid profile in lanes 2 through 4. Lane 1 is the molecular standard.

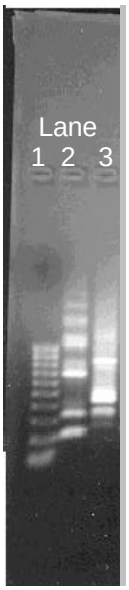


Figure 13. PCR fingerprint of the only plasmid from day 1, pig 19, isolate 2. Lane 1 is a molecular standard, lane 2 is primer OPB-17, and lane 3 is primer 23L.

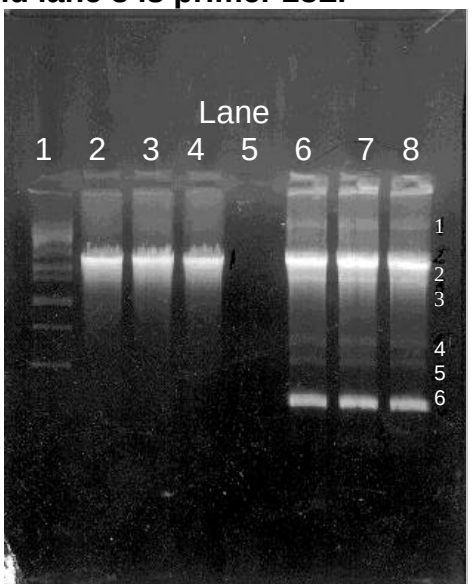


Figure 14. Day 2, pig 23, isolate 3 (sensitive strain) plasmid profile in lanes 6 through 8. Lane 1 is the molecular standard. Six plasmids, numbered to the right of the lanes, were used for the next PCR fingerprints.

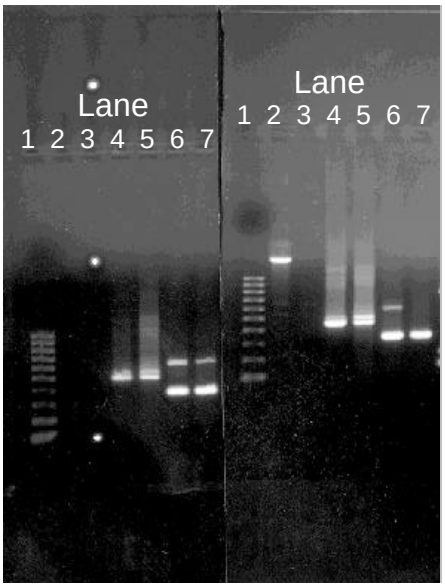


Figure 15. Day 2, pig 23, isolate 3, plasmid 1 (on the left) and plasmid 2 (on the right) PCR fingerprints. Lanes 1 on both gels are molecular standards, lanes 4 and 5 are primer 23L, and lanes 6 and 7 are primer OPB-17.

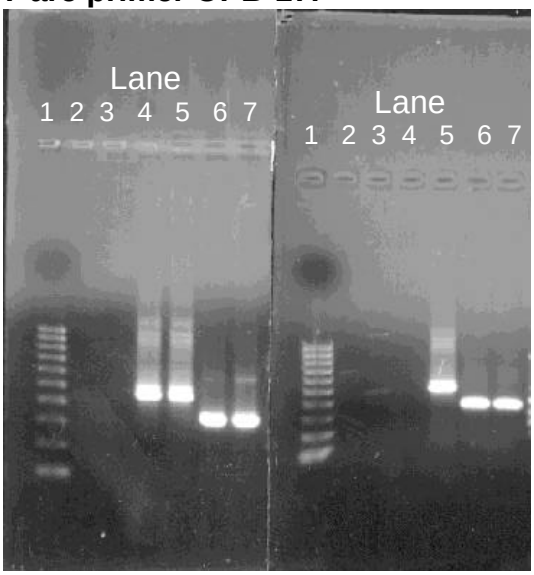


Figure 16. Day 2, pig 23, isolate 3, plasmid 3 (on the left) and plasmid 4 (on the right side) PCR fingerprints. Lanes 1 on both gels are molecular standards, lanes 4 and 5 are primer 23L, and lanes 6 and 7 are primer OPB-17.

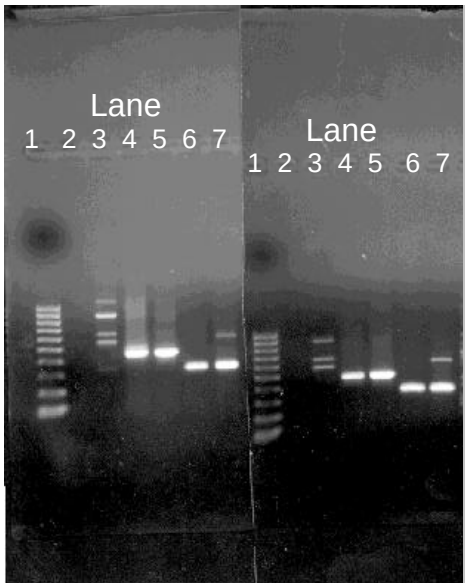


Figure 17. Day 2, pig 23, isolate 3, plasmid 5 (on the left side of the split) and plasmid 6 (on the right side) PCR fingerprints. Lanes 1 on both gels are molecular standards, lanes 4 and 5 are primer 23L, and lanes 6 and 7 are primer OPB-17.

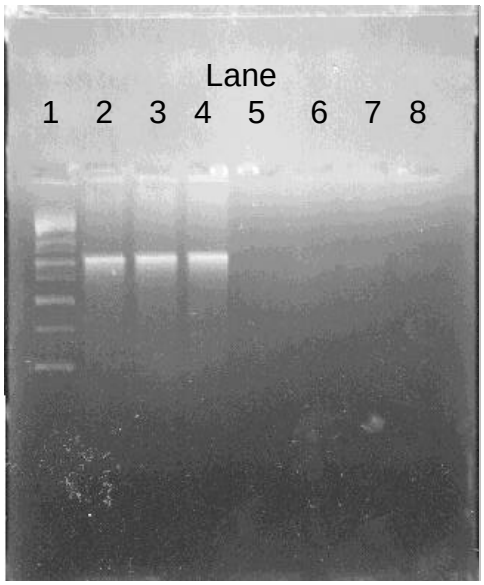


Figure 18. Day 3, pig 22, isolate 1 (sensitive strain) plasmid profile in lanes 6-8 (very faint, about the same level as lanes 2-4 plasmids). Lane 1 is the molecular standard.

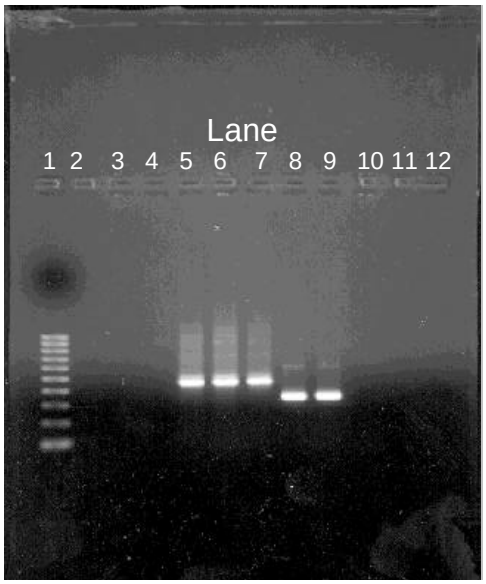


Figure 19. PCR profile of the only plasmid from day 3, pig 22, isolate 1. Lane 1 is a molecular standard, lanes 5-7 are primer 23L, and lanes 8 and 9 are primer OPB-17.

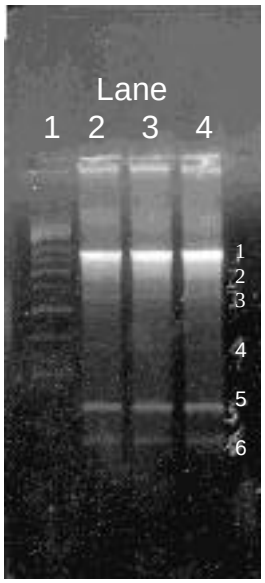


Figure 20. Plasmid profile of day 3, pig 23, isolate 1 (resistant strain). Lane 1 is the molecular standard, Lanes 2-4 are the plasmids and are numbered 1-6.

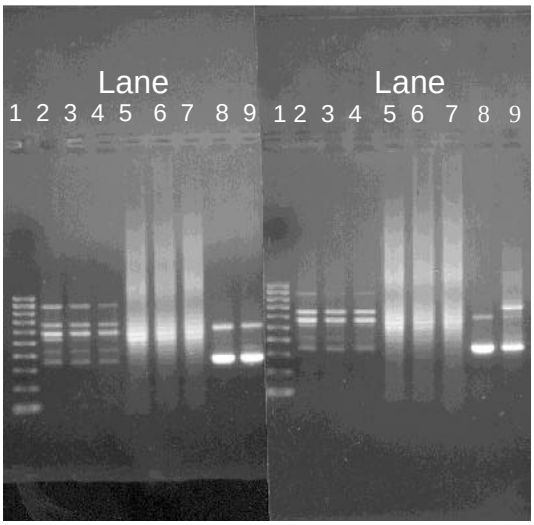


Figure 21. PCR fingerprints of day 3, pig 23, isolate 1, plasmid 1 (left of the split) and plasmid 2 (on the right). Lanes 1 for both gels are molecular standards, lanes 5-7 are primer 23L, and lanes 8-9 are primer OPB-17.

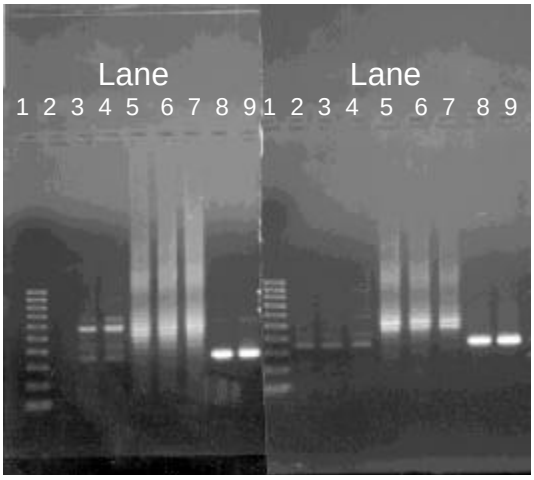


Figure 22. PCR fingerprints of day 3, pig 23, isolate 1, plasmid 3 (left of the split) and plasmid 4 (on the right). Lanes 1 for both gels are molecular standards, lanes 5-7 are primer 23L, and lanes 8-9 are primer OPB-17.

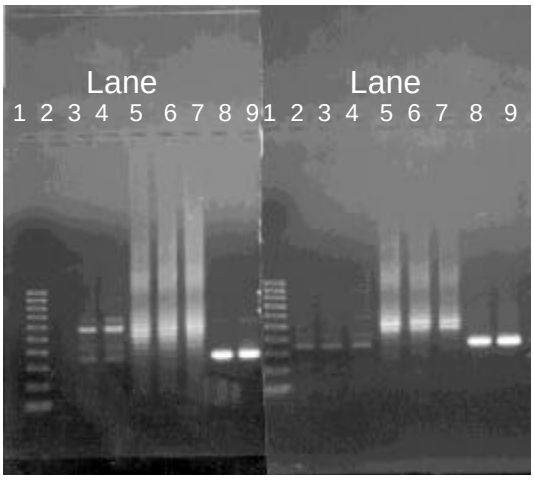


Figure 23. PCR fingerprints of day 3, pig 23, isolate 1, plasmid 5 (left of the split) and plasmid 6 (on the right). Lanes 1 for both gels are molecular standards, lanes 5-7 are primer 23L, and lanes 8-9 are primer OPB-17.

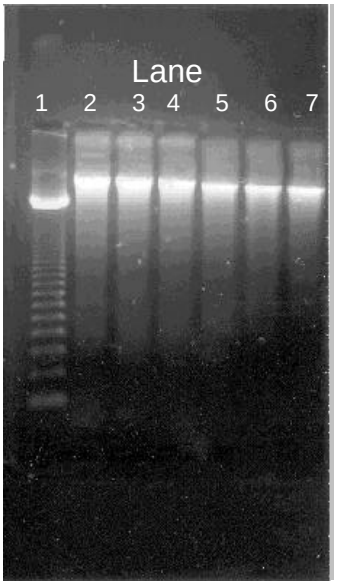


Figure 24. Day 4, pig 19, isolate 2 (resistant isolate) plasmid profile in lanes 5 through 7. Lane 1 is a molecular standard.

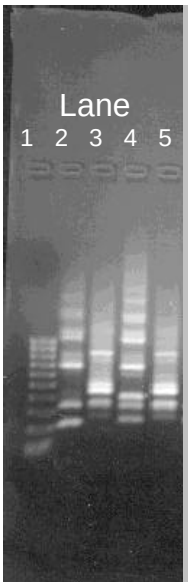


Figure 25. PCR fingerprint of the only plasmid from day 4, pig 19, isolate 2, in lanes 4-5. Lane 1 is the molecular standard, lane 4 is primer OPB-17, and lane 5 is primer 23L.

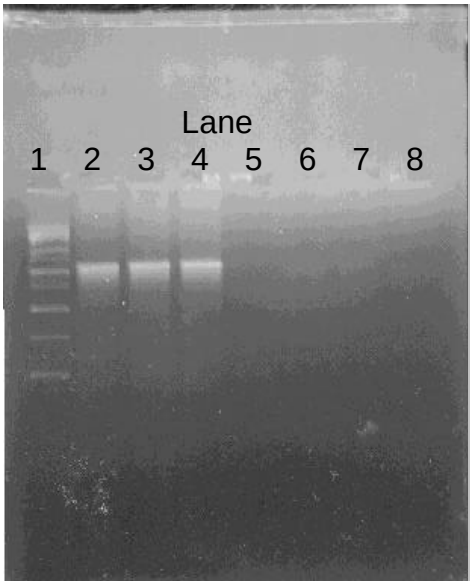


Figure 26. Plasmid profile of day 5, pig 20, isolate 3 (sensitive strain) in lanes 2-4. Lane 1 is a molecular standard.



Figure 27. PCR fingerprint of the only plasmid from day 5, pig 20, isolate 3. Lane 1 is the molecular standard, lanes 5-7 are primer 23L, and lanes 8-9 are primer OPB-17.

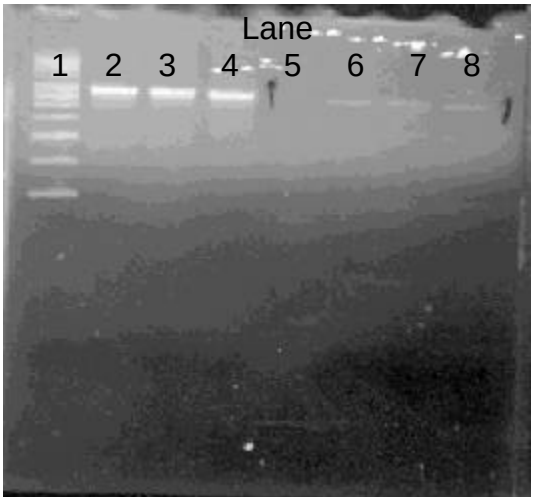


Figure 28. Plasmid profile from day 6, pig 24, isolate 1 (sensitive isolate) in lanes 2-4, and from day 6, pig 24, isolate 2 (resistant isolate) in lanes 5-7. Lane 1 is the molecular standard.



Figure 29. PCR fingerprint of the only plasmid from day 6, pig 24, isolate 1. Lane 1 is the molecular standard, lanes 5-7 are primer 23L, and lanes 8-10 are primer OPB-17.

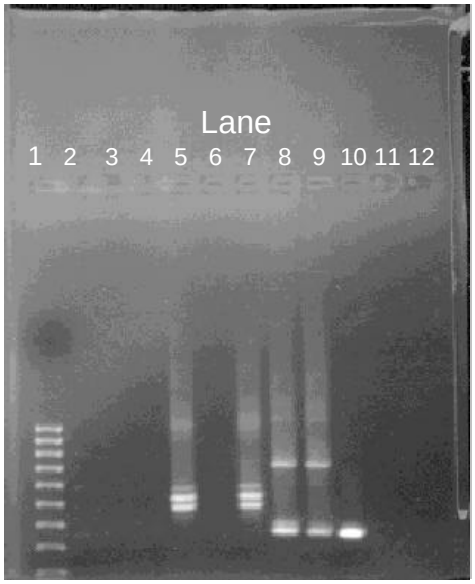


Figure 30. PCR fingerprint of the only plasmid from day 6, pig 24, isolate 2. Lane 1 is the molecular standard, lanes 5-7 are primer 23L, and lanes 8-10 are primer OPB-17.

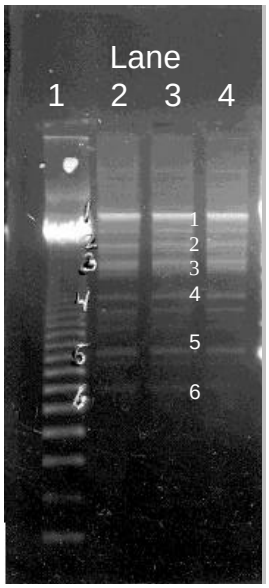


Figure 31. Plasmid profile from day 7, pig 20, isolate 2 (sensitive isolate) in lanes 2-4. Plasmids used in the following fingerprints are labeled 1-6. Lane 1 is the molecular standard.

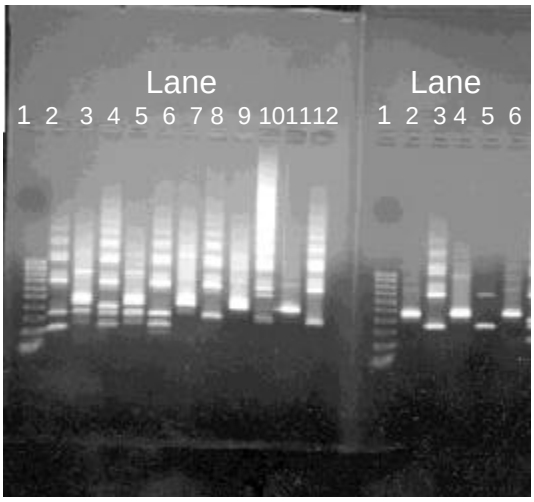


Figure 32. PCR fingerprints of day 7, pig 20, isolate 2 plasmids. Lane 1 is the molecular standard for both gels. On the left gel, lane 6 is plasmid 1, primer OPB-17; lane 7 is plasmid 1, primer 23L; lane 8 is plasmid 2, primer OPB-17; lane 9 is plasmid 2, primer 23L; lane 10 is plasmid 3, primer OPB-17; lane 11 is plasmid 3, primer 23L; and lane 12 is plasmid 4, primer OPB-17. On the right gel, lane 2 is plasmid 4, primer 23L; lane 3 is plasmid 5, primer OPB-17; lane 4 is plasmid 5, primer 23L; lane 5 is plasmid 6, primer OPB-17 and lane 6 is plasmid 6, primer23L.

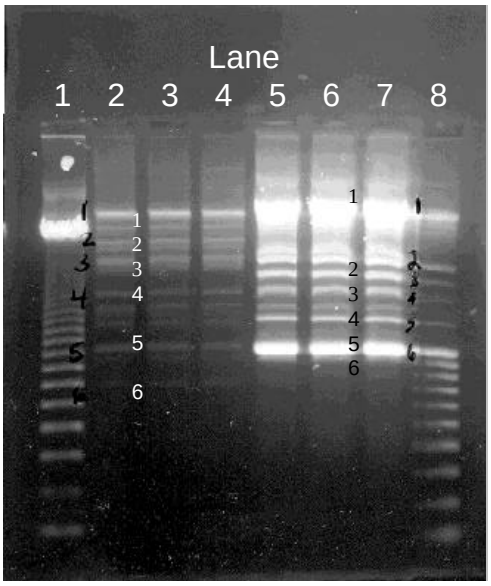


Figure 33. Plasmid profile of day 8, pig 22, isolate 4 (sensitive isolate) in lanes 5-7. The six numbered plasmids are the ones used in the following fingerprints. Lane 1 is the molecular standard.

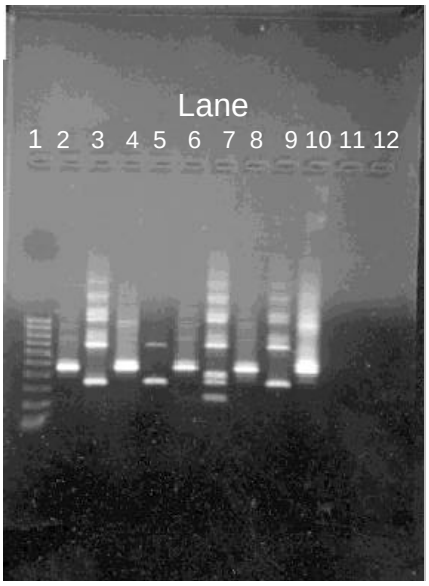


Figure 34. PCR fingerprints of plasmids from day 8, pig 22, isolate 4. Lane 1 is the molecular standard; lane 7 is plasmid 1, primer OPB-17; lane 8 is plasmid 1, primer 23L; lane 9 is plasmid 2, primer OPB-17; lane 10 is plasmid 2, primer 23L.

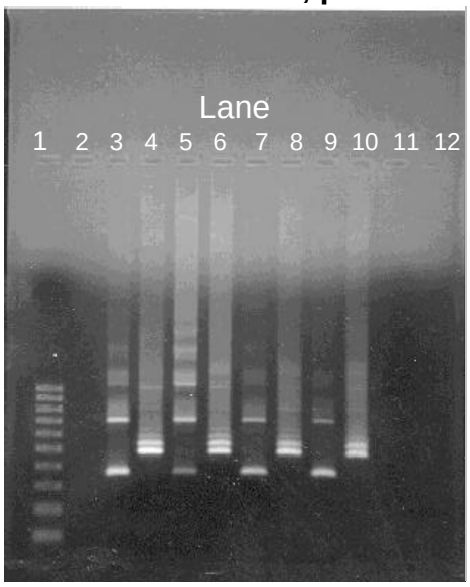


Figure 35. PCR fingerprints of plasmids from day 8, pig 22, isolate 4, continued. Lane 1 is the molecular standard; lane 3 is plasmid 3, primer OPB-17; lane 4 is plasmid 3, primer 23L; lane 5 is plasmid 4, primer OPB-17; lane 6 is plasmid 4, primer 23L; lane 7 is plasmid 5, primer OPB-17; lane 8 is plasmid 5, primer 23L; lane 9 is plasmid 6, primer OPB-17; and lane 10 is plasmid 6, primer 23L.

IIC. Pulsed-Field Gel Electrophoresis

Following are macrorestriction profiles (MRPs) generated by pulsed-field gel electrophoresis (PFGE) (Figures 36 and 37). Two different restriction enzymes were used on all twelve isolates in different runs. In both photographs, the first and last lanes are a control strain, and the eighth lane is a lambda DNA ladder.

IID. Analysis of AP PCR and PFGE Fingerprints

IIDa. Analysis by Eye

Visual analysis of plasmid fingerprints revealed some similarities among plasmids. Table 4 shows sizes of bands that were found for each plasmid and both primers. Table 5 shows plasmids that were deemed related by eye (same number and size of bands) for each primer separately and together, used in AP PCR. Again, these were scored by two different people.

Tables 6 and 7 show bands that were found when PFGE MRPs were analyzed by eye for each restriction enzyme.

IIDb. Analysis by Computer

Dendrograms created by the Molecular Analyst software for AP PCR plasmid profiles can be seen in Figures 38 and 39. Figures 40 and 41 are dendrograms created by the Molecular Analyst software for PFGE profiles of chromosomal DNA exposed to two different restriction enzymes.

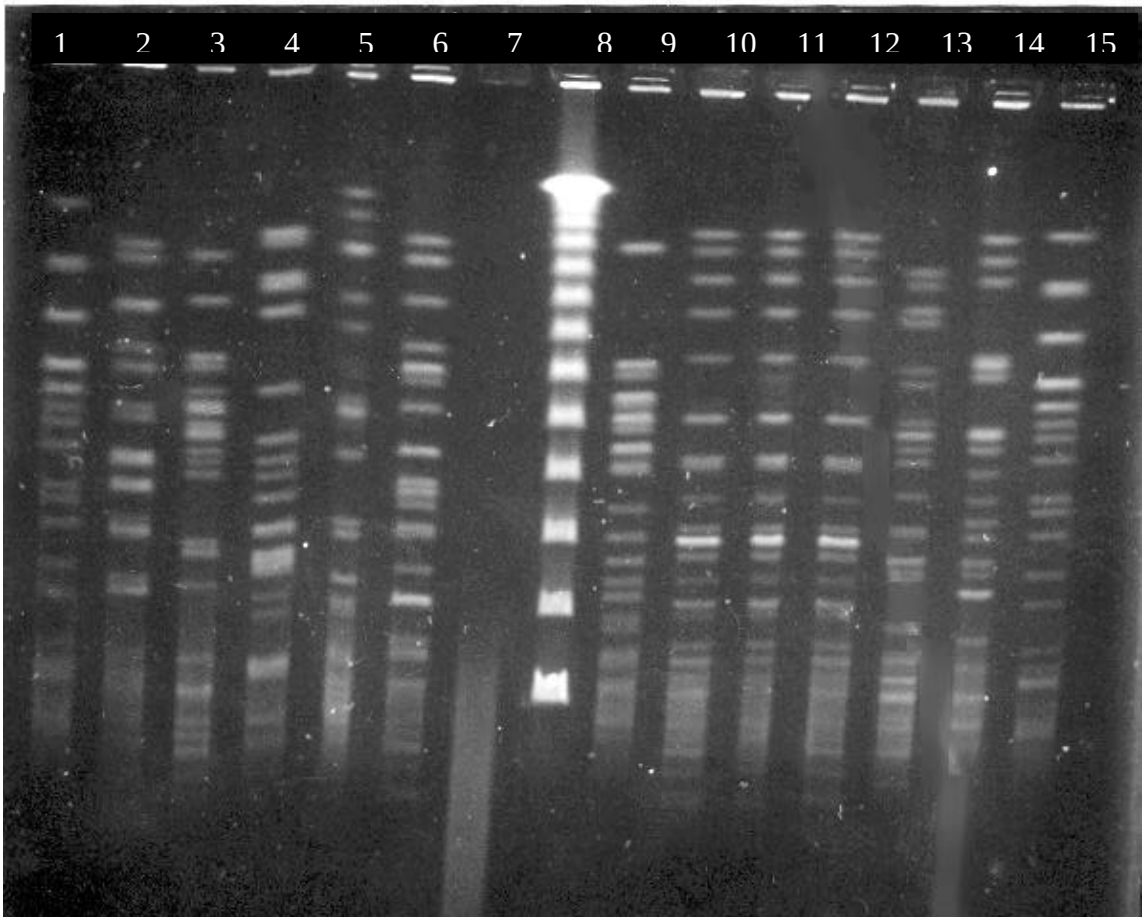


Figure 36. DNA MRPs generated by PFGE using restriction enzyme *Xba* 1 (Roche, Indianapolis, IN). Lanes 1 and 15 are a control strain, *E. coli* 0157:H7, #G524, from the CDC in Washington. Lane 8 is a lambda ladder. Lane 2 is d1, 23-1 (sensitive), lane 3 is d1, 19-2 (resistant), lane 4 is d2, 23-3 (sensitive), lane 5 is d3, 22-1 (sensitive), lane 6 is d3, 23-1 (resistant), lane 7 is d4, 19-2 (resistant), lane 9 is d5, 20-3 (sensitive), lane 10 is d5, 23-2 (resistant), lane 11 is d6, 24-1 (sensitive), lane 12 is d6, 24-2 (resistant), lane 13 is d7, 20-2 (sensitive), and lane 14 is d8, 22-4 (sensitive).

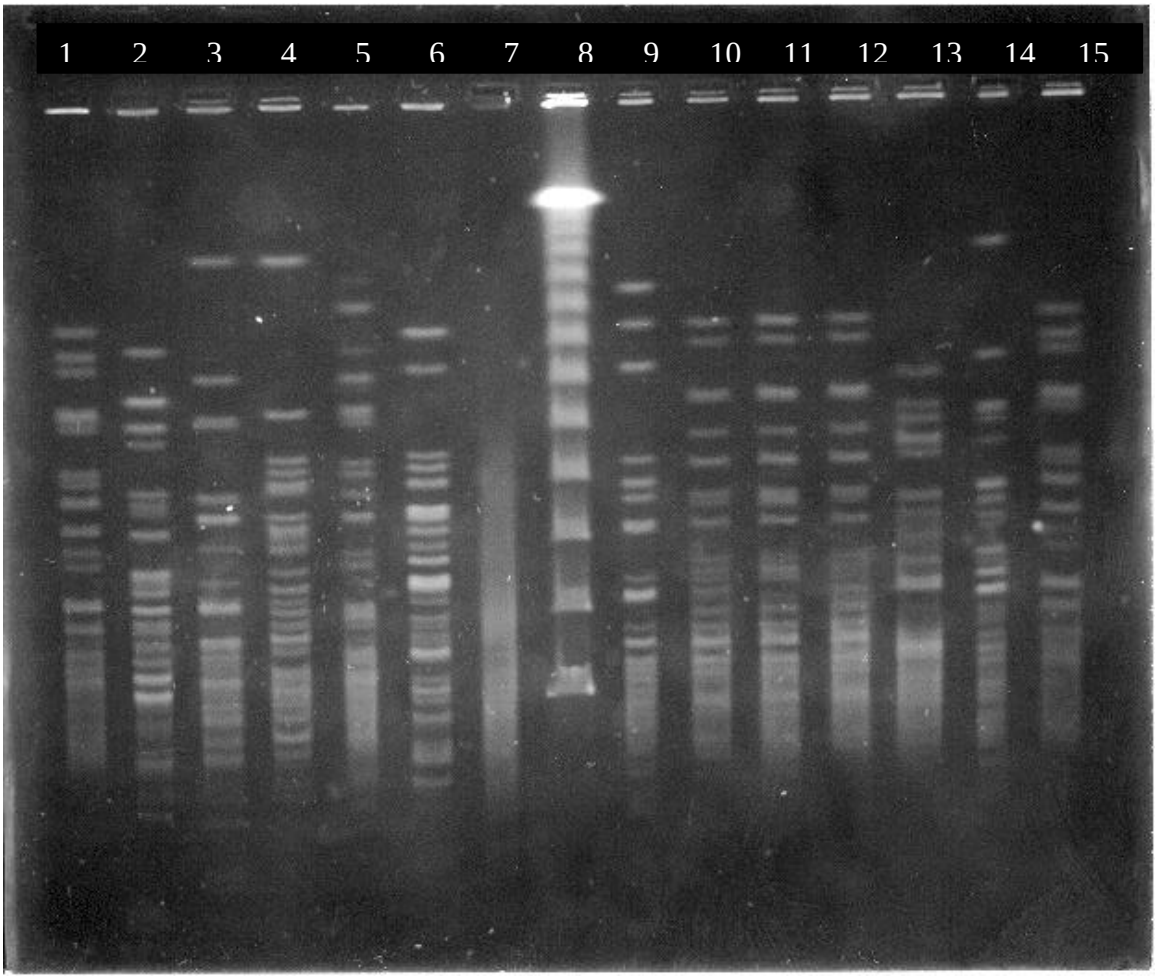


Figure 37. DNA MRPs generated by PFGE using restriction enzyme *Spe* 1 (Roche, Indianapolis, IN). Lanes 1 and 15 are a control strain, *E. coli* 0157:H7, #G524, from the CDC in Washington. Lane 8 is a lambda ladder. Lane 2 is d1, 23-1 (sensitive), lane 3 is d1, 19-2 (resistant), lane 4 is d2, 23-3 (sensitive), lane 5 is d3, 22-1 (sensitive), lane 6 is d3, 23-1 (resistant), lane 7 is d4, 19-2 (resistant), lane 9 is d5, 20-3 (sensitive), lane 10 is d5, 23-2 (resistant), lane 11 is d6, 24-1 (sensitive), lane 12 is d6, 24-2 (resistant), lane 13 is d7, 20-2 (sensitive), and lane 14 is d8, 22-4 (sensitive).

Table 4. Bands found for each plasmid and both primers for AP PCR, as analyzed by eye by two different people

PLASMID	PRIMER OPB-17 BANDS (# BASE PAIRS)	PRIMER 23L BANDS (# BASE PAIRS
d1, 19-2, #1	250, 350, 700, 1000	250, 350, 450, 500, 850
d1, 23-1, #1	350	450, 800, 850
d1, 23-1, #2	350	450
d1, 23-1, #3	350	450
d2, 23-3, #1	350, 550, 650	500, 550
d2, 23-3, #2	350, 600	450, 500, 550, 700, 1000
d2, 23-3, #3	300, 550	450, 500, 700, 1000
d2, 23-3, #4	450	550, 600
d2, 23-3, #5	300, 550	450
d2, 23-3, #6	400, 650	500
d3, 22-1, #1	350, 600	450, 500, 700, 1000
d3, 23-1, #1	350, 600	450, 500, 550, 600, 650
d3, 23-1, #2	350, 600	400, 450, 500, 550, 600, 650
d3, 23-1, #3	350, 600	400, 450, 500, 550, 600, 650
d3, 23-1, #4	350	400, 450, 500, 550, 600
d3, 23-1, #5	300	400, 450, 500, 700, 1000
d3, 23-1, #6	350	400, 450, 500, 550, 700, 1000
d4, 19-2, #1	250, 350, 450, 600, 700, 1000	300, 350, 400, 450, 500, 800, 900
d5, 20-3, #1	320, 550, 650, 950	400, 450, 500, 550, 600
d5, 23-2, #1	350, 600	450
d6, 24-2, #1	400, 650, 750	450, 500, 950
d6, 24-2, #1	350	450, 500, 550, 1000
d7, 20-2, #1	250, 350, 450, 650, 700, 850, 1000	400, 500, 550, 800
d7, 20-2, #2	350, 600, 650, 700, 800, 1000	450, 500, 550, 700, 1000
d7, 20-2, #3	350, 400, 550, 650, 1000	400, 450, 700, 900
d7, 20-2, #4	300, 650, 700, 950, 1000	450, 500, 800
d7, 20-2, #5	350, 700, 800, 1000	450, 500, 800, 1000
d7, 20-2, #6	350, 700	500, 550, 800
d8, 22-4, #1	250, 350, 400, 700, 800, 1000	450, 500, 650, 900
d8, 22-4, #2	350, 700, 800, 1000	450, 500, 700, 950
d8, 22-4, #3	350, 700, 1000	500, 550, 600, 650, 800, 1000
d8, 22-4, #4	350, 700, 1000	500, 550, 600, 650, 800, 1000
d8, 22-4, #5	350, 700, 1000	500, 550, 600, 650, 800, 1000
d8, 22-4, #6	350, 700	500, 550, 600, 800, 1000

Table 5. Groups of plasmids that were deemed "related" by eye for primer OPB-17, primer 23L, and both primers used in AP PCR.

Primer 17	Primer 23L	Both primers
d1, 23-1, #1 d1, 23-1, #2 d1, 23-1, #3 d3, 23-1, #4	d1, 23-1, #2 d1, 23-1, #3 d2, 23-3, #5 d5, 23-2, #1	d1, 23-1, #2 d1, 23-1, #3
d3, 23-1, #6 d6, 24-2, #1	d2, 23-3, #2 d7, 20-2, #2	d3, 23-1, #2 d3, 23-1, #3
d2, 23-3, #2 d3, 22-1, #1	d2, 23-3,#3 d3, 22-1, #1	d8, 22-4, #3 d8, 22-4, #4 d8, 22-4, #5
d3, 23-1, #1 d3, 23-1, #2	d3, 23-1, #2 d3, 23-1, #3	
d3, 23-1, #3 d5, 23-2, #1	d3, 23-1, #4 d5, 20-3, #1	
d2, 23-3, #3 d2, 23-3, #5	d8, 22-4, #3 d8, 22-4, #4 d8, 22-4, #5	
d7, 20-2, #6 d8, 22-4, #6		
d8, 22-4, #3 d8, 22-4, #4 d8, 22-4, #5		
d7,20-2, #5 d8, 22-4, #2		

Table 6. Bands identified by eye for PFGE MRPs for each isolate digested with enzyme *Xba* I.

Isolate	Sizes of bands found (# of kilobases)
d1, 23-1	460, 450, 365, 325, 305, 260, 250, 195, 185, 160, 155, 118, 110, 68
d1, 19-2	452, 370, 315, 307, 277, 260, 245, 240, 220, 195, 190, 135, 130, 115, 65
d2, 23-3	520, 515, 410, 405, 365, 277, 235, 195, 190, 176, 155, 130, 125, 120, 102, 95, 65, 60
d3, 22-1	620, 560, 525, 460, 375, 357, 310, 304, 270, 260, 250, 220, 160, 155, 120, 100
d3, 23-1	485, 440, 368, 330, 302, 298, 265, 230, 190, 186, 180, 155, 150, 125, 102, 72, 67, 55
d4, 19-2	-- did not work --
d5, 20-3	480, 308, 291, 260, 250, 238, 220, 200, 165, 148, 132, 128, 110, 95, 90, 60
d5, 23-2	495, 480, 405, 378, 311, 243, 238, 210, 205, 170, 150, 145, 128, 105, 95, 63, 57, 50
d6, 24-1	495, 480, 405, 378, 311, 291, 243, 238, 210, 205, 170, 150, 145, 128, 105, 95, 63, 57, 50
d6, 24-2	495, 480, 405, 378, 311, 243, 238, 210, 205, 170, 150, 145, 128, 105, 95, 63, 57, 50
d7 20-2	452, 445, 378, 373, 310, 302, 257, 244, 230, 220, 185, 160, 133, 125, 121, 85, 65, 53, 48
d8, 22-4	495, 455, 405, 311, 305, 250, 235, 198, 180, 162, 157, 133, 125, 108, 70, 60, 48

Table 7. Bands identified by eye for PFGE MRPs for each isolate digested with enzyme *Spe* I.

Isolate	Sizes of bands found (# of kilobases)
d1, 23-1	450, 315, 276, 266, 230, 219, 212, 182, 150, 130, 120, 105, 98, 72, 65, 55, 45
d1, 19-2	630, 340, 281, 225, 190, 176, 140, 120, 72, 68, 50
d2, 23-3	630, 285, 261, 246, 235, 190, 182, 176, 150, 130, 125, 120, 102, 82, 68
d3, 22-1	600, 590, 450, 340, 285, 281, 250, 245, 223, 190, 162, 157, 122, 118
d3, 23-1	510, 342, 261, 256, 249, 192, 189, 180, 176, 160, 130, 124, 102, 99, 68, 53, 50, 40
d4, 19-2	-- did not work --
d5, 20-3	610, 515, 340, 250, 223, 215, 180, 105, 100, 89, 80, 70
d5, 23-2	515, 450, 315, 266, 250, 215, 210, 183, 109, 106, 103, 100, 95, 80, 70, 63, 57
d6, 24-1	515, 450, 315, 266, 250, 215, 210, 183, 109, 106, 103, 100, 95, 80, 70, 63, 57
d6, 24-2	515, 450, 315, 266, 250, 215, 210, 183, 109, 106, 103, 100, 95, 80, 70, 63, 57
d7 20-2	325, 288, 278, 256, 252, 213, 192, 140, 109, 100, 65
d8, 22-4	635, 345, 288, 278, 254, 223, 212, 180, 175, 115, 113, 105, 100, 93, 88, 80, 76, 72

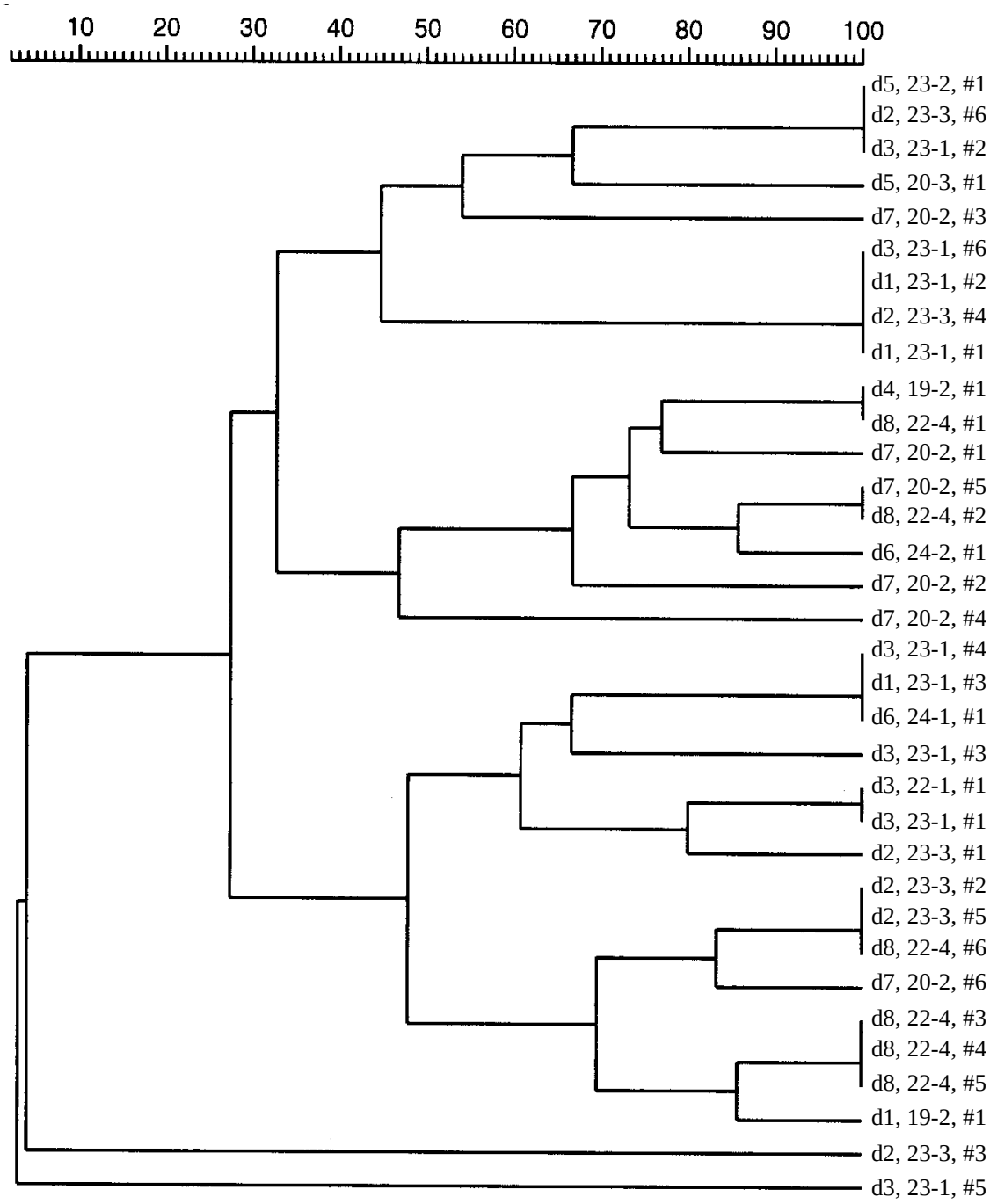


Figure 38. Dendrogram generated by Molecular Analyst software for AP PCR on all plasmids using primer OPB-17. The Dice coefficient, UPGMA clustering, and 1% tolerance in band position differences were used. The scale at the top indicates % correlation between plasmids.

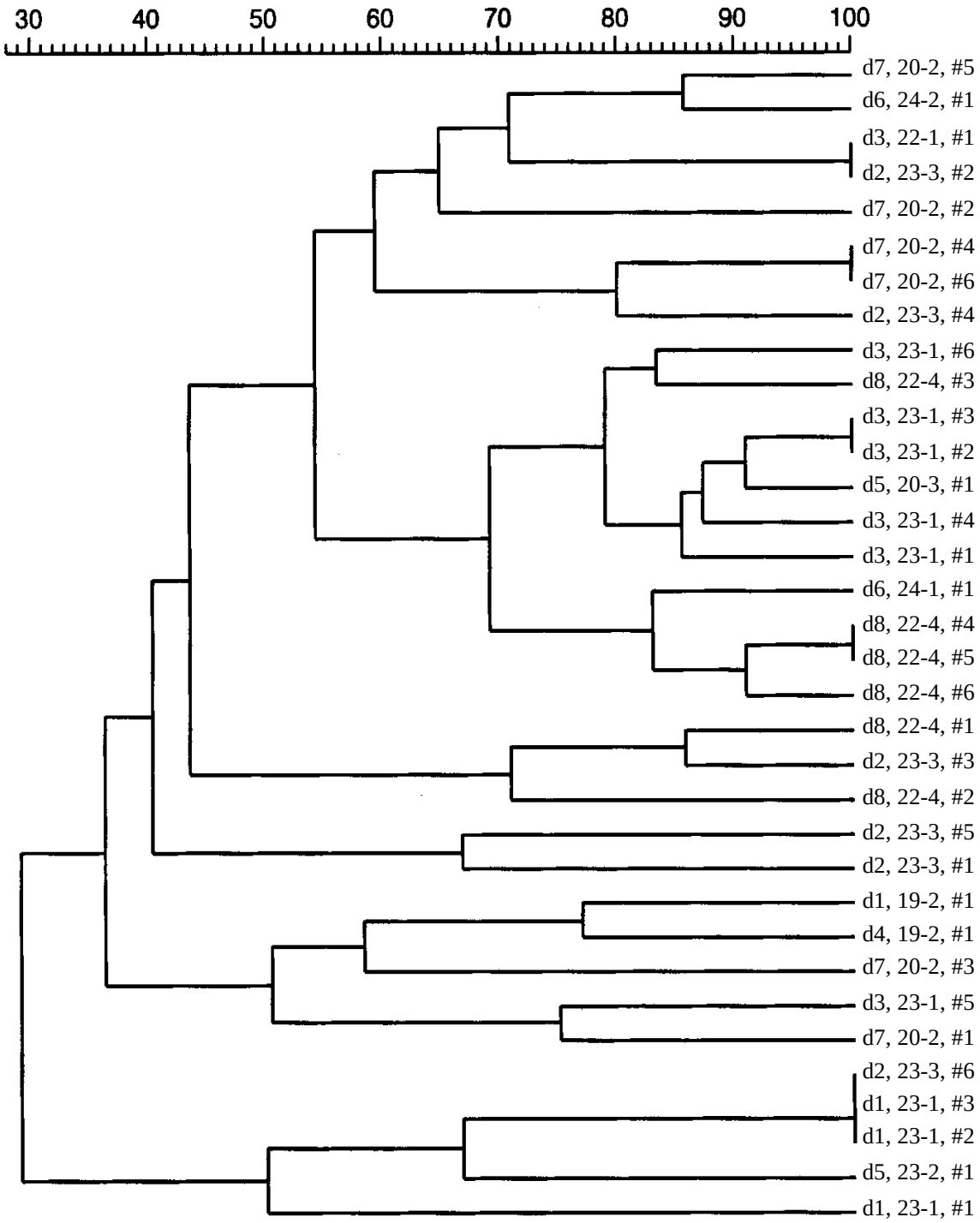


Figure 39. Dendrogram generated by Molecular Analyst software for AP PCR on all plasmids using primer 23L. The Dice coefficient, UPGMA clustering, and 1% tolerance in band position differences were used. The scale at the top represents % correlation between plasmids.

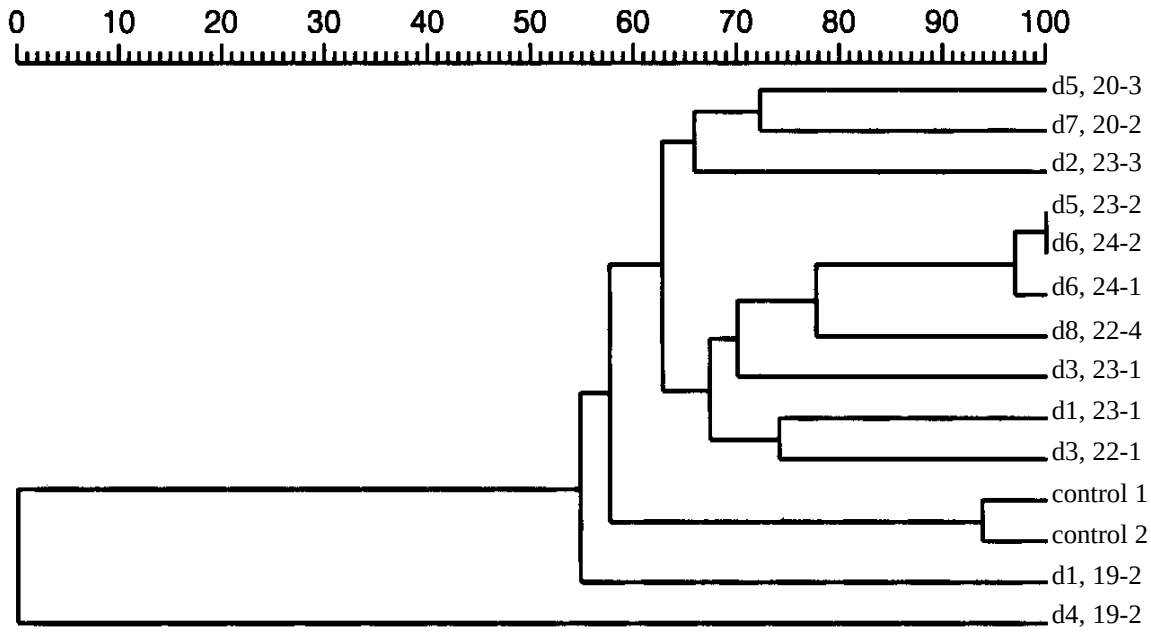


Figure 40. Dendrogram generated by Molecular Analyst software for PFGE on all chromosomal DNA using restriction enzyme *Xba* 1. The Dice coefficient, UPGMA clustering, and 1% tolerance in band position differences were used. The scale at the top represents % correlation.

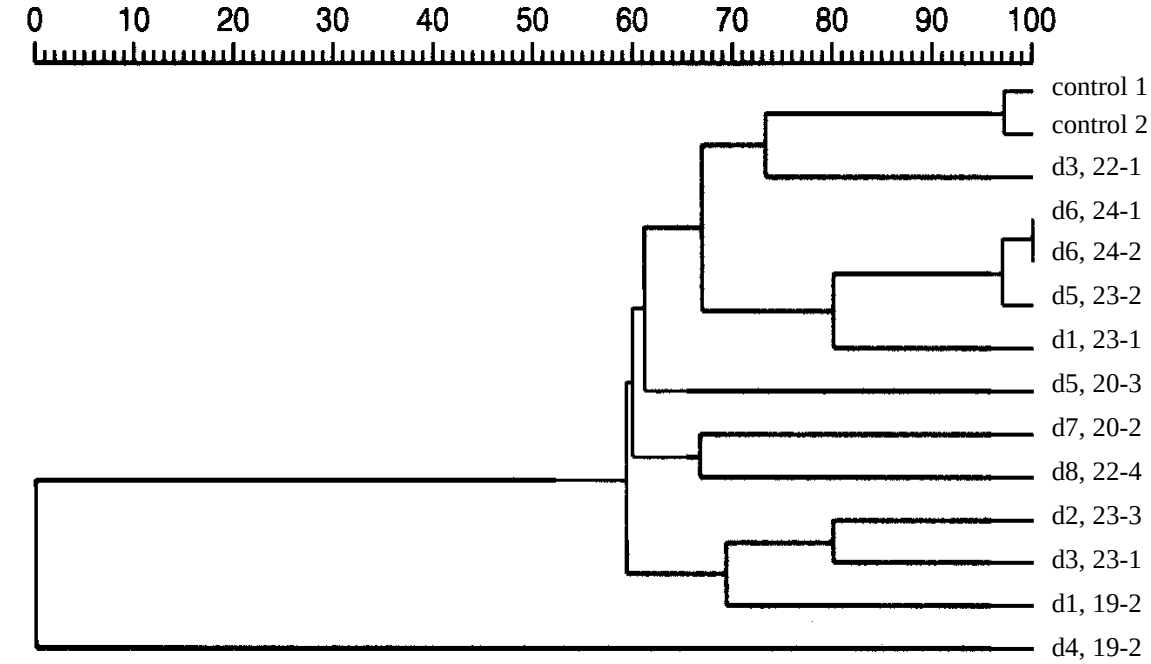


Figure 41. Dendrogram generated by Molecular Analyst software for PFGE on all chromosomal DNA using restriction enzyme *Spe* 1. The Dice coefficient, UPGMA clustering, and 1% tolerance in band position differences were used. The scale at the top represents % correlation.

4. DISCUSSION

I. ELECTROPORATION

This study was an attempt to determine if development of resistance to apramycin sulfate in *E. coli* isolated from cold-stressed swine could be linked to a plasmid or plasmids. If so, and if possible, it was desired that the plasmid/s be identified in some way. Electroporation was attempted first, and plasmids isolated from resistant bacteria were placed into receptive, nonresistant bacteria to see if antibiotic resistance would develop in these electroporated bacteria as a result. In doing so, plasmids responsible for resistance in the bacteria could possibly be identified by running plasmid preps on any resulting growth.

After the first electroporation study (involving the ATCC strain and plating on only 0 and 128 μg/ml apramycin plates), it was decided that a gradient, rather than extremes, of apramycin levels on nutrient agar plates was needed. Even though levels on MIC plates from the previous study were considered resistant at $\geq$128 and sensitive at $\leq$2, resistant bacteria could still possibly grow on plates at slightly lower levels of apramycin. Electroporation could possibly reduce the effectiveness of the electroporated plasmid to confer resistance, since it was a source of stress to the cells. Thus, the levels of apramycin in the second electroporation study included 0, 16, 48, 80, 96, and 128 μg/ml apramycin plates.

Since no growth was observed with either of the first two electroporation studies, it was thought that perhaps petri dishes were not the best culture method for the electroporated bacteria, since electroporation is stressful for bacteria, and maybe a nourishing broth would serve as a better substance for growth. This

would also be advantageous for the following plasmid prep, since it would mean avoiding the extra step of having to inoculate broth from growth on plates, which may contribute to the loss of the electroporated plasmid. Thus both plates and nutrient broth, with gradients of apramycin levels, were used as means of growth in the third electroporation study. Since growth occurred only in the broth and on plates with 0 μg/ml apramycin, a plasmid prep was performed only on that broth; however, no electroporated plasmid was found.

Then it was thought that perhaps the ATCC strain of bacteria was too "different" from the bacteria from which the plasmids were isolated; in other words, perhaps incompatibility was occurring and the plasmids were being lost from the electroporated cells. New electrocompetent cells were made from a sensitive strain from the previous study, since the sensitive strain should be more "similar" to the bacteria from which the plasmids were extracted. Once again, however, the electroporated plasmid could not be found in the plasmid preparation.

The idea for the last two electroporation studies, which involved using SOC with differing levels of apramycin, came about after reviewing a paper by Miller (1994) on electroporation. In that work he states that for plasmids encoding resistance, outgrowth in subinhibitory concentrations of antibiotic could perhaps improve recovery of transformants. Thus, differing, small levels of apramycin were added to the SOC (the outgrowth media) to see if that would improve recovery. Yet again, no electroporated plasmids were found. In all of these studies, it could not be determined whether the electroporation was not

working, or whether for some reason the cells would not retain the plasmids. As a result, electroporation was abandoned and alternate means of identification of resistant plasmids were explored.

II. AP PCR

It was thought that resistant plasmids could possibly be identified instead with the use of arbitrarily primed PCR. Plasmids could be isolated from all strains, both resistant and sensitive, and PCR fingerprints generated of all plasmids found. If one or more plasmids were responsible for resistance, then their fingerprints should be similar for the same primer (and the same fingerprints should be similar by both primers) and should be found only in the resistant isolates. Plasmids from different sensitive isolates could have the same fingerprint as well, and some sensitive isolate plasmids could be the same as those found in resistant isolates, but of course it could not be assumed that those plasmids were responsible for resistance.

First it should be noted that AP PCR has been used with varying degrees of success. Ideally, the PCR should be performed on all isolates at once in the thermal cycler, using the same “master mix” of reagents, and the products should ideally be visualized all on the same gel (Burr et al., 1997). A high level of standardization is necessary to obtain reproducible profiles, and variations in several parameters can cause aberrations in the profiles (Niederhauser et al., 1994). These parameters include concentration of magnesium, for example (Ellsworth et al., 1993), and the thermal cycler that is used (Penner et al., 1993).

In this study, although an attempt was made to ensure that all master mixes and parameters were the same, it was not possible to run all samples at once, particularly on the same gel. Once plasmids are individually isolated from agarose gels, they degrade rapidly and should be used for PCR reactions as soon as possible. It is not possible to extract more than a few plasmids simultaneously, as DNA yields decrease significantly if too much time is taken during the steps of extraction.

IIA. Analysis by Eye

It was decided that two fingerprints can be said to be related if patterns are identical (same number and size of bands, when compared to the molecular standard). By the decided method, only three sets of plasmids were found to be similar by both primers when analyzed by eye. These were d1, 23-1, plasmids 2 and 3 (sensitive); d3, 23-1, plasmids 2 and 3 (resistant); and d8, 22-4, plasmids 3, 4, and 5 (sensitive). Since a single plasmid can give rise to more than one band on gel electrophoresis because of different conformations of the plasmids (for example, supercoiled or relaxed), it is likely that for all of these sets, the plasmids that were found to be similar are actually the same plasmid, given that everything else worked with the PCR reactions and analysis.

However, analysis by eye can be very subjective, and could differ from person to person. Different results could be found depending on the researcher's decision as to what standards to accept for similarity among fingerprints. Thus it

cannot be said that analysis by eye is entirely reliable. In either case, identification of a resistant plasmid could not be found with this analysis.

IIB. Analysis by Computer

Computer analysis offers the possibility of comparison of large numbers of patterns, formation of databases, and the use of cluster analysis (Koeleman et al., 1998). However, Burr and Pepper (1997) found that even when conditions have been optimal for AP PCR, variations in human band scoring affect the ability of computers to correctly correlate fingerprints. Despite these findings, many researchers have used computers with success to score results of AP PCR; in these cases, either human band scoring was not considered to be a detrimental factor, or computer programs were more accurate with automatic band searching (Hilton and Penn, 1998; Koeleman et al., 1998; Hilton et al., 1996).

The Molecular Analyst® Software can compare bands in two different ways; by quantification, where concentrations of bands are compared, and by band-based similarity coefficients, where presence or absence of bands is scored. Quantification was not of interest here since some bands were more intensely visible and others less intense, thus band-based similarity coefficients were used. When comparing presence or absence of bands, there are several coefficients to choose from in the program, including coefficient of Jaccard, the Dice coefficient, an area-sensitive coefficient, a “fuzzy logic” coefficient, and Jeffrey’s x coefficient. The Dice coefficient was chosen because many other

researchers have successfully analyzed AP PCR in this way (Hilton et al., 1998; Burr et al., 1997; On et al., 1997; Markogiannakis et al., 2000).

Once the coefficient is chosen, three different clustering algorithms can be utilized. These include unweighted pair group method using arithmetic averages (UPGMA) clustering, the Ward clustering algorithm, and the Neighbour Joining (NJ) method. The NJ method was not chosen, since it is used more for creating biological evolution trees, and the resulting dendrogram trees are drawn as fluent curves instead of perpendicular lines. Many researchers use the UPGMA clustering (Hilton et al., 1998; Burr et al., 1997; On et al., 1997; Markogiannakis et al., 2000), thus this method was chosen as well.

Plasmid profiles were deemed similar if, for both primers, two plasmid profiles were 100% correlated (according to the dendrogram). The computer analysis showed that only one set of plasmids were similar; d8, 22-4, plasmid 4, and d8, 22-4, plasmid 5 (sensitive isolate). Since both plasmids were from the same isolate and even the same day, again it can be said that these are probably identical plasmids that simply occurred in two different formations on the gel. Additionally, since both of these plasmids were found to be similar by both eye analysis and computer analysis, it can be said with a fair amount of certainty that these plasmids are indeed the same. Once again, however, neither method of analysis is entirely accurate, and no resistant plasmid was identified by either method of analysis.

III. PULSED-FIELD GEL ELECTROPHORESIS

IIIA. Analysis by Eye

According to the method of Tenover et al. (1995), lanes d5, 23-2 (resistant), d6, 24-1 (sensitive) and d6, 24-2 (resistant), are considered to be identical for both restriction enzymes. All other chromosomal DNA patterns differed by six or more bands, so all other patterns were deemed unrelated to each other by both of the restriction enzymes used in PFGE when analyzed by eye. It is interesting to note that a sensitive and resistant isolate from the same pig and the same day have identical macrorestriction profiles.

IIB. Analysis by Computer

Once again, dendrograms were created using the Dice coefficient and the UPGMA clustering method according to the method of other researchers. The dendrograms for PFGE macrorestriction profiles (MRPs) showed that the two control strain lanes, which should be 100% correlated (because they are the exact same bacteria), were 95% correlated on the *Xba* I gel, and about 97% correlated on the *Spe* I gel. Thus, the computer seemed to be fairly accurate in reflecting correlations, though not entirely. The analysis also revealed the same three MRPs to be similar (by 97% for both gels) as those that were found to be similar by the eye analysis. So it can be said that these three MRPs are indeed related. All other profiles were shown by computer to be no more than 80% related.

IV. OVERALL RESULTS

Given that the plasmid PCR fingerprints are accurate despite differing "master mixes" and gels, the correct bands were manually identified for the computer analysis, the analysis by eye is accurate even when performed by two different people, the correct decisions were made as to which fingerprints to accept as being correlated by eye, and the correct coefficients used for computer analysis, then it can be said that a resistant plasmid was not identified with the PCR tests in this study. However, it is not certain that all of the above were correct decisions, though everything was performed with as much accuracy as possible. Thus it is possible that existing resistance plasmids were not identified by the above analyses. The PFGE results, which showed that a resistant and sensitive isolate had the same macrorestriction, chromosomal profiles, indicate that most likely, at least for those isolates, plasmids should have been identified that could be linked to antibiotic resistance, as it is most likely that resistance is found on either the chromosome or plasmid.

Development of resistance to apramycin sulfate in *E. coli* isolated from cold-stressed swine could not be linked to a plasmid or plasmids, nor was the genetic source of the resistance ever identified with this study.

V. WHAT COULD HAVE BEEN DONE DIFFERENTLY?

Instead of electroporation, conjugation studies could have been performed, where sensitive and resistant isolates could be plated side-by-side to see whether the sensitive isolates would become resistant. If this happened it

could be hypothesized that transfer of plasmids was taking place between the two different colonies. Resulting plasmid preps and PCR, again with the aminoglycoside-resistance-recognizing primer, could have been used to identify specific plasmids to determine whether or not they were the source of resistance.

More than two primers should probably have been used on AP PCR of plasmids. Koeleman et al. (1998) found that no less than five primers were needed for each isolate in order to obtain high discriminatory power for RAPD analysis. Perhaps then more matching resistance plasmids could be found with AP PCR, and one or several resistance plasmids identified from the resistant isolates. Again, though, this assumes that all samples could be run with the same master mix, and ideally, on the same gel.

Alternatively, PCR could be performed on bacteria with primers that identify antibiotic resistance genes. Aminoglycoside resistance genes have already been identified (Shaw et al., 1993). It may have been advantageous to find the sequence of that gene, formulate a specific primer, then run PCR on both plasmids and chromosomal DNA to try to detect the gene. The resistance gene should have been present on some DNA element since the MICs from the previous study indicate that resistance was definitely occurring. In this way the exact location of the resistance could be identified.

In the same manner, with the resistance gene identified, a Southern Blot analysis could be performed on both plasmid and chromosomal DNA. A probe could be made with the sequence of the gene for apramycin resistance. In this manner, the location of the resistance gene could again possibly be identified.

Further studies will be necessary to identify the genetic source of apramycin resistance in cold-stressed swine.

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