Diversity of *Escherichia coli* and *Salmonella* spp Isolates from Playa Waters and Sediments.

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ABSTRACT. *Preliminary work indicates that the CAFO environment is populated by various strains of Escherichia coli and Salmonella sp, numerous other members of the Enterobacteriaceae along with a wide variety of naturally occurring microorganisms. A central question to be addressed, are CAFO playa environments populated by resident genotypes and associated antibiotic resistant phenotypes or is there a shifting spectrum of genotypes and pathotypes? Answers to this question may influence management practices concerning the handling of animal waste. We report on the genetic structure of Escherichia coli and Salmonella sp that occupy the beef CAFO environment of the high plains of Texas. Research was conducted at seven feedyards and three control playas with periodic summer and winter samples collected over a two year period. Selective and nonselective media were used to isolate Escherichia coli and Salmonella sp and other microorganisms. Genetic analysis of Escherichia coli and Salmonella strains using for example repetitive element PCR (Rep-PCR) based assays indicate presence of five to seven dominate DNA fingerprint patterns for each genus. Serological and phenotypic analysis of 239 Salmonella sp indicates the presence of approximately 15 serotypes of Salmonella sp. Playa waters and sediments represent an environment suitable for the habitation of a small number of dominant Escherichia coli and Salmonella sp along with a wide variety of less frequently occurring strains as indicated by genotype.*

Keywords. *Pathogen diversity, DNA typing, serotype, beef cattle feedyard, playa*

INTRODUCTION

Escherichia coli O157:H7 and other pathogenic *Escherichia coli* strains are major food-borne infectious pathogens and, due to their worldwide distribution, pose a serious threat to public health systems. *Escherichia coli* O157:H7 was first recognized following two outbreaks in Michigan and Oregon in 1982 (Riley et al 1983) and is capable of causing diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. In the United States close to 75,000 cases of O157:H7 infections are now estimated to occur annually (Mead et al. 1999). The genus *Salmonella*, comprises one of the most important pathogen groups involved in human foodborne illness, is divided into two species: *S. enterica* that is subdivided into over 2,000 serovars, and *S. bongori*. One serovar, *S. enterica* serovar Typhi CT18 is host restricted, infecting only humans (typhoid fever), while the other serovar, *S. enterica* serovar Typhimurium LT2, is a host generalist that infects humans (gastroenteritis) and other mammalian species. Domestic animals act as a reservoir of non-typhoid *Salmonella* infections and are responsible for millions of infections and multiple deaths in the human population costing billions of dollars (range 4 to 23) yearly (Todd 1990). Thus, the epidemiological relationships amongst various *Salmonella* serotypes; especially as related to outbreaks of human salmonellosis due to consumption of contaminated dairy, poultry and meat products; is a key concern in monitoring the presence and spread of disease.

One question of interest regarding beef cattle confined animal feeding operation (CAFO) environments is the population structure of various isolates of *Escherichia coli* and *Salmonella* spp. Are these CAFO environments dominated by a few resident genotypes or are there ever-shifting spectrums of genotypes? Playa water and sediment samples within the CAFO environment were

obtained from seven feedyard and three control playas via the periodic collection of summer and winter samples over a four year period. Selective media were used for the isolation of members of the family *Enterobacteriaceae*, *Escherichia coli* and *Salmonella* sp. Research results indicate that beef CAFOs are populated by various strains of *Escherichia coli* and *Salmonella* sp along with numerous other members of the family *Enterobacteriaceae* (based on the use selective chromogenic media and biochemical evaluation). We report on the genetic structure of *Escherichia coli* and *Salmonella* strains that occupy the beef CAFO environment of the high plains of Texas. Genetic analysis of 221 *Escherichia coli* and 239 *Salmonella* strains was conducted using Rep-PCR assay employing the BOXA1R primer. Rep-PCR has proven to be a sensitive and rapid DNA typing method with broad applicability to analyzing the genetic variability of a broad range of microbial organisms (Versalovic et al. 1991, Olive and Bean 1999). Unknown playa Escherichia coli strains were referenced against the DEC A set of Escherichia coli strains obtained from the Dept. of Toxicology, Michigan State University. This reference collection is comprised of known enterohemorrhagic *Escherichia coli* (EHEC) isolates and enteropathogenic *Escherichia coli* (EPEC) isolates such as various serotypes; O157:, O128:, O111:, etc. *Salmonella* sp were serotype and compared on the basis of serology. Results of this study are reported below.

MATERIALS AND METHODS

ENVIRONMENTAL SAMPLING

Environmental samples consisting of feedyard playa water and sediments were sampled at all four cardinal locations (N, S, E, and W) within a feedyard playa primarily during the summer and winter of 2001 and 2002 (with the addition of a previously obtained 1998 sample). Duplicate one liter water samples were collected within 3 meters of one another at each location and pooled. Sediments were collected into 300 ml polypropylene containers using a round-nosed shovel at each location.

E. COLI ISOLATION PROCEDURE

Water samples (100 ml aliquot) were diluted 1:1 in Novobiocin (25 µg/ml) – Tryptose broth (NTB), incubated overnight at 37 ˚C followed by a 1:10 dilution in NTB and again incubated overnight at 37 ˚C. Aliquots (0.1 ml) of serial dilutions were plated out on sorbitol MacConkey agar plates and incubated overnight at 37 ˚C. Alternatively water samples were mixed 1:1 with 2X EC modified enrichment broth plus Novobiocin (25 µg/ml) / potassium tellurite (0.25 ug) and incubated overnight at 37 ˚C followed by direct plating on sorbitol MacConkey agar plates. Sediments were diluted 1:10 (10 g sediment / 90 ml NTB) and incubated overnight, and then aliquots (0.1 ml) of serial dilutions were plated out on sorbitol MacConkey agar plates and incubated overnight at 37 ˚C. White and pink/red colonies were selected, grown overnight and stored frozen in milks at -80 ˚C until further analysis. Suspect *Escherichia coli* strains stored at -80 ˚C were reisolated on HiCrome Agar™ (Sigma Chemical Co, St. Louis, MO) a chromogenic agar for differentiation of various *Enterobacteriaceae* species. All dark blue colonies (suspect *E. coli* species) were confirmed biochemically using the indole test.

SALMONELLA SP ISOLATION PROCEDURE

Water samples (100 ml aliquot) were diluted 1:1 in 100 ml of 2X strength sulfur-brilliant-green (SBG) enrichment broth plus novobiocin (25 µg/ml) and incubated for 24 h at 37 ˚C. A 100 ml aliquot of SBG enrichment culture was mixed with 100 ml of fresh 1X SBG plus novobiocin enrichment broth and cultured for 24 h at 37 ˚C. Sediments were diluted 1:10 (10 g sediment / 90 ml SBG plus

novobiocin) and incubated in a shaker incubator at 37 ˚C for 24 h. The sediment-enrichment culture was transferred to 100 ml of 1X SBG plus novobiocin and incubated at 37 ˚C for 24 h. After the second incubation, samples were cultured for *Salmonella* spp on 3 types of differential media (MacConkey agar, brilliant green agar, and xylose-lysine-desoxycholate agar) at 37 ˚C for 24 h. Suspect *Salmonella* spp were evaluated on additional differential media (triple-sugar-iron, lysine-iron agar, motility indole ornithine, and urease). Suspect *Salmonella* spp revealed from these tests were evaluated on nutrient agar plates plus 5% bovine serum albumin for the absence of hemolytic activity. Suspect *Salmonella* spp were shipped to the National Veterinary Services Laboratory in Ames, IA for verification as *Salmonella enterica* sp and serotyping. Suspect *Salmonella* strains stored at -80 ˚C were reisolated on Rainbow Salmonella Agar™ (Biolog, Hayward, CA) a chromogenic agar for detection and evaluation of various *Salmonella* spp.

DNA PREPARATION AND BOXA1R PCR

Bacterial genomic DNA preparations were made using PUREGENE® DNA isolation kits according to manufactures instructions (Gentra Systems, Minneapolis, MN). PCR reactions (25 µl) contained the following: 40 ng of template DNA, 1X JumpStart reaction buffer (Sigma Chemical Co, St. Louis, MO), 1.5 mmol 1-1 $MgCl_2$, 200 µmol 1-1 dNTP, 40 pmol 1-1 primer, and 1 U JumpStart Taq DNA polymerase (Sigma Chemical Co, St. Louis, MO). PCR temperature profile of 2 min at 94 ˚C followed by 30 cycles of 30 s at 94 ˚C, 30 s at 50 ˚C, and 1 min at 65 ˚C . The 30 cycles were followed by one cycle of 7.5 min at 72 ˚C. Aliquots (12 µl) of each reaction were run on 1.4% agarose gels and stained with ethidium bromide (Sambrook *et al*. 1989).

IMAGE ANALYSIS AND DENDROGRAM CONSTRUCTION

Gel images were captured and analyzed by using a computerized video image analysis system (Kodak image workstation 440CF, Eastman Kodak, Rochester, NY). Molecular weight standards were included in each gel to allow for normalization of gel images for valid between-gel comparisons of fingerprints. BOXA1R DNA fingerprint profiles were digitally compared using the Pearson and Dice coefficients and dendrograms were construct using the UPGMA method employing various programs within Bionumerics version 4.0 (Applied Maths, Austin, TX).

RESULTS AND DISCUSSION

Escherichia coli strains were recovered from all feedyard and one control playas while *Salmonella* spp were recovered only from feedyard playas. Over 800 suspect *Escherichia coli* strains were obtained from seven feedyards and one control playa during the sampling period. Approximately 37% were confirmed to be Escherichia coli isolates based on dark blue colony phenotype (Table 1) when plated on HiCrome Agar™ and confirmed by a positive indole assay result. Other *Enterobacteriaceae* bacterial strains noted are indicated by the following phenotypes that were observed on HiCrome Agar and they are indicated as follows: DBV, dark blue – *E coli*; SR, salmon red – *Enterobacter cloacae* or *Citrobacter freundii*; LP, light pink – *Klebsiella pneumonia*; CL, colorless – *Salmonella enteritidis* or *Shigella flexneri* and WH, white – undefined phenotype.

Table 1. Phenotype of suspect *E. coli* strains*

Phenotype	No of Isolates
CL	125
DBV	224
LP	48
NG^	235
SR	147
WH	46

*on HiCrome Agar
^no growth

A broad diversity of DNA fingerprint patterns and / or *Salmonella* serotypes were observed within the *Escherichia coli* and *Salmonella* strains isolated from the Texas high plains beef cattle CAFO playa environment. This is similar to other reports documenting a broad range of serotypes of *Escherichia coli* and *Salmonella* strains isolated from cattle-pastureland operations and beef cattle CAFO from different geographical locations and climatic environments (Beach *et al*. 2002; Dargatz *et al*. 2000; Purdy *et al*. 2004; Renter *et al*. 2003, 2004). Genetic analysis of 221 suspect *Escherichia coli* strains using Rep-PCR (BOXA1R) assay indicates the presence of at least five overall dominate DNA fingerprint types amongst those observed diverging from 30 % to 70 % similarity level (genetic variability) along with respective distinctive subtypes (Fig 1 and 2). A total of 42 subtypes comprising 100 isolates were observed. The number of strains per DNA fingerprint subtype ranged from 2 to 6. A total of 121 unique DNA fingerprint patterns were observed although some are similar to other dominant DNA fingerprints (range of 75 % to 95 % similarity). One DNA fingerprint pattern was confined primarily to playa F11 (Fig 1) while numerous DNA patterns were more broadly distributed amongst three to five playas (combinations of playas F2, F3, F5, F7 and F8) in both water and / or sediment samples (Fig 2). Identical DNA fingerprints were observed in different playas at different times, while playa F11 also contained several different DNA fingerprint broadly distributed throughout both water and sediment samples. *Escherichia coli* isolates from the feedyard playas were similar to the DEC A reference strain set in the range of 10 % to 95 % similarity (data not shown). No *Escherichia coli* isolate yielded an identical DNA fingerprint to known *Escherichia coli* O157:H7 strains. However, twelve *Escherichia coli* O157:H7 isolates were obtained by enrichment and the use of immunomagnetic separation technology from these playa water and sediment samples that were sent to other investigators (personal communication).

A total of 239 *Salmonella* strains were confirmed either by serological analysis or by growth and phenotypic analysis on Rainbow Salmonella agar. Phenotypic results on Rainbow Salmonella agar indicate the presence of either *S gallinarum* or *S pullorum* sp. A total of 15 confirmed *Salmonella* serotypes were determined by serological and or phenotypic analysis. Rep-PCR DNA fingerprint pattern analysis indicates the possibility of an additional three to five *Salmonella* sp (data not shown). The distribution of *Salmonella* serotypes across CAFO playas ranged from 4 serotypes that occur only in a single playa to two serotypes that are present in four playas (Table 2). With the exception of the serotype *S typhimurium* the genetic variability of the observed *Salmonella* spp ranged from the 23% to 81% similarity levels (Table 2).

Table 2. Distribution of *Salmonella* serotypes across feedyard playas

Salmonella sp	No. of strains	No. of DNA types	Playa (no of strains)	Similarity (%) amongst Strains
S agona	3	2	F1 (1), F5 (2)	49.3
S anatum	6	3	F1 (4), F2 (2)	78.0
S cerro	5	3	F11 (4), F3 (1)	47.7
S give	3	2	F11	79.8
S infantis	7	NA`	F1	NA
S kentucky	33	3*	F3	69.4
S kiambu	10	3	F1	80.9
S mbandaka	51	5*	F2 (8), F3 (16), F7 (21), F11 (6)	23.2
S montevideo	22	3*	F1 (19), F7 (3)	42.2
S oranienburg	3	2	F2	66.5
S reading	12	3	F5 (3), F7 (5), F8 (1), F11 (3)	66.7
S thomasville	3	2	F5	53.3
S typhimurium	28	4	F8 (26), F11 (2)	0.1

`pending
*no of major, several minor

S mbandaka was the most dominant serotype observed (51 isolates) while four serotypes occurred at a frequency of three isolates per serotype. The number of *Salmonella* serotypes occurring within a feedyard ranged from a minimum of three serotypes to a maximum of five serotypes per feedyard playa (Table 3). The number of recovered *Salmonella* isolates per playa ranged from 8 in playa F5 to 63 from playa F3.

Table 3. Distribution of *Salmonella* serotypes within feedyard playas

Feedyard playa	*Salmonella* sp	*Salmonella* serotypes
F1	44	*S agona, S anatum, S infantis, S kiambu, S montevideo*
F2	12	*S anatum, S mbandaka, S oranienburg*
F3	63	*S cerro, S kentucky, S mbandaka,*
F5	8	*S agona, S reading, S thomasville*
F7	49	*S mbandaka, S meleagridis, S montevideo, S reading*
F8	29	*S montevideo, S reading, S typhimurium*
F11	25	*S cerro, S give, S mbandaka, S reading, S typhimurium*

S mbandaka was broadly distributed in playa waters and sediments of several playas and exhibited considerable genetic diversity (Fig 3). The genetic diversity of ten *Salmonella* serotypes recovered from three high population density playas indicates that identical genotypes are distributed across feedyard playas and that some serotypes are genetically diverse e.g. *S mbandaka* (Fig 4, 5, and 6). Feedyard playa waters and sediments represent an environment suitable for the habitation of a small number of dominant *Escherichia coli* and *Salmonella* spp along with a wide variety of less frequently occurring strains. It also appears that an ever-shifting spectrum of *Escherichia coli* and *Salmonella* spp can coexists along with a small number of dominant isolates. The long range temporal stability of dominant serotypes remains to be established.

Conclusion

We have determined the genetic population structure of *Escherichia coli* strains and *Salmonella* spp present in feedyard playas by the use of repetitive DNA fingerprint method. A database of environmental isolates (reference strain collections) comprised of members of *Enterobacteriaceae*, *Escherichia coli* and *Salmonella* spp. has been established that will allow for the long term monitoring of microbiological populations associated with CAFO environments. We can evaluate this reference collection of *Enterobacteriaceae*, *Escherichia coli* and *Salmonella* strains from the Texas high plains to evaluate the movement of antibiotic resistance genes, the distribution of virulence genes, and to evaluate the fate and transport of pathogens in the environment. Thus, this 'environmental library' of microbial isolates will allow for the long term assessment of the impact of beef CAFO on various downstream agricultural production systems.

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APPENDIX

The following figures 1-6 consist of genetic analysis of bacterial DNA isolated from the *Escherichia coli* and *Salmonella* spp. The column format is as follows: dendrogram, DNA fingerprint, strain designation, genus and species (Figs 3-6 only), feedyard playa, sample date, sample source and direction.

Fig 1. Diversity of *E coli* isolates from feedyards F1, F11 and control playa C10.

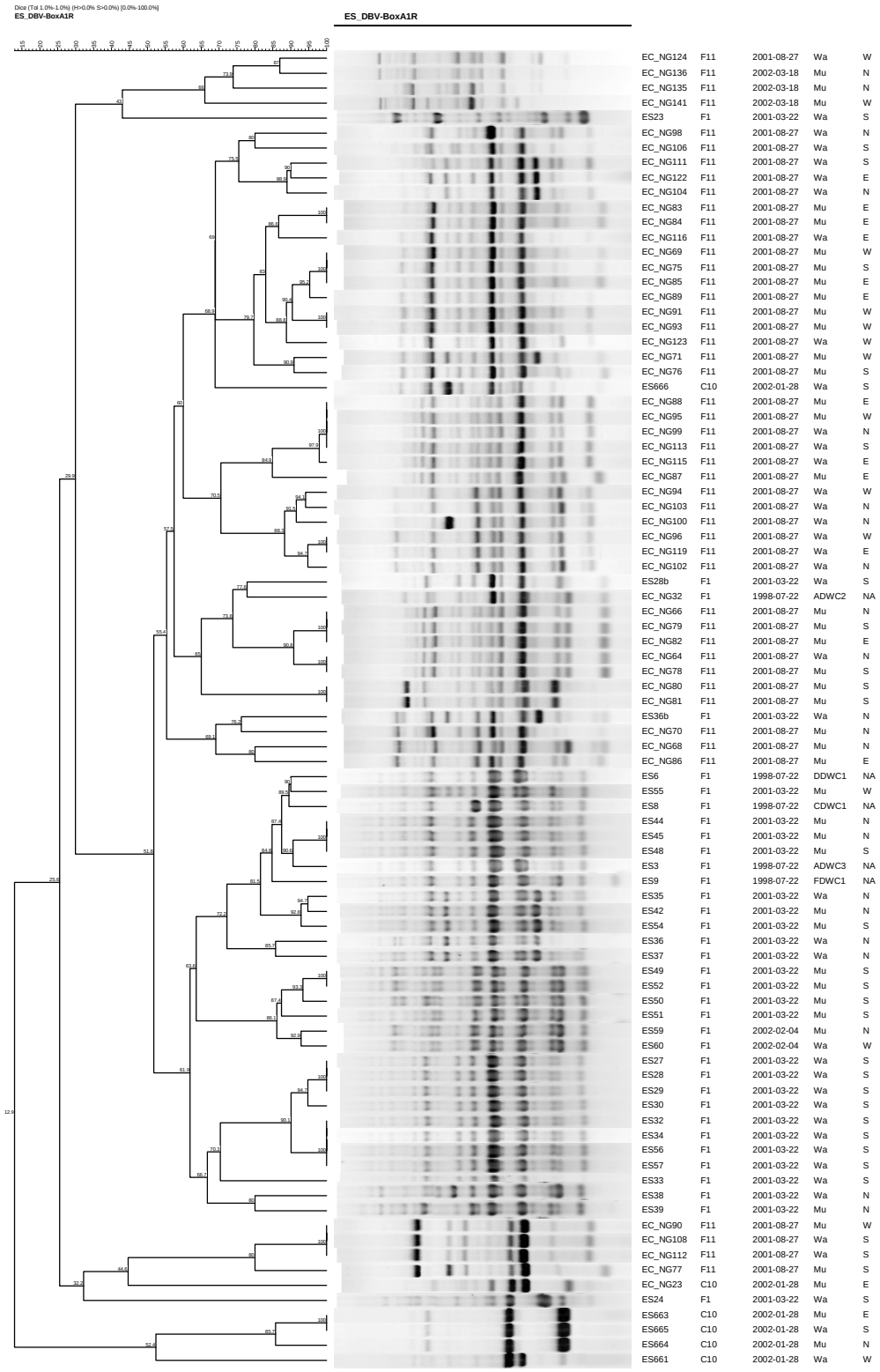


Fig 2. Diversity of *E coli* isolates from feedyards F2, F3, F5, F7, and F8

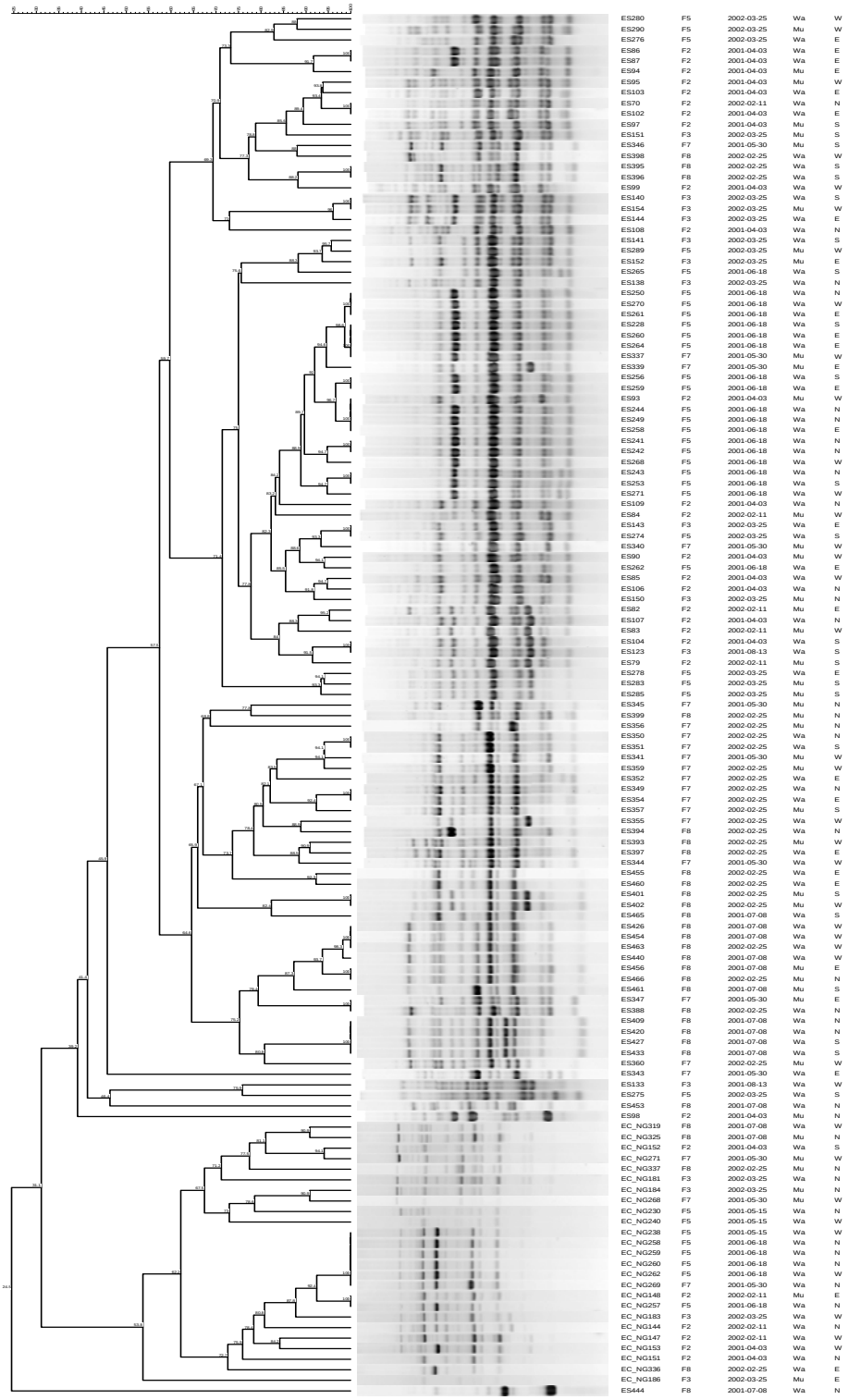


Fig 3. Diversity of *S mbandaka* isolates from feedyard playas F2, F3, F7, and F11

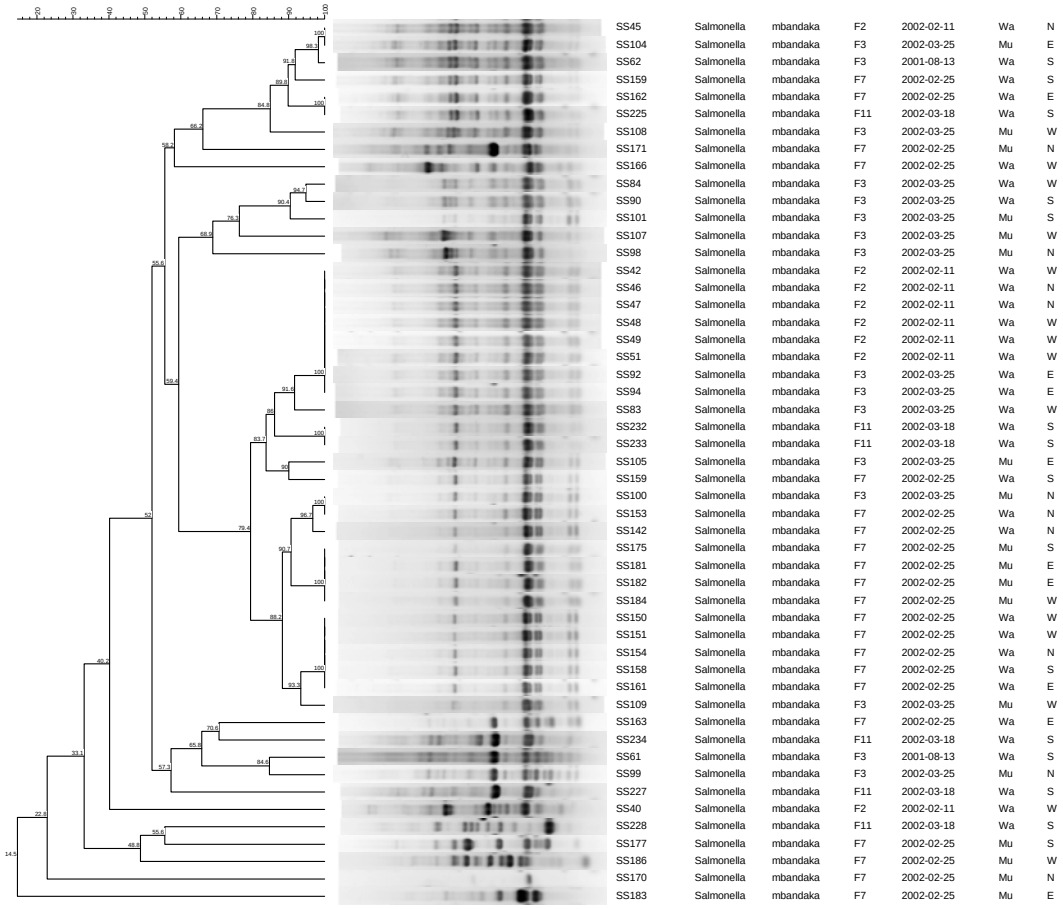


Fig 4. Diversity of *Salmonella* spp within F1 as determined by Rep-PCR

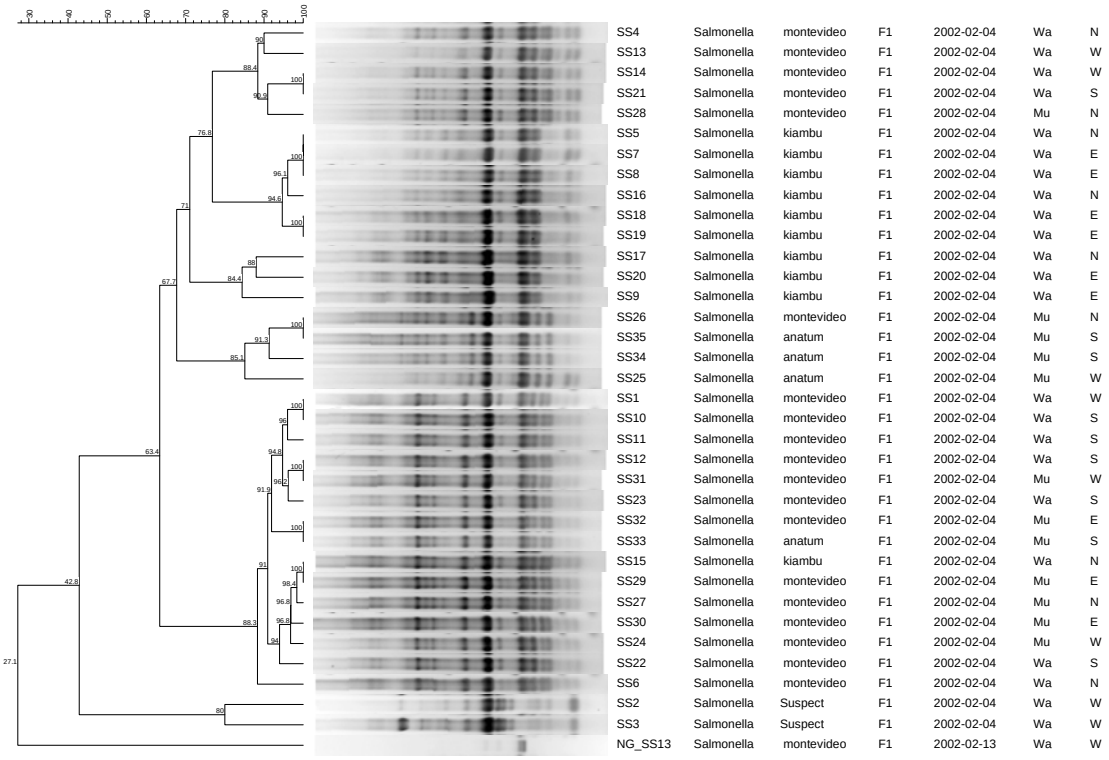


Fig 5. Diversity of *Salmonella* spp within F3 as determined by Rep-PCR

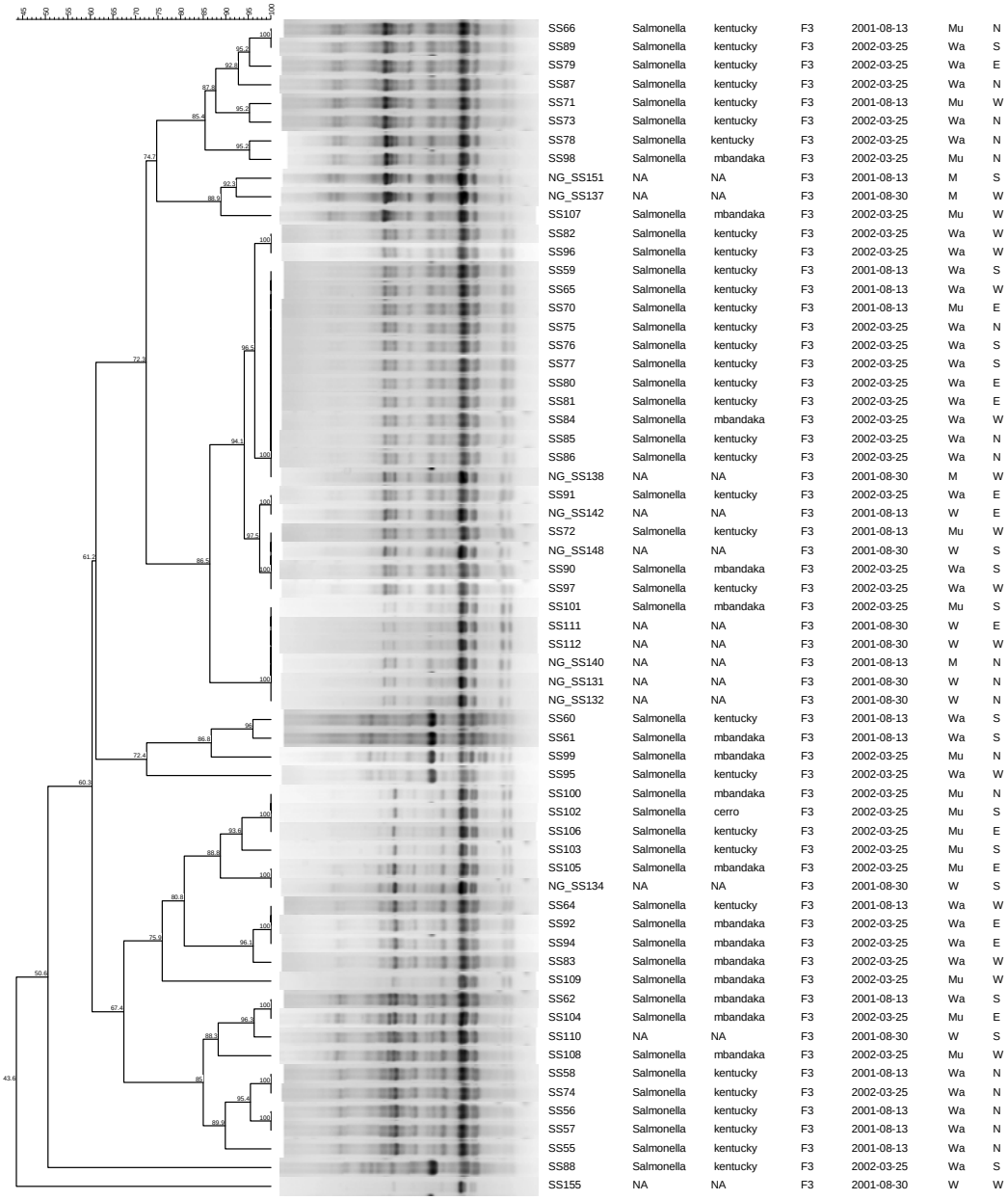
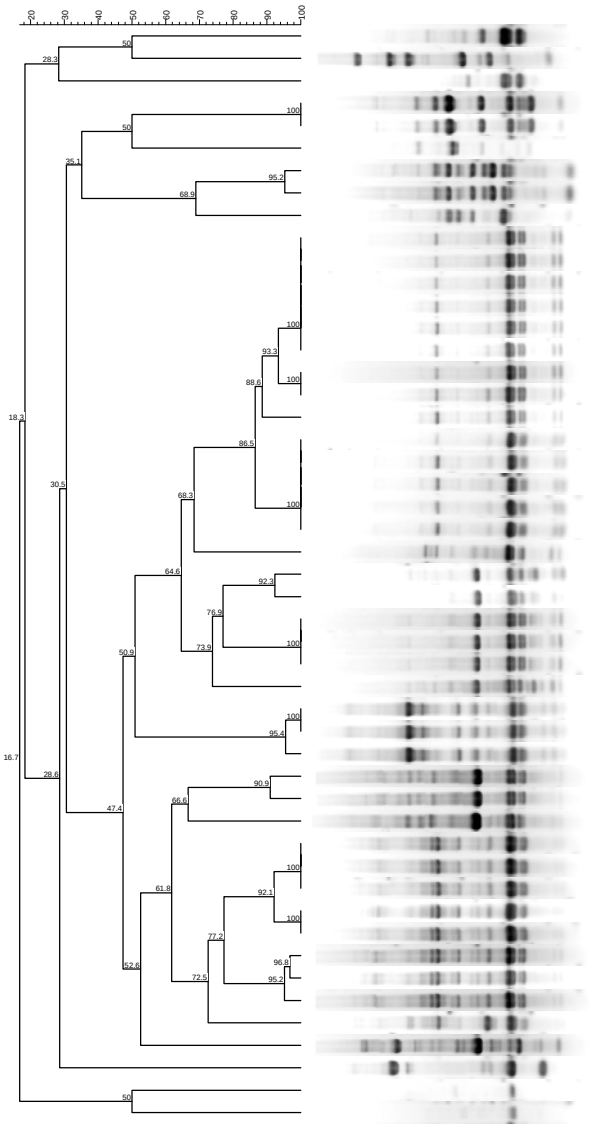


Fig 6. Diversity of *Salmonella* spp within F7 as determined by Rep-PCR



SS183	Salmonella	mbandaka	F7	2002-02-25	Mu	E
SS134	NA	NA	F7	2001-05-29	Wa	E
SS189	Salmonella	suspect	F7	1998-01-10	na	E
SS125	NA	NA	F7	2001-05-29	Mu	N
SS177	Salmonella	mbandaka	F7	2002-02-25	Mu	S
SS176	Salmonella	meleagridis	F7	2002-02-25	Mu	S
SS186	Salmonella	mbandaka	F7	2002-02-25	Mu	W
SS132	NA	NA	F7	2001-05-29	Wa	N
SS135	NA	NA	F7	2001-05-29	Wa	W
SS150	Salmonella	mbandaka	F7	2002-02-25	Wa	W
SS151	Salmonella	mbandaka	F7	2002-02-25	Wa	W
SS152	Salmonella	montevideo	F7	2002-02-25	Wa	N
SS154	Salmonella	mbandaka	F7	2002-02-25	Wa	N
SS158	Salmonella	mbandaka	F7	2002-02-25	Wa	S
SS161	Salmonella	mbandaka	F7	2002-02-25	Wa	E
SS142	Salmonella	mbandaka	F7	2002-02-25	Wa	N
SS153	Salmonella	mbandaka	F7	2002-02-25	Wa	N
SS159	Salmonella	mbandaka	F7	2002-02-25	Wa	S
SS175	Salmonella	mbandaka	F7	2002-02-25	Mu	S
SS181	Salmonella	mbandaka	F7	2002-02-25	Mu	E
SS182	Salmonella	mbandaka	F7	2002-02-25	Mu	E
SS184	Salmonella	mbandaka	F7	2002-02-25	Mu	W
SS185	Salmonella	meleagridis	F7	2002-02-25	Mu	W
NG_SS170	NA	NA	F7	2001-05-29	Wa	W
SS163	Salmonella	mbandaka	F7	2002-02-25	Wa	E
SS190	Salmonella	suspect	F7	1998-01-10	na	E
SS140	Salmonella	reading	F7	2002-02-25	Wa	N
SS145	Salmonella	reading	F7	2002-02-25	Wa	E
SS146	Salmonella	reading	F7	2002-02-25	Wa	E
SS148	Salmonella	montevideo	F7	2002-02-25	Wa	W
SS164	Salmonella	montevideo	F7	2002-02-25	Wa	W
SS165	Salmonella	montevideo	F7	2002-02-25	Wa	W
SS166	Salmonella	mbandaka	F7	2002-02-25	Wa	W
SS139	Salmonella	reading	F7	2002-02-25	Wa	N
SS147	Salmonella	reading	F7	2002-02-25	Wa	E
SS171	Salmonella	mbandaka	F7	2002-02-25	Mu	N
SS128	NA	NA	F7	2001-05-29	Mu	S
SS129	NA	NA	F7	2001-05-29	Mu	E
SS133	NA	NA	F7	2001-05-29	Wa	S
SS162	Salmonella	mbandaka	F7	2002-02-25	Wa	E
SS126	NA	NA	F7	2001-05-29	Mu	W
SS127	NA	NA	F7	2001-05-29	Mu	N
SS159	Salmonella	mbandaka	F7	2002-02-25	Wa	S
SS123	NA	NA	F7	2001-05-29	Wa	S
SS137	NA	NA	F7	2001-05-29	Wa	S
SS138	NA	NA	F7	2001-05-29	Wa	E
SS136	NA	NA	F7	2001-05-29	Wa	N
SS170	Salmonella	mbandaka	F7	2002-02-25	Mu	N
SS131	NA	NA	F7	2001-05-29	Mu	W