Utilization of Both Phenotypic and Molecular Analyses to Investigate an Outbreak of Multidrug-Resistant *Salmonella anatum* in Horses

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ABSTRACT

Phenotypic and molecular techniques, including antimicrobial susceptibility testing, plasmid analysis, and pulsed-field gel electrophoresis (PFGE) were used to characterize 15 isolates of multidrug-resistant (MDR) *Salmonella anatum* cultured during a 16 mo period from horses and a veterinary clinic environment. The isolates were resistant to multiple antimicrobial agents and could be placed into 4 groups based on their antimicrobial resistance patterns. The isolates contained multiple plasmids ranging in size from 2 to >100 kb that could be grouped into 3 different plasmid profile patterns; these patterns did not correlate with the antimicrobial resistance groupings. Furthermore, antimicrobial resistance was conjugatively transferable. Digestion of genomic DNA from the 15 isolates with 3 different restriction endonucleases, *Sfi*I, *Spe*I, and *Xba*I followed by PFGE revealed a highly conserved restriction endonuclease digestion pattern. In contrast, diverse banding patterns were observed with *S. anatum* obtained from other sources. These observations suggest that the MDR *S. anatum* isolates represent a common outbreak strain even though they possess different, albeit similar, antibiograms and plasmid profiles. The study showed that PFGE is a useful epidemiological tool for discriminating between unrelated and outbreak-related strains of *S. anatum*. In conclusion, epidemiological studies of outbreaks caused by MDR isolates of *S. anatum* should consist of both genotypic and phenotypic methods of analysis.

RÉSUMÉ

Des méthodes d'analyses phénotypique et génotypique ont été utilisées dans le but de caractériser 15 isolats de *Salmonella anatum* multi-résistants aux antibiotiques isolés sur une période de 16 mois à partir de chevaux et d'une clinique vétérinaire. Les isolats étaient résistants à une multitude d'agents antimicrobiens et pouvaient être regroupés en quatre groupes sur la base de leurs profils de résistance. Les isolats possédaient plusieurs plasmides variant en dimension de 2 à >100 kb qui pouvaient être groupés en trois patrons de profils plasmidiques différents; ces patrons n'étaient pas corrélés avec les regroupements basés sur les patrons de résistance aux antibiotiques. De plus, la résistance aux antimicrobiens étai transférable par conjugaison bactérienne. La digestion de l'ADN génomique des 15 isolats à l'aide de trois enzymes de restriction, *Sfi*I, *Spe*I et *Xba*I, suivie d'une électrophorèse en gel par champs pulsés (EGCP) a permis de démontrer un patron de digestion très conservé contrairement à ce qui était observé avec des isolats de *S. anatum* obtenus d'autres sources. Ces observations suggèrent que les isolats de *S. anatum* multi-résistants représentent une souche épidémique commune et ce, même s'ils possèdent des antibiogrammes et des profils plasmidiques différents, mais similaires. Cette étude a démontré que l'EGCP est un outil épidémiologique utile pour distinguer les isolats de *S. anatum* reliés et non-reliés à une épidémie. En conclusion, lors d'épidémies de *S. anatum* causées par des isolats multi-résistants, il serait utile d'effectuer la caractérisation des isolats à l'aide de méthodes d'analyse phénotypique et génotypique.

(Traduit par docteur Serge Messier)

INTRODUCTION

Between April 1991 and August 1992, 9 isolates of multidrug-resistant (MDR) *Salmonella anatum* were cultured from horses treated at the Veterinary Medical Teaching Hospital (VMTH) of the University of Wisconsin-Madison and at a private veterinary clinic (Clinic A). An additional 6 isolates of MDR *S. anatum* were cultured from the environment of Clinic A. The *S. anatum* isolates were resistant to ampicillin, carbenicillin, ticarcillin, tetracycline, chloramphenicol and 1 or more of the following drugs: gentamicin, tobramycin, trimethoprim/sulfamethoxazole, and cephalothin. Because of the increased incidence of MDR *S. anatum* isolations and because horses infected with species of *Salmonella* can serve as a significant reservoir of infection for both humans and animals (1,2), a potential existed for continued transmission of the MDR *S. anatum*. Therefore, it was important that an epidemiological investigation of this outbreak be conducted to determine the relationship of the MDR *S. anatum* isolates and to prevent any potential spread to other animals and humans.

Epidemiological investigations into outbreaks caused by species of *Salmonella* have typically utilized only phenotypic methods including serotyping, biotyping, phagetyping, and antimicrobial susceptibility testing to identify an outbreak strain.

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TABLE I. Characteristics of *S. anatum* isolates studied by plasmid and PFGE analysis

Isolate	Source	Origin	Geographic location[a]	Date of Isolation (mo/y)	Antimicrobial Group[b]	Plasmid pattern[c]	PFGE pattern[d]
P1	Patient	Liver	Clinic A/VMTH	4/1991	IV	2/R6	A
P2	Patient	Blood	Clinic A/VMTH	5/1991	IV	2/R6	A
E1	Environmental	Stall C6	Clinic A	5/1991	I	3/R1	C
P3	Patient	Fecal	Clinic A	5/1991	I	3/R1	C
E2	Environmental	Mineral oil	Clinic A	5/1991	II	2/R2	A
P4	Patient	Fecal	Clinic A	5/1991	II	2^+/R5	A
E3	Environmental	Stall C2C3	Clinic A	5/1991	III	2/R3	A
E4	Environmental	Stall A1	Clinic A	5/1991	III	2/R3	A
E5	Environmental	Stall A1	Clinic A	5/1991	III	3/R4	A
E6	Environmental	Stall A1	Clinic A	5/1991	III	3/R4	A
P5	Patient	Necropsy	Clinic A	5/1991	III	2/R3	A
P6	Patient	Fecal	IL/VMTH	6/1991	III	2/R3	A
P7	Patient	Fecal	IL/VMTH	6/1991	III	2/R3	A
P8	Patient	Fecal	WI/VMTH	7/1992	III	3/R4	B
P9	Patient	Jejunum	WI/VMTH	8/1992	III	2/R3	B
C1	Control	Fecal	WI/VMTH	9/1988	R	NT	D
C2	Control	Fecal	WI/VMTH	11/1989	S	NT	E
C3	Control	Colon	WI/VMTH	4/1990	S	NT	F
C4	Control	Fecal	PA				
C5	Control	Fecal	CA	10/1990	NT	NT	G
C6	Control	Fecal	TX	11/1990	NT	NT	H
C7	Control	Fecal	AR	2/1991	NT	NT	I
C8	Control	Other	FL	5/1991	NT	NT	J
C9	Control	Fecal	OK	4/1992	NT	NT	K

[a] Place of orgin: /VMTH, patient referred to VMTH; IL, Illinois; WI, Wisconsin; PA, Pennsylvania; CA, California; TX, Texas; AR, Arizona; FL, Florida; OK, Oklahoma

[b] I = resistant to tetracycline (Te), chloramphenicol (Ch), ampicillin (Am), carbenicillin (Cb), ticarcillin (Ti); II = resistant to Te, Ch, Am, Cb, Ti, gentamicin (Gm), tobramycin (Tm); III = resistant to Te, Ch, Am, Cb, Ti, Gm, Tm, timethoprim/sulfamethoxazole (SXT); IV = resistant to Te, Ch, Am, Cb, Ti, Gm, Tm, SXT, cephalothin; R = resistant to TE, Ch; S = susceptible to antibiotics tested; NT = not tested

[c] The numbers 2, 2^+, and 3 indicate the profile pattern of plasmids present: 2 = 100 and >100 kb; 2^+ = 2-, 100, and >100 kb; 3 = 55, 100, and >100 kb; NT = not tested; /R1-R6 indicate the different restriction endonuclease fragment patterns observed after digestion with *Eco*RI (Figure 3).

[d] A,B,C,D,E,F,G,H,I,J, and K refer to unique restriction fragment length polymorphism patterns observed

These methods suffer from 1 or more of the following disadvantages: they rely on phenotypes that may not be stably expressed, the necessary reagents may not be commercially available, or the sensitivity may be insufficient to discriminate between strains (3). In addition, these methods do not always distinguish between phenotypically similar, but genetically, unrelated organisms. Recent developments in molecular techniques such as plasmid analysis and genome typing have reduced the dependence on phenotypic characterization. Plasmid analysis has successfully traced epidemic strains of *Salmonella* in several outbreaks (4,5,6). However, plasmid analysis is only useful in tracing those strains that possess plasmids. Additionally, the spontaneous loss or acquisition of plasmids by a strain can affect the reliability of this technique. To overcome these limitations, genetic methods that examine the bacterial genome such as pulsed-field gel electrophoresis (PFGE) have been developed. This technique has good discriminatory power and has been used successfully in epidemiological studies of several bacterial pathogens (7,8,9,10,11). In this study, our goal was to determine the genetic relationship of the MDR *S. anatum* isolates using in addition to phenotypic methods previously presented (12), PFGE and plasmid analysis.

MATERIALS AND METHODS

BACTERIAL STRAINS

The source, year of isolation, place of origin, and antimicrobial resistance pattern group of the *S. anatum* isolates is shown in Table I. Fifteen outbreak associated isolates were cultured from patients and the environment. Nine isolates unassociated with the outbreak were included as control strains. Control strains C1-C3 were clinical strains of *S. anatum* cultured from 2 horses and a cow at the VMTH prior to the outbreak. Control strain C4 was from a horse and kindly provided by Dr. Benson of the New Bolton Center, Pennsylvania. Control strains C5-C9 and 8 additional strains not shown were all isolated from horses and kindly provided by the National Veterinary Service Laboratory, Ames, Iowa. All bacteria were cultured and identified as previously described (12). All isolates were stored at −70°C.

A spontaneous rifampicin resistant mutant of *Escherichia coli* K-12 CF1648 (F^-, Lac^+) was obtained by plating on L-agar containing 100 μg/mL of rifampicin.

PLASMID PROFILE ANALYSIS OF THE MDR *S. anatum* ISOLATES

The MDR *S. anatum* isolates were passaged twice to L-agar plates containing the appropriate antibiotics according to their antimicrobial resistance phenotype group (Table I) (12) as follows: Group I, ampicillin (32 μg/mL) and tetracycline (16 μg/mL); Group II, ampicillin (32 μg/mL), tetracycline (16 μg/mL), and gentamicin (16 μg/mL); Group III and IV, ampicillin (32 μg/mL), tetracycline (16 μg/mL), gentamicin (16 μg/mL), and trimethoprim/sulfamethoxazole (64/320 μg/mL).

Plasmid DNA was isolated using a modification of the Kado and Liu procedure (13). One cubic centimeter of

growth from each plate was emulsified in 200 μL of 1X TAE buffer (40 mM Tris acetate-1 mM Na_2-EDTA [pH 8.0]). The bacterial cells and lysing solution (3% SDS-50 mM Tris, pH 12.6) were incubated for 2 h at 56°C and extracted with an equal volume of Tris-buffered (pH 8.0) phenol and chloroform:isoamyl alcohol (24:1). Using blunt cut pipet tips, the upper aqueous phase was transferred to a microcentrifuge tube containing 2 μL of RNase, 10 mg/mL (Sigma), incubated at room temperature for 1 h, and then stored at 4°C. Plasmid DNA (90 μL) was electrophoresed through a 0.7% agarose gel in 1X TAE. Small molecular weight plasmids were barely visible by this method; therefore, an alternative method was used (14). Supercoiled plasmid DNA standards were prepared from *E. coli* V517 (15) and *S. typhimurium* LT2-Z strain χ3000 (16).

RESTRICTION ENDONUCLEASE ANALYSIS OF PLASMID DNA FROM THE MDR *S. anatum* ISOLATES

Plasmid DNA was prepared using a modification of the procedure described by Olsen (17). The isolates were removed from −70°C freezer storage, plated, lysed, and incubated in the lysing buffer as stated above for the modified Kado and Liu procedure. After incubation, 300 μL of 1.5 M potassium acetate, pH 5.2 was added and the tubes were placed on ice for 10 min followed by centrifugation at 16 000 x *g* for 10 min. The supernatant was extracted with an equal volume of Tris-buffered (pH 8.0) phenol and chloroform:isoamyl alcohol (24:1) 1:1 vol. The tubes were gently inverted 5 times and centrifuged at 16 000 x *g* for 10 min. The aqueous layer was removed with a blunt cut pipet tip. The phenol and chloroform treatment was repeated as described above. After centrifugation, the aqueous layer was transferred to a fresh microcentrifuge tube containing 700 μL of room temperature isopropanol and incubated at room temperature for a minimum of 25 min. The DNA was pelleted by centrifugation at 16 000 x *g* for 20 min. The DNA pellet was air dried overnight and resuspended in TE buffer (10 mM Tris-Cl-0.1 mM Na_2-EDTA [pH 8.0]). The resuspended DNA pellet was digested with the restriction endonuclease *Eco*RI (Gibco BRL, Gaithesberg, Maryland) according to the manufacturer's instructions and electrophoresed through a 0.7% agarose gel in 1X TAE for 4 h at 72 V. DNA was visualized by staining overnight in ethidium bromide (0.5 μg/mL) followed by destaining in distilled water.

MDR *S. anatum* PLASMID TRANSFER PROCEDURE

A spontaneous rifampicin resistant mutant of *E. coli* CF1648 (F^-, Lac^+, Rif^R) which did not contain plasmids was used as the recipient in conjugal matings with 4 rifampicin susceptible MDR *S. anatum* isolates representative of each antimicrobial resistance group (E1, E2, E5, P2). Equal volumes (1 mL) of an 18 h brain heart infusion broth (Difco) culture of the donor and recipient strains were mixed in the presence of 50 U of DNase I (Promega). The mating mixtures were incubated for 3 h at 37°C without shaking and were plated on MacConkey agar (Difco Laboratories, Detroit, Michigan) containing rifampicin (25 μg/mL) and 1 of the following antibiotics: ampicillin (100 μg/mL), carbenicillin (100 μg/mL), cephalothin (32 μg/mL), gentamicin (16 μg/mL), tetracycline (25 μg/mL), chloramphenicol (32 μg/mL), or trimethoprim/sulfamethoxazole (64/320 μg/mL). To determine if plasmid transfer could occur in the absence of intact donor cells either by transduction or the assimilation of naked DNA, matings were performed by adding equal volumes (1 mL) of the *Salmonella* donor supernatants that had been chloroform lysed and filtered through a 0.2 μM filter to recipient *E. coli* CF1648 cells in the presence and absence of 50 U of DNase I. These mixtures were incubated and plated as described above.

PULSED-FIELD GEL ELECTROPHORESIS

Genomic DNA was prepared by a modification of the procedure described by Grothous and Tümmler (18). The *S. anatum* isolates were removed from −70°C freezer storage to trypticase soy agar containing 5% sheep blood (Remel, Lenexa, Kansas) and incubated for 24 h at 36°C. The cells were harvested using a sterile cotton swab and suspended in 1 mL of SE buffer (75 mM NaCl, 25 mM Na_2-EDTA [pH 8.0]) to a turbidity resembling skim milk. 125 μL of this suspension was mixed with an equal volume of preheated 2% low melting point preparative grade agarose (Bio-Rad Laboratories, Richmond, California), transferred to a 100 μL mold (Bio-Rad Laboratories) and allowed to solidify at 4°C for 15 min. The plugs were extruded and incubated in 2 volumes of lysing solution (0.5 M Na_2-EDTA and 0.5% N-laurylsarcosine) supplemented with 1.3 mg/mL of proteinase K (Calbiochem-Novabiochem Corp, La Jolla, California) per volume of agarose plug and incubated for 48 h at 50°C with gentle shaking. The plugs were then transferred to a sterile 15 mL conical centrifuge tube and washed for 1 h at room temperature in 10 mL of TE buffer with gentle shaking. The wash solution was replaced with 2 mL of TE buffer-1 mM phenylmethylsulfonyl fluoride and incubated for 1 h at room temperature. The plugs were then washed 3 times in 10 mL TE buffer for 1 h at room temperature with gentle shaking. The agarose plugs were stored in TE buffer at 4°C for no more than 1 mo. For restriction endonuclease digestion, an 8th of a 10 mm agarose plug was placed in a microcentrifuge tube with 135 μL of sterile distilled water, 15 μL of the appropriate 10X enzyme buffer and 20 U of *Xba*I (Boehringer Mannheim, Indianapolis, Indiana), *Sfi*I (Boehringer Mannheim), or *Spe*I (New England Biolabs, Beverly, Massachusetts) and incubated overnight at 37°C with gentle shaking. The digestion reaction was replaced with 150 μL of 0.5X TBE (45 mM Tris, 45 mM boric acid and 1.0 mM Na_2-EDTA [pH 8.0]) before electrophoresis. The plugs were loaded into a 1.0% SeaKem LE agarose (FMC Bioproducts) gel prepared in 0.5X TBE running buffer. Bacteriophage lambda concatemers (Promega Corp, Madison, Wisconsin) were used as size standards. The DNA fragments were separated by PFGE using a contour-clamped homogeneous electric field apparatus (CHEF-DR III, Bio-Rad, Richmond, California). The pulse ranges for *Xba*I and *Spe*I were 15–30 s for 20 h and 1–30 s for 18 h, respectively, at 6 V/cm and a 120° angle. The pulse range for *Sfi*I was 1–15 s for 8 h and 1–30 s for 12 h at 6 V/cm and 120° angle. The gels were

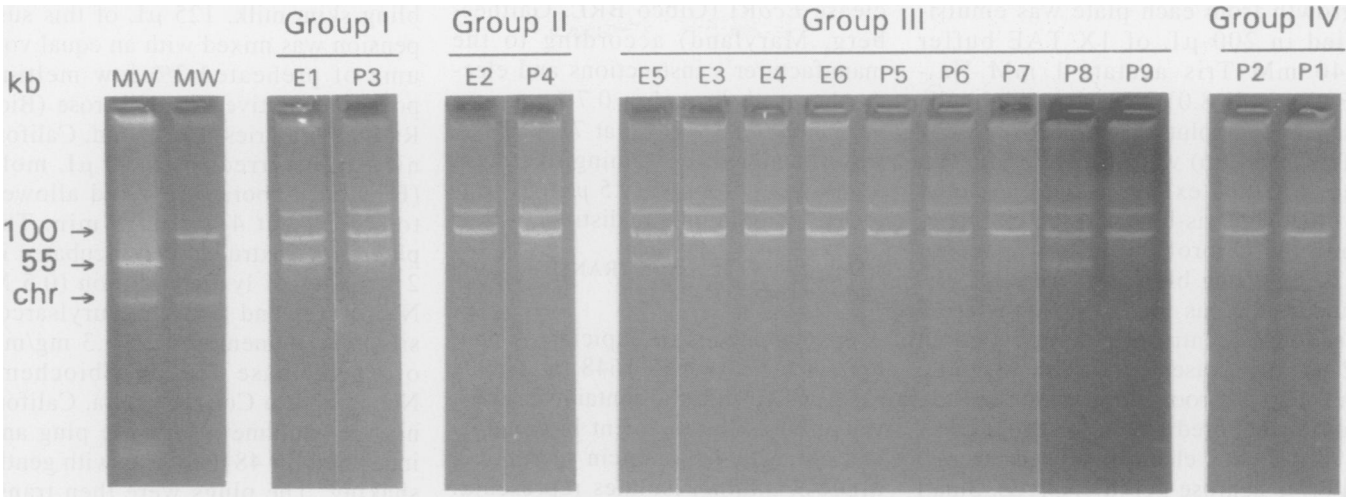

Figure 1. Agarose gel electrophoresis of purified plasmid DNA from the MDR *S. anatum*. Isolates are grouped according to antimicrobial resistance group as given in Table I. The 2 lanes on the left, labeled MW, contain supercoiled size markers derived from *E. coli* strain V517 and from *S. typhimurium* LT2-Z strain χ3000, respectively. Molecular weights (in kilobases) are indicated on the left. Contaminating chromosomal DNA is indicated by "chr."

stained in 0.5 μg/mL of ethidium bromide overnight, destained in distilled H_2O, and photographed with UV illumination. The DNA bands were sized by comparison of migration distances with those of the DNA standard. PFGE was performed in triplicate.

RESULTS

INVESTIGATION OF OUTBREAK

In the spring of 1991, MDR *S. anatum* were cultured for the 1st time from 2 septicemic foals referred to the VMTH from Clinic A. An epidemiological investigation conducted between April 1991 and August 1992 resulted in the recovery of 6 isolates of MDR *S. anatum* from horses treated at the VMTH and 9 isolates of MDR *S. anatum* from horses and various environmental sources at Clinic A. A detailed description of this outbreak investigation is published elsewhere (19). All 15 isolates of MDR *S. anatum* were resistant to ampicillin, tetracycline, chloramphenicol, ticarcillin, and carbenicillin. In addition, the isolates were separated into 4 different groups based on their resistance to gentamicin, tobramycin, cephalothin, and/or trimethoprim/sulfamethoxazole (12).

PLASMID CONTENT OF MDR *S. anatum*

To determine the number and sizes of plasmids carried by individual isolates, we analyzed the plasmid content of the MDR *S. anatum* (Table I and Fig. 1). All 15 isolates contained a 100 kb and >100 kb plasmid. In addition, the more sensitive isolates belonging to antimicrobial Group I contained a plasmid of approximately 55 kb in size. The antimicrobial Group II isolate, P4, also contained a small molecular weight plasmid, approximately 2 kb in size, which was better visualized when the isolates were analyzed by an alternative procedure (data not shown). In addition to the 100 kb and >100 kb plasmids, the antimicrobial Group III isolates E5, E6, and P8 also contained a 55 kb plasmid; therefore, these isolates possessed a plasmid profile pattern similar to that of the antimicrobial Group I isolates. Thus, the 15 isolates of MDR *S. anatum* possessed 3 different plasmid profile patterns that did not correspond to their antimicrobial resistance groupings.

To detect genetic similarities in the plasmids, polymorphisms in the size of *Eco*RI restriction fragments were studied. A total of 6 different plasmid restriction fragment patterns were observed; examples of the digestion patterns obtained are shown in Figure 2. A different restriction fragment pattern was observed for each different plasmid profile within an antimicrobial group and for each different antimicrobial group. Plasmid restriction fragment pattern, R1 was associated with the antimicrobial Group I isolates E1 and P3; these 2 isolates possessed the same antimicrobial resistance and plasmid profile.

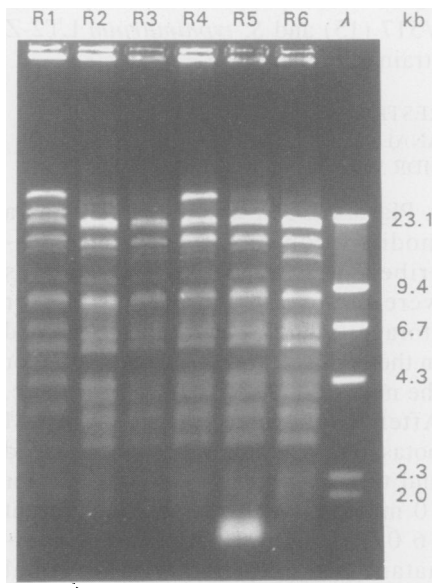

Figure 2. Agarose gel electrophoresis of *Eco*RI digested plasmid DNA from 6 representative MDR *S. anatum* isolates for each different restriction fragment pattern observed. Isolate designations are indicated above each lane: R1 = E1; R2 = E2; R3 = E3; R4 = E5; R5 = P4; R6 = P1. The lane marked λ contains bacteriophage lambda DNA digested with *Hin*dIII. The size of each fragment in kilobases is given on the right.

The antimicrobial Group II isolates E2 and P4 possessed 2 different restriction fragment patterns, R2 and R5, respectively. Restriction fragment pattern R5 of isolate P4 differed from that of R2 by an additional small plasmid that was not digested by *Eco*RI restriction endonuclease. The isolates belonging to antimicrobial Group III possessed 2 different plasmid profiles and 2 different restriction fragment

TABLE II. Summary of MDR *S. anatum* conjugative plasmid characteristics

Isolate	Antimicrobial group	Antimicrobial resistance[a]	Plasmid number /RFP group[b]	Transferred plasmid (Kb)	Antibiotic selection[a]	Resistance markers transferred[b]
E1	I	Am Cb Ti	3/R1	100, >100	Am	Am Cb Ch Te
		Ch Te		100, >100	Cb	Am Cb Ch Te
				100, >100	Ch	Am Cb Ch Te
				100	Te	Te
E2	II	Am Cb Ti	2/R2	100	Am	Am Cb Ch Te
		Ch Te		100, >100	Cb	Am Cb Ch Te
		Gm Tm		>100	Ch	Am Cb Ch Te
				>100	Te	Am Cb Ch Te
				100	Gm	Am Cb Gm
E5	III	Am Cb Ti	3/R4	>100	Am	Am Cb Ch Te Sxt
		Ch Te		>100	Cb	Am Cb Ch Te Sxt
		Gm Tm		>100	Ch	Am Cb Ch Te Gm Sxt
		Sxt		>100	Te	Am Cb Ch Te Gm Sxt
				>100	Gm	Am Cb Ch Te Gm Sxt
				55, >100	Sxt	Am Cb Ch Te Gm Sxt
P2	IV	Am Cb Ti	2/R6	100, >100	Am	Am Cb Ch Te Gm Sxt
		Ch Te		100, >100	Cb	Am Ac Ch Te Gm Sxt
		Gm Tm		>100	Ch	Am Cb Ch Te Gm Sxt
		Sxt		>100	Te	Am Cb Ch Te Gm Sxt
		Cf		100, >100	Gm	Am Cb Ch Te Gm Sxt
				100, >100	Sxt	Am Cb Ch Te Gm Sxt

[a] Am, ampicillin; Ch, chloramphenicol; Cb, carbenicillin; Ti, ticarcillin; Te, tetracycline; Gm, gentamicin; Tb, tobramycin; Sxt, trimethoprim/sulfamethoxazole; Cf, cephalothin

[b] Plasmid number indicates the plasmid content of the isolate: 2 = 100 and >100 kb plasmids; 3 = 55, 100, and >100 kb plasmids; RFP group refers to the plasmid DNA restriction fragment pattern shown in Figure 3

patterns. Isolates E5, E6 and P8 contained an additional 55 kb plasmid and had restriction fragment pattern R4 compared to isolates E3, E4, P5, P6, P7, and P9 which had restriction fragment pattern R3. The 2 antimicrobial Group IV isolates, P1 and P2, shared the same plasmid profile and the same restriction fragment pattern, R6. Therefore, 6 unique restriction fragment patterns were observed for the 15 isolates of MDR *S. anatum*. However, the majority of the plasmid restriction fragments were shared indicating that a high degree of genetic similarity exists among the plasmids.

TRANSFER OF ANTIMICROBIAL RESISTANCE MARKERS AND PLASMIDS

We evaluated whether the plasmids associated with a representative MDR *S. anatum* isolate from each antimicrobial group could be transferred to another organism and by which mechanism the transfer occurred. Each of the 4 representative MDR *S. anatum* isolates (E1, E2, E5, and P2) was able to transfer antimicrobial resistance to *E. coli* CF1648 (Table II). The mechanism of transfer appeared to be conjugation because the transfer of antimicrobial resistance was resistant to treatment with DNAase I and did not occur in the absence of direct cell-to-cell contact. To determine which antimicrobial resistance markers were acquired by each transconjugate, we plated each transconjugate on MacConkey agar containing each of the 7 different antimicrobial agents mentioned previously in Materials and Methods. Except for cephalothin resistance, all antimicrobial resistance markers were transferred.

The plasmid content of the *E. coli* transconjugates was characterized to determine which antimicrobial resistance marker(s) were associated with a particular plasmid (Table II). Isolate E1 contained 3 plasmids of approximately 55, 100, and >100 kb in size. Both the 100 and >100 kb plasmids were transferred to the *E. coli* recipient strain; the 55 kb plasmid was not transferred. The 100 kb plasmid possessed resistance to tetracycline. The >100 kb plasmid was co-transferred with the 100 kb plasmid and together they had an associated phenotype of resistance to ampicillin, carbenicillin, chloramphenicol, and tetracycline. Transfer of a single >100 kb plasmid was not obtained and therefore, the resistance phenotype of this plasmid could not be determined. Isolate E2 of antimicrobial Group II possessed 2 plasmids of approximately 100 and >100 kb both of which were transferable to the *E. coli* recipient strain. The >100 kb plasmid possessed resistance to ampicillin, carbenicillin, chloramphenicol, and tetracycline. However, 2 different resistance phenotypes were associated with the transfer of a 100 kb plasmid; resistance to ampicillin, carbenicillin, chloramphenicol, and tetracycline or resistance to ampicillin, carbenicillin, and gentamicin. Isolate E5 of antimicrobial Group III possessed 3 plasmids of approximately 55, 100, and >100 kb in size. Only the 55 and >100 kb plasmids were transferred to the recipient *E. coli* strain. Two different resistance phenotypes were associated with the >100 kb plasmid; resistance to ampicillin, carbenicillin, chloramphenicol, tetracycline, and trimethoprim/sulfamethoxazole or resistance to these same agents plus gentamicin. The 55 kb plasmid was only transferred with the >100 kb plasmid; therefore, its associated antimicrobial resistance could not be determined. Antimicrobial Group IV isolate P2 contained 2 plasmids of approximately 100 and >100 kb in size which were both transferred to the *E. coli* recipient strain. Resistance to ampicillin, carbenicillin, chloramphenicol, tetracycline, gentamicin, and trimethoprim/sulfamethoxazole was associated with the >100 kb plasmid. Because the 100 kb plasmid of isolate P2 co-transferred with the >100 kb plasmid, determination of its associated antibiotic resistance could not be made.

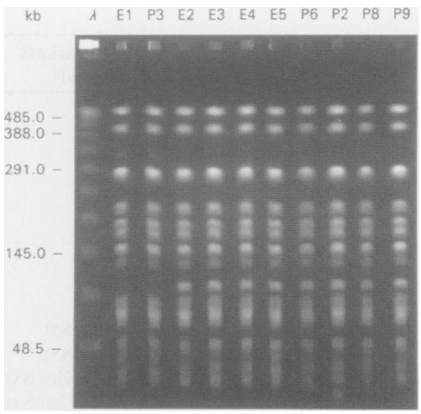

Figure 3. PFGE of *Spe*I-digested genomic DNA of selected MDR *S. anatum* isolates. Isolate designations are indicated above each lane. The lane marked λ contains oligomers of bacteriophage lambda DNA. Molecular weights (in kilobases) are indicated on the far left.

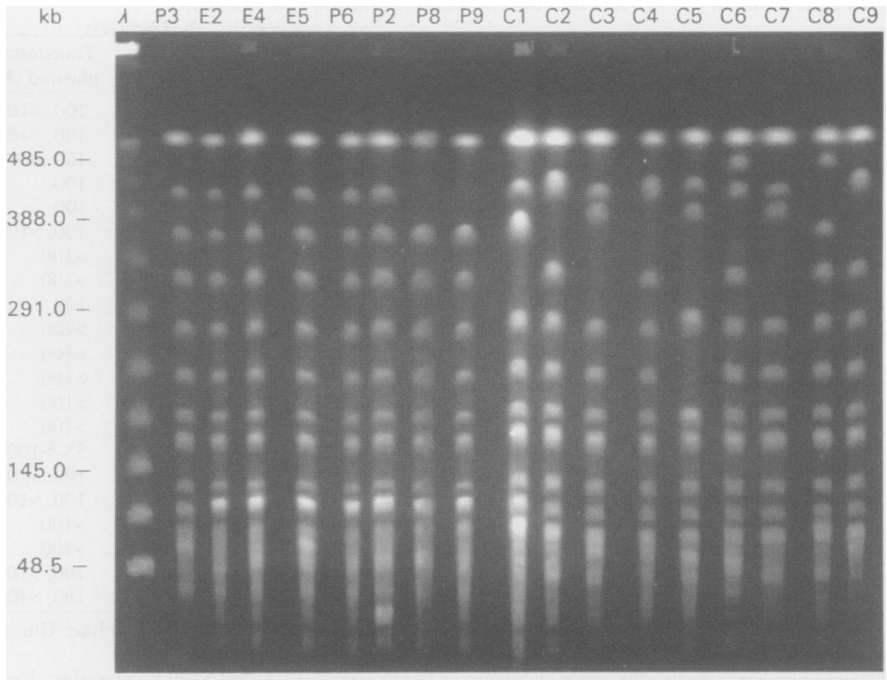

Figure 4. PFGE of *Xba*I-digested genomic DNA of selected MDR *S. anatum* isolates and selected unrelated control isolates. Isolate designations are indicated above each lane. Lane marked λ contains oligomers of bacteriophage lambda DNA. Molecular weights (in kilobases) are indicated on the far left.

PULSED-FIELD GEL ELECTROPHORESIS

To determine the chromosomal relatedness of the MDR *S. anatum* isolates, PFGE patterns after DNA digestion by *Spe*I, *Xba*I, and *Sfi*I were compared. An identical restriction pattern for all 15 isolates was generated by PFGE of *Sfi*I-digested fragments of genomic MDR *S. anatum* DNA (data not shown). A common PFGE pattern following *Spe*I digestion was also observed. Examples of the PFGE patterns obtained by digestion of DNA with *Spe*I are shown in Figure 3. With *Spe*I, 13 of the 15 isolates possessed a PFGE pattern consisting of 11 bands ranging in size from 48 to 485 kb. However, isolates E1 and P3, the least resistant isolates belonging to antimicrobial Group I, were missing a band of approximately 100 kb. All 15 isolates were also subjected to *Xba*I digestion; examples of the digestion patterns obtained are shown in Figure 4. A shared PFGE pattern with bands ranging in size from 100 to 400 kb is shown for 6 representative isolates (P3, E2, E4, E5, P6, and P2) in Figure 4. Seven additional isolates (P1, E1, P4, E3, P5, P6, and P7) shared this same pattern (data not shown). A minor difference, absence of a 400 kb band, is present for isolates P8 and P9; these isolates were cultured 1 y after the initial isolation of MDR *S. anatum* at the VMTH. The outbreak associated MDR *S. anatum* isolates were also compared to 17 epidemiologically unrelated control strains of *S. anatum*. Seventeen different PFGE patterns compared to the outbreak associated isolates were observed and examples of 9 of the control isolates are shown in Figure 4. The PFGE patterns, generated by digestion with *Xba*I, produced for control strains C1-C3 (cultured prior to 1991 at the VMTH), for control strains C4-C9 (isolated from different geographic locations throughout the United States during 1991–1992), and for an additional 8 control strains (isolated from different geographic locations throughout the United States during 1991-1993) (data not shown) were different from those seen with the outbreak associated MDR *S. anatum* isolates P3-P9. However, the differences in the *Xba*I PFGE pattern observed for control strain C1 (absence of a 300 kb band and a more intense approximately 90 kb band) compared to the PFGE pattern of the outbreak associated isolates appeared to be minor. Therefore, control strain C1 was subjected to PFGE following *Spe*I digestion. Three major differences were observed in the PFGE pattern of control strain C1 (absence of a band > 485 kb and 90 kb and presence of an additional 388 kb band) compared to the *Spe*I PFGE pattern of the outbreak associated isolates (data not shown).

DISCUSSION

The recent outbreak of MDR *S. anatum* at the VMTH caused much concern, particularly because of the MDR associated with the isolates. Prior to this outbreak, MDR *S. anatum* had not been cultured at the VMTH. Efficient containment and prevention of such outbreaks depends on our ability to identify their source(s) and their mechanism of transmission. This information is obtained from epidemiological studies. Frequently, epidemiological studies of *Salmonella* transmission have relied on the characterization of phenotypic traits such as serotype, phagetype, and antimicrobial resistance. Serotyping and phagetyping are only successful when an outbreak is caused by a rare or unusual serotype or phagetype, respectively (20). Antimicrobial resistance detection may also be insufficient at discriminating accurately between strains and may yield misleading information when resistance to a particular antibiotic or to several antibiotics is conferred by genetically different plasmids which together produce the same phenotypic resistance pattern in chromosomally unrelated strains (20). Therefore, due to the inability of

phenotypic typing techniques to discriminate between phenotypically identical but genotypically different strains, DNA-based typing methods are being used more frequently for epidemiological investigations. DNA-based typing methods such as determination of plasmid profiles, fingerprinting of chromosomal genotypes by IS200 analysis, and PFGE analysis have successfully been used for investigations of *Salmonella* outbreaks (20,21,22,23,24). In our study, we found that both phenotypic analysis, such as antimicrobial susceptibility testing, and molecular typing, including plasmid analysis and PFGE of genomic DNA, were necessary to determine the epidemiological relationship of the MDR *S. anatum* isolates obtained during this outbreak.

Plasmid analysis of the 15 MDR *S. anatum* identified 3 different plasmid content patterns; these patterns did not correspond to the 4 antimicrobial resistance patterns (Table I). This is in contrast to several studies which have demonstrated that plasmid profile analysis alone or in combination with phagetyping and antimicrobial susceptibility testing can sufficiently identify related or unrelated strains from outbreaks caused by species of *Salmonella* (4,21,25,26,27,28). In addition, restriction analysis of the MDR *S. anatum* plasmid DNA also identified differences among the plasmids. Six unique plasmid restriction fragment patterns were observed for the 15 MDR *S. anatum* isolates. These different patterns can be attributed to differences in the number of plasmids carried by each isolate in each antimicrobial group and to differences in the antimicrobial resistance markers carried on each plasmid. For example, the antimicrobial Group II isolates E2 and P4 have the same antimicrobial resistance pattern but contain a different number of plasmids; isolate P4 contains an additional approximately 2 kb plasmid, which accounts for the 2 different plasmid restriction fragment patterns R2 and R5 (Table I). In addition, antimicrobial Group I isolates E1 and P3 have the same plasmid content as the antimicrobial Group III isolates E5, E6, and P8. However, these 2 groups have 2 different plasmid restriction fragment patterns R1 and R3, respectively and are resistant to different antimicrobial agents; isolates E5, E6, and P8 are also resistant to gentamicin, tobramycin and trimethoprim/sulfamethoxazole. Therefore, the possession of these additional antimicrobial resistance markers may account for the different plasmid restriction fragment patterns R1 and R3, possessed by the antimicrobial Group I isolates E1 and P3 and the antimicrobial Group III isolates E5, E6, and P8, respectively. Utilization of only the plasmid analysis and previously presented phenotypic data would have indicated that 6 different strains of MDR *S. anatum* were associated with the outbreak (Table I).

We also observed variability in the antimicrobial resistance carried by each conjugatively transferred plasmid. For example, 2 different resistance phenotypes were associated with the 100 kb plasmid of isolate E2 (antimicrobial Group II) and with the >100 kb plasmid of isolate E5 (antimicrobial Group III). These differences could be due to the presence of 2 different 100 or >100 kb plasmids in isolates E2 and E5, respectively. However, such differences should be detected by plasmid restriction analysis. We detected only minor differences when the plasmids were subjected to *Eco*RI restriction analysis indicating that significant portions of these plasmids were similar. Alternatively, the differences in the antimicrobial resistance markers transferred could be a result of acquisition or loss of specific antimicrobial resistance markers by a plasmid during the conjugation process.

The variability detected in the plasmid content may have been a result of the intense antibiotic use at Clinic A (19). Schwalbe et al (29) identified in vivo acquisition of resistance by *S. typhi* and commensal intestinal flora after antibiotic treatment. Therefore, the extensive use of penicillin, gentamicin, tetracycline, amikacin, chloramphenicol and ceftiofur by clinicians at Clinic A may have selected for drug-resistant strains of *S. anatum* as well as other commensal enteric bacteria in patients. This speculation can be made because of the presence of MDR, multiple high molecular weight plasmids ranging in size from 55 kb to > 100 kb, and numerous smaller plasmids in *C. freundii* (4 isolates), *E. cloacae* (5 isolates), and *E. coli* (3 isolates) cultured from the Clinic A environment (data not shown). In addition, the isolates of MDR *S. anatum* cultured from the horses were resistant to more antimicrobial agents than the environmental isolates of MDR *S. anatum* suggesting that acquisition of these additional resistance markers may have occurred in vivo from the commensal MDR enteric flora. This observation is further substantiated by the isolation, from the feces of the same horse at Clinic A, of the antimicrobial Group I *S. anatum* isolate P3 and an *E. coli* isolate that had the same resistance pattern as the most resistant Group IV *S. anatum* isolates (data not shown).

We used PFGE to determine whether the 15 MDR *S. anatum* isolates represented the same chromosomal outbreak strain. Recently, PFGE of large restriction fragments has demonstrated its usefulness as an excellent technique for discriminating chromosomal differences between related and unrelated strains of *Salmonella* (8,11). We observed 3 different PFGE patterns for the 15 isolates of MDR *S. anatum* (Table I), however, these differences were attributed to the absence of only a single band for isolates E1 and P3 digested with *Spe*I and for isolates P8 and P9 digested with *Xba*I. A single band difference consistent with a single genetic event (e.g. a point mutation resulting in the loss or gain of a restriction site, an insertion, a deletion, or a chromosomal inversion) is not a reliable basis for concluding that 2 isolates that are epidemiologically linked represent different strains (30). In fact, increasingly discriminatory techniques are, by definition, able to detect smaller and less frequent variation (31). Therefore, we conclude that all 15 MDR *S. anatum* isolates represent a single chromosomally related strain that have a distinct PFGE pattern which is different from the patterns obtained from the epidemiologically unrelated control strains of *S. anatum* (Table I).

Ease of operation, speed, cost, the discriminatory power, and reproducibility of a method are all factors that need to be considered when choosing a suitable typing technique for an epidemiological investigation (31). Phagetyping, antimicrobial susceptibility patterns and plasmid

profile analysis are simple, inexpensive and, in certain situations, have good discriminatory power (15,21). In addition, It appears that PFGE used as a sole technique is rapidly becoming the gold standard for epidemiological investigations. However, the findings of the present study demonstrated the necessity to include both phenotypic analysis, plasmid analysis, and a genomic technique, such as PFGE, which could identify chromosomal differences to determine the epidemiology of the MDR outbreak associated isolates. The antimicrobial susceptibility testing and plasmid analysis suggested that the outbreak was caused by multiple strains of *S. anatum* each with a different pattern of antimicrobial resistance and plasmid content; however, PFGE suggested that the outbreak was caused by a single chromosomal strain, but did not provide any information about plasmids or antimicrobial resistance. Thus, all 3 approaches were necessary to establish that the outbreak was caused by a single strain containing 1 or more related plasmids carrying various combinations of antimicrobial resistance markers. Therefore, the present study illustrates the necessity to use a combination of phenotypic and molecular methods to fully determine and understand the mechanism of an outbreak caused by a MDR species of *Salmonella*.

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