

## Phenotypic and Genotypic Changes in *Salmonella enterica* subsp. *enterica* Serotype Typhimurium during Passage in Intestines of Broiler Chickens Fed on Diets That Included Ionophore Anticoccidial Supplements

Salvatore Scalzo,<sup>1</sup> John E. Corkill,<sup>2</sup> David J. Shanks,<sup>1</sup> Timothy G. Rowan,<sup>1\*</sup> José Delaval,<sup>3</sup> Andrew Fleetwood,<sup>1</sup> Martin Murphy,<sup>4</sup> and C. Anthony Hart<sup>2</sup>

Veterinary Medicine Research and Development, Pfizer Animal Health, Sandwich, Kent CT13 9NJ,<sup>1</sup> and Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool, Liverpool, Merseyside L69 3GA,<sup>2</sup> United Kingdom; RBK House, Irishtown, Athlone, County Westmeath, Ireland<sup>4</sup>; and Laboratoire de Touraine, Parçay-Meslay, 37023 Tours, France<sup>3</sup>

Received 1 December 2003/Returned for modification 5 March 2004/Accepted 16 April 2004

**The effect of continuous in-feed administration of anticoccidial agents on antimicrobial sensitivity and the level of bacterial shedding in poultry experimentally infected with *Salmonella enterica* subsp. *enterica* serotype Typhimurium definitive type 104 (DT104) were investigated. On day 0, 1,200 1-day-old *Salmonella*-free broiler chicks were placed into 50 pens, and the pens were randomly allocated to one of five treatments: nonsupplemented (negative control; T1), monensin at 120 mg/kg of diet (T2), salinomycin at 60 mg/kg of diet (T3), semduramicin at 20 mg/kg of diet (T4), or semduramicin at 25 mg/kg of diet (T5). Each bird was inoculated with a well-characterized strain of serotype Typhimurium DT104 on day 10. On day 49, the birds were euthanatized humanely. Bulk fecal samples were collected on days 13, 43, and 48 and were examined for organisms which had acquired resistance. The genetic basis of acquired resistance was determined from representative samples of isolates. Of 784 *Salmonella*-selective plates supplemented with antimicrobial agents, only 33 showed growth. These isolates came from all treatment regimens, including the nonsupplemented control. A number of phenotypic changes were observed; these included changes in motility, phage type, and agglutination properties. Supplementation of the diet with an anticoccidial drug does not appear to affect antimicrobial resistance or the level of excretion of salmonellae. Most of the changes observed do not seem to be related to the presence of a supplement in feed. Salmonellae appear to be capable of acquiring antimicrobial resistance and phenotypic changes independently of specific antimicrobial selection pressures.**

The increase in antimicrobial resistance reported for human pathogens and the forecast by some commentators that a post-antibiotic era is on the horizon (4) have greatly stimulated research into the origins of antimicrobial resistance and into the possible factors that are likely to affect the prevalence and distribution of antimicrobial resistance genes in human pathogens. Some investigators have suggested that pathogenic or commensal bacteria in animals might acquire antimicrobial resistance genes and then either directly infect humans (zoonotic pathogens) or transmit their resistance genes to human pathogens (29).

It is commonly accepted that in the presence of selective pressure (for example, treatment with antimicrobial agents), *Salmonella enterica* easily acquires antimicrobial resistance (11, 26), probably through the acquisition or exchange of plasmids with other *Enterobacteriaceae* within the intestinal lumen. Selective pressure has also been included in the “drug resistance equation,” which states that the resistance problem is the result of the selective pressure (amount and time of antimicrobial use in a defined area) and the prevalence of resistance

traits and resistance genes against the drug under consideration (15).

Among the salmonellae capable of causing human disease, *S. enterica* subsp. *enterica* serotype Typhimurium is well known for acquiring antimicrobial resistance, especially phage type 104 (definitive type 104 [DT104]) (19, 20, 26, 27). Human infection with serotype Typhimurium DT104 can be acquired from cattle, pigs, poultry, and other animals. Infection of humans usually occurs through ingestion of insufficiently cooked meat and predominantly causes diarrheal disease (salmonellosis). However, extra-intestinal salmonellosis is not rare in Africa (7). In one study, serotype Typhimurium could be isolated from the blood of febrile children more commonly than *Streptococcus pneumoniae* (14). In poultry, serotype Typhimurium is responsible for various disease manifestations, usually in the form of mild gastroenteritis with low mortality, but it can cause septicemia with high mortality.

Ionophore anticoccidial agents are chemicals that have antimicrobial properties and that are used in poultry for the prevention of coccidiosis, a ubiquitous and economically important disease caused by protozoa of the family *Eimeriidae*. Anticoccidial agents are continuously administered to chickens from the day after hatching until the beginning of the withdrawal period (i.e., a period preceding slaughter during which these compounds are not included in the diet). Ionophore

\* Corresponding author. Mailing address: Veterinary Medicine Research and Development, Pfizer Animal Health, IPC 896, Ramsgate Rd., Sandwich, Kent CT13 9NJ, United Kingdom. Phone: 44 1304 646504. Fax: 44 1304 656257. E-mail: tim.rowan@pfizer.com.

anticoccidial agents (e.g., monensin, salinomycin, and semduramicin) are thought to act by disrupting cationic cross-membrane gradients (17) by interfering with the ion transport system ( $K^+$ ,  $Na^+$ ,  $Mg^{2+}$ , and  $Ca^{2+}$ ) and arresting the development of coccidia early in their life cycle, before the host-cell-damaging stages are reached.

In this study, we investigated the possible role of continuous in-feed administration of ionophore anticoccidial agents on the acquisition of an antimicrobial resistance phenotype and resistance genes by serotype Typhimurium. A DT104 strain of avian origin was used. This strain had the same phage type as an important zoonotic *Salmonella* strain that caused epidemics during the 1990s. This strain, although able to colonize chickens and cause disease like other DT104 strains, was unusual in that it did not carry a multiresistance phenotype and was sensitive to most tested antimicrobial agents. The use of such a sensitive strain should facilitate the assessment of the acquisition of antimicrobial resistance.

#### MATERIALS AND METHODS

**Experimental design and procedure.** On day 0, 1,200 1-day-old *Salmonella*-free broiler chicks were placed into 50 pens within a new purposely built unit, and the pens were randomly allocated to one of five diets with or without anticoccidial supplement: nonsupplemented diet (negative control; T1), monensin at 120 mg/kg of diet (T2), salinomycin at 60 mg/kg of diet (T3), semduramicin at 20 mg/kg of diet (T4), or semduramicin at 25 mg/kg of diet (T5). The birds were housed in floor pens with internal dimensions of 2 by 1.2 by 2.3 m (length by width by height) and accommodating 24 birds. Ten pens (240 birds) were allotted to each experimental treatment. Birds in groups T2, T3, T4, and T5 were given supplemented diets from day 0 until day 44, at which time dietary supplements were withdrawn and all birds were maintained with nonsupplemented diet until day 49 in order to simulate field conditions in commercial practice. Birds in group T1 received the nonsupplemented diet throughout the study. All birds were euthanized humanely on day 49.

The birds were provided from a *Salmonella*-free hatchery and, upon arrival at the study site, approximately 10 chicks from each batch were sent to a laboratory and shown to be negative for *Salmonella* carriage. Extremely strict environmental and hygienic measures were implemented throughout the study to prevent any cross-contamination between pens (details are given in the Appendix). Prior to administration, all food was sterilized by irradiation. Postirradiation feed analysis confirmed that each of the ionophores was present at the expected level in each diet. Chicks were given drinking water that was suitable for human consumption and that also had been sterilized by UV light treatment. No drugs, other than the anticoccidial dietary supplements described above, including therapeutic or subtherapeutic antimicrobial agents, were given. Wood shavings used as litter were sterilized and were pesticide free. All animal welfare requirements were strictly observed.

On day 10, a cloacal swab sample was collected from each bird and cultured to confirm the absence of *Salmonella*. In addition, bulk fecal samples were collected from each pen and cultured to show the presence or absence of *Salmonella*. After collection of the samples, each bird was inoculated with serotype Typhimurium DT104 (approximately  $10^7$  CFU per bird) by oral gavage. Following challenge, the acquisition of antimicrobial resistance was assessed through screening of bulk fecal samples and confirmation that the isolated strain was identical to the inoculated strain by serotyping, phage typing, and pulsed-field gel electrophoresis (PFGE) of macrorestricted chromosomal DNA.

For the experimental challenge, we used a recent field isolate of serotype Typhimurium DT104 of avian origin, kindly provided by C. Wray and R. Davis, Central Veterinary Laboratory, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, United Kingdom.

**Preparation and administration of the infective inoculum.** The *Salmonella* inoculum was prepared from an overnight culture in tryptone soy broth which had been inoculated with a single colony. After centrifugation, the bacterial pellet was resuspended in sterile phosphate-buffered saline (pH 7.2) and diluted as appropriate with phosphate-buffered saline (pH 7.2) to a concentration of approximately  $2 \times 10^7$  CFU/ml, as determined by nephelometry. Birds were inoculated with 0.5 ml of the bacterial suspension, thus ensuring that each bird received approximately  $10^7$  CFU of serotype Typhimurium. The dose of the

inoculum was confirmed by viability counts undertaken on the same day as the challenge by making serial decimal dilutions from aliquots of the bacterial suspension used to challenge the birds. Additional aliquots of the inoculum were used for MIC determinations and for PFGE. The MIC and PFGE profiles of the inoculum were used for comparing the strains isolated after challenge.

**Enumeration of CFU and screening for acquisition of resistance.** Postchallenge bulk fecal samples were collected on days 13, 43, and 48, diluted 1:10 in buffered peptone water, and sedimented or slightly centrifuged. The supernatant, corresponding to a dilution of  $10^{-1}$ , was used both for enumeration of CFU and for screening for acquisition of resistance.

(i) **Collection of fecal samples.** Bulk fecal samples were collected by placing on the floor of each pen a plastic sheet covering approximately 90% of the surface of each pen for 2 h on the day of sample collection. Fresh feces were collected from the plastic sheet, mixed, and weighed; samples of at least 5 g of feces were placed in sterile containers and immediately sent to the laboratory under refrigeration. Care was taken to ensure that each sample reached the laboratory within 24 h of sample collection.

(ii) **Enumeration of CFU.** An aliquot of the supernatant was used to prepare serial decimal dilutions from  $10^{-2}$  to  $10^{-10}$ . The dilutions were first incubated for 18 to 24 h at  $37^\circ\text{C} \pm 1^\circ\text{C}$ . A 100- $\mu\text{l}$  aliquot of each dilution was plated in duplicate on motility-selective Rappaport-Vassiliades plates, incubated at  $42^\circ\text{C} \pm 1^\circ\text{C}$ , and subcultured on brilliant green agar. The highest dilution that showed growth was recorded as the result.

(iii) **Screening for acquisition of resistance.** The  $10^{-1}$  dilution from samples collected on days 13 and 48 was used for screening for the acquisition of resistance to *Salmonella*. A 2-ml aliquot of the  $10^{-1}$  dilution was inoculated into 20 ml of Rappaport-Vassiliades enrichment broth and incubated for 18 to 24 h at  $42^\circ\text{C} \pm 1^\circ\text{C}$ . After incubation, plates of Rambach agar each supplemented with one antimicrobial agent (i.e., ciprofloxacin, trimethoprim-sulfamethoxazole, cefotaxime, amoxicillin-clavulanic acid, gentamicin, amoxicillin, nalidixic acid, or tetracycline) at four times the MIC for the challenge bacterium before infection or at four times the lowest tested concentration each were inoculated with 100  $\mu\text{l}$  of enrichment broth and incubated again. Any growth on the supplemented plates was recorded.

(iv) **Confirmation of identification.** Representative colonies growing on supplemented plates were further characterized by biochemical tests (indole test, urease test, triple sugar iron agar slope, lysine decarboxylase test, Voges-Proskauer test, and/or API ID32, as appropriate), serotyping, MIC determination in liquid medium, phage typing, and PFGE analysis to confirm that the isolates were identical to the inoculum (10, 25). In addition, representative samples of the isolates were analyzed to determine the genetic nature of the acquired resistance.

(v) **Characterization of bacterial resistance.** Isolates identified at the diagnostic laboratory were sent to the laboratory of the Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool, Liverpool, United Kingdom, for confirmation that they were identical to the challenge strain by genomic profiling. Samples of the *Salmonella* isolates that were confirmed as being indistinguishable from the inoculum and that showed an antimicrobial resistance shift were analyzed for plasmid carriage by an alkaline lysis technique and for transfer of resistance by conjugation. *Escherichia coli* K-12 (Nal<sup>r</sup> Amp<sup>s</sup> Tet<sup>s</sup> Gen<sup>s</sup>) was used as the recipient strain for conjugation. The aminoglycoside resistance mechanism was determined by using a battery of antimicrobial disks containing gentamicin, netilmicin, tobramycin, amikacin, apramycin, kanamycin, and neomycin and by measuring the inhibition zones around each of the disks.  $\beta$ -Lactamase activity was determined by isoelectric focusing of enzymes from cell-free sonicates (5).

(vi) **PFGE.** Bacteria were harvested from solid media and embedded in lysozyme-containing agarose (Sigma). Lysis was achieved with a bile detergent buffer, and nucleases were neutralized with proteinase K (Sigma). Chromosomal DNA was initially digested with restriction endonuclease XbaI (40 U per block), and the digestion fragments were separated by PFGE (CHEF 3D; Bio-Rad Ltd.). Electrophoretic parameters were as follows: initial switch time, 3.0 s, with a linear increase to 30.0 s after 20 h; buffer temperature,  $14^\circ\text{C}$ ; 6 V/cm; and pulsing angle,  $120^\circ$ . In addition to XbaI digestion, chromosomal DNA was also digested separately with restriction enzyme SpeI, and the fragments were analyzed with an initial switch time of 5 s increasing to 33 s. Gels were stained with ethidium bromide and photographed under UV light. Clonal relatedness was determined according to the criteria of Tenover et al. (25), which postulate that related strains should not differ by more than three fragment bands.

**Statistical analyses.** An individual pen was taken as the experimental unit for all statistical analyses. For each antimicrobial agent and each organism, the proportion of pens in which isolates showed a change in the MIC of four times or greater than the MIC for the inoculum was determined, and Fisher's exact test

TABLE 1. Levels of shedding of *Salmonella* in bulk fecal samples

Treatment	CFU/g of feces <sup>a</sup> on day:		
	13	43	48
T1	$3.2 \times 10^8$	$6.3 \times 10^7$	$5.0 \times 10^8$
T2	$2.0 \times 10^8$	$7.9 \times 10^7$	$2.0 \times 10^8$
T3	$5.0 \times 10^8$	$2.5 \times 10^8$	$1.0 \times 10^9$
T4	$3.2 \times 10^9$	$7.9 \times 10^8$	$1.0 \times 10^8$
T5	$3.0 \times 10^8$	$1.8 \times 10^9$	$6.5 \times 10^8$

<sup>a</sup> Estimated geometric mean CFU per gram of feces in all pens for each treatment.

was used to determine whether or not this proportion had been affected by treatment. For the concentration of *Salmonella* in bulk fecal samples, the log dilution on days 13, 43, and 48 was analyzed by using a mixed linear model for repeated measurements. The model included fixed effects for treatment, day, and their interaction and random effects for block and pen. The 5% level of significance ( $P \leq 0.05$ ) was used.

## RESULTS

**Level of shedding.** Enumeration of CFU in bulk fecal samples collected on days 13, 43, and 48 of the study was carried out. Levels of shedding in feces of birds in different treatment groups were measured by estimating the number of CFU per gram of feces from the last dilution showing the growth of *Salmonella*. Levels of shedding ranged from  $10^6$  (T4; semduramicin at 20 mg) to  $10^{12}$  (T1; nonsupplemented control). Geometric means ranged from  $6.3 \times 10^7$  (T1; nonsupplemented control; day 43) to  $3.2 \times 10^9$  (T4; semduramicin; day 13) (Table 1).

Analysis of variance for a total of 147 bulk fecal samples on days 13, 43, and 48 showed that there were no significant differences in the concentrations of serotype Typhimurium excreted between treatments ( $P = 0.2972$ ).

**Acquisition of resistance.** The inoculated serotype Typhimurium was fully sensitive to the eight tested antimicrobial agents (with MICs of 1 mg of gentamicin/liter, 2 mg of tetracycline/liter, and <1 mg of amoxicillin/liter) (Table 2). *Salmonella* colonies were confirmed in 33 out of 784 examined antimicrobial agent-supplemented plates containing bulk fecal samples originating from 22 pens; 1 of the plates contained amoxicillin, 2 contained tetracycline, and the remainder con-

TABLE 2. Growth of *Salmonella* from bulk fecal samples on plates containing selected antimicrobial agents

Antimicrobial agent on plate <sup>a</sup>	No. of plates showing growth <sup>b</sup>	Original MIC ( $\mu\text{g/ml}$ )
CIP	0	<0.5
SXT	0	<0.5/9.5
CTX	0	<2
AMC	0	<2/1
GEN	30	1
AMX	1	<1
NAL	0	4
TET	2	2

<sup>a</sup> Used at a concentration corresponding to four times the MIC before inoculation. CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; CTX, cefotaxime; AMC, amoxicillin-clavulanic acid; GEN, gentamicin; AMX, amoxicillin; NAL, nalidixic acid; TET, tetracycline.

<sup>b</sup> Out of 98 tested.

tained gentamicin. No growth was observed on plates that had been supplemented with any of the other antimicrobial agents, indicating that the acquisition of resistance to antimicrobial agents had occurred only for gentamicin, tetracycline, and amoxicillin (Table 3). The pens from which samples yielding positive results on plates originated were distributed similarly across all treatment groups. The isolates came from samples collected on day 13 ( $n = 10$ ) and on day 48 ( $n = 23$ ) (Table 3).

Analysis of the MIC profiles of the isolates showed an increase in the MIC of gentamicin, and in most instances, this increase was also associated with an increase in the MIC of tetracycline. According to the magnitude of the increase for gentamicin, the isolates were classified into two groups: high-level resistance (MIC of 16 mg/liter or higher) and low-level resistance (MIC of 4 mg/liter or lower). Representatives of each of these two groups were further characterized by biochemical analysis of the enzymes involved and by conjugation experiments.

The enzyme conferring high-level gentamicin resistance was shown to be a phenotype consistent with AAC(3)-1, which was able to inactivate only gentamicin and closely related antimicrobial agents (astromycin and sisomicin) (23, 24) and did not confer cross-resistance against other important aminoglycosides, such as kanamycin, neomycin, apramycin, or netilmicin. The gene coding for the AAC(3)-1 enzyme was located on a plasmid that could be transferred to recipient *E. coli* K-12. The gene conferring tetracycline resistance was located on the same plasmid as the gene conferring gentamicin resistance (cotransfer of resistance by conjugation). A minority (three) of the isolates showed only a low-level increase in the MIC of gentamicin; they did not carry any plasmid, and the low level of resistance was not specific and could have resulted from changes in permeability barriers due to the possibility of chromosomal mutations affecting the genes coding for the outer membrane proteins. One of the serotype Typhimurium isolates with low-level gentamicin resistance also showed resistance to amoxicillin (sample 11068). Another sample showed only amoxicillin resistance (sample 10053). For both samples, the enzyme conferring amoxicillin resistance was characterized by isoelectric focusing of cell-free sonicates. Both enzymes focused at pI 5.4, the same position as the TEM-1 control, and were identified as TEM-1. The gene coding for this enzyme was located on a plasmid.

PFGE analysis was carried out on all of the above isolates, and they were all demonstrated to be indistinguishable from the original inoculated serotype Typhimurium DT104. Isolates showing an increase in the MIC were distributed throughout the treatment groups, including the nonsupplemented control group (T1). Statistical analysis of the results showed no significant differences in susceptibility changes for serotype Typhimurium between treatment groups ( $P = 1.000$ ).

**Plasmid profile analysis.** With the exception of the inoculum and of samples 11021, 11064, and 11106, all tested antimicrobial agent-resistant isolates carried a large, 60-MDa, low-copy-number plasmid (20). Smaller plasmids (<5 MDa) were present in five of the isolates. The large plasmid conferred resistance to gentamicin plus tetracycline, to gentamicin alone, or to amoxicillin alone.

**Phenotypic changes.** In addition to an increase in the MIC, a number of other phenotypic changes were noted for the

TABLE 3. Resistance phenotypes, treatment groups, and PFGE genotypes of *Salmonella* isolates grown on plates supplemented with antimicrobial agents

Resistance phenotype <sup>a</sup>	Sample	Treatment <sup>b</sup>	Day	CFU/g	PFGE genotype (SpeI)
GEN64/AMX2/TET>16	10051-GEN	MON	13	10 <sup>8</sup>	1
GEN64/AMX<1/TET>16	10073-GEN	SEM25	13	10 <sup>7</sup>	1A
	11056-TET	MON	48	10 <sup>8</sup>	1
	11115-GEN	SEM25	48	10 <sup>10</sup>	1
GEN32/AMX2/TET>16	10052-GEN	SAL	13	10 <sup>9</sup>	1
	10054-GEN	CTRL	13	10 <sup>9</sup>	1
	10055-GEN	SEM20	13	10 <sup>9</sup>	1
	11060-GEN	SAL	48	10 <sup>7</sup>	1A
	11061-TET <sup>c</sup>	SEM25	48	10 <sup>8</sup>	1
	11061-GEN <sup>e</sup>	SEM25	48	10 <sup>8</sup>	1D
	11122-GEN	MON	48	10 <sup>7</sup>	1
GEN32/AMX<1/TET>16	10070-GEN	SAL	13	10 <sup>8</sup>	1
	11053-GEN	SEM25	48	10 <sup>8</sup>	1
	11054-GEN	CTRL	48	10 <sup>12</sup>	1B
	11062-GEN <sup>d</sup>	SAL	48	10 <sup>11</sup>	1
	11069-GEN	CTRL	48	10 <sup>8</sup>	1A
	11123-GEN	CTRL	48	10 <sup>7</sup>	1B
	11072-GEN	SAL	48	10 <sup>7</sup>	1
	11103-GEN <sup>e</sup>	SEM25	48	10 <sup>9</sup>	1
	11112-GEN	SEM20	48	10 <sup>7</sup>	1
	GEN16/AMX<1/TET>16	10059-GEN	CTRL	13	10 <sup>7</sup>
11055-GEN		SEM20	48	10 <sup>6</sup>	1
11059-GEN <sup>e</sup>		CTRL	48	10 <sup>8</sup>	1
11063-GEN		MON	48	10 <sup>8</sup>	1A
11066-GEN		MON	48	10 <sup>11</sup>	1C
11070-GEN		SAL	48	10 <sup>11</sup>	1
GEN16/AMX<1/TET2		10061-GEN	SEM25	13	10 <sup>9</sup>
GEN16/AMX2/TET>16	11071-GEN	SEM20	48	10 <sup>9</sup>	1A
GEN4/AMX<1/TET<1	11021-GEN <sup>f</sup>	SEM20	13	10 <sup>7</sup>	1
GEN2/AMX<1/TET2	11064-GEN <sup>g</sup>	SEM20	48	10 <sup>8</sup>	1
GEN2/AMX<1/TET<1	11106-GEN	CTRL	48	10 <sup>8</sup>	1
GEN2/AMX>128/TET<1	11068-GEN <sup>g</sup>	SEM20	48	10 <sup>8</sup>	1B
GEN1/AMX64/TET2	10053-AMX <sup>h</sup>	SEM25	13	10 <sup>10</sup>	1

<sup>a</sup> GEN, gentamicin; TET, tetracycline; AMX, amoxicillin. Numerals indicate MICs in milligrams per liter.

<sup>b</sup> SEM, semduramicin; SAL, salinomycin; MON, monensin; CTRL, control. Numerals indicate concentrations in milligrams per kilogram of diet.

<sup>c</sup> Sample grew on plates supplemented with tetracycline as well as plates supplemented with gentamicin.

<sup>d</sup> Flagellar phase 2.

<sup>e</sup> Phage type 302.

<sup>f</sup> Negative with phase 2 antisera, poor growth and smaller colonies.

<sup>g</sup> Did not agglutinate with polyvalent H antisera; nonmotile.

<sup>h</sup> Phage type 120.

*Salmonella* isolates recovered from fecal samples. Of 32 isolates analyzed, 3 showed a change in phage type (phage type 302, isolate 11103 and isolate 11059; or phage type 120, isolate 10053). While phage type 302 is closely related to DT104, phage type 120 is not. Some of the isolates showed a change in agglutination pattern; isolate 11021 agglutinated with polyvalent H antisera but did not react with specific I or phase 2 antisera. Two of the isolates (11064 and 11068) showed a loss of motility. Most of the isolates showing phenotypic changes came from bulk fecal samples collected on day 48, but two of them (10053 and 11021) were from fecal samples collected on day 13, only 3 days after challenge.

Together with the isolates showing changes in antimicrobial profiles, all of the isolates showing changes in phage type, colony morphology, or motility were confirmed to be identical to the inoculum by PFGE analysis. Isolates showing phenotypic changes were distributed throughout the treatment groups.

## DISCUSSION

Serotype Typhimurium DT104 is a widespread and internationally recognized human and animal pathogen. Described isolates from epidemic episodes frequently show resistance to five different antimicrobial agents (ampicillin, chlorampheni-

col, streptomycin, sulfonamides, and tetracyclines), although isolates resistant to up to nine antimicrobial agents have been described. This microorganism is a zoonotic pathogen which is widespread in Europe, North America, and the Middle East (12).

A number of studies on experimental infection with *Salmonella* have shown that the microorganism can colonize the intestinal tract and then invade and disseminate in the host to reach numerous internal organs (6, 9). In this study, the experimental infection was successful, as demonstrated by the high levels of excretion of *Salmonella* across treatment groups and for the duration of the study. Birds infected on day 10 with an inoculum of  $10^7$  CFU of serotype Typhimurium showed high receptivity to infection; colonization was established, with consequent shedding of *Salmonella* in feces for the duration of the study for most birds, on some occasions at high levels. In this study, the presence or absence of ionophore anticoccidial agents in the diet did not affect the frequency or magnitude of shedding.

In contrast to a previous study (2), a comparison of results obtained for different treatment groups showed that the presence of anticoccidial drugs in feed did not influence *Salmonella* shedding. This finding was confirmed by statistical analysis; the presence or absence of anticoccidial agents in the diet did not affect significantly the acquisition of antimicrobial resistance ( $P = 1.000$ ).

Previous studies with the use of commercial feed containing resistant bacteria have shown that some *E. coli* strains can colonize the intestines of chickens to various levels (8). The in vivo exchange of plasmids between different *E. coli* strains has also been demonstrated in experimental settings with germ-free chickens (13, 21).

In this study, we achieved an experimental setting closely resembling field conditions (use of commercial birds supplied from a high-quality commercial hatchery, with naturally acquired gut flora; use of commercial feed; and duration of study) while keeping very strict control on the target organism (irradiated food, sterile water, new building, and comparison of the inoculated strain with bacteria isolated from feces).

The results on the acquisition of antimicrobial resistance and the observations made for *Salmonella* isolates during this study suggest that changes in antimicrobial sensitivity can be observed independently of the presence of anticoccidial drugs in the diets of birds. Although anticoccidial agents are not expected to exert any direct selection pressure on *Salmonella*, their potential activity against gram-positive bacteria could have an impact on the availability of intestinal niches and, consequently, on competitive exclusion (18, 22), thereby influencing the level of shedding in different treatment groups. The fact that shedding, changes in antimicrobial sensitivity, and the acquisition of phenotypic changes were seen in all treatment groups, including the nonsupplemented control group, suggests that the simple passage of *Salmonella* through the intestine of poultry is able to induce phenotypic and genotypic changes, including the acquisition of antimicrobial resistance plasmids, changes in sensitivity to bacteriophages, and changes in motility. The reduced sensitivity to some antimicrobial agents does not appear to be related to anticoccidial selection pressure. In fact, similar changes were found in the nontreated group, but ionophore anticoccidial agents are not believed to

have any direct activity against *Salmonella* and other gram-negative bacteria because of permeability barriers.

Anticoccidial resistance determinants are very poorly characterized and are not well described for bacteria. To our knowledge, a resistance mechanism has been characterized only on one occasion, for *Streptomyces longisporoflavus* (16). The resistance mechanism reported involved an ABC transporter system, and it has been postulated that efflux mechanisms are involved in the other described cases of resistance to ionophores (3). We believe that it is unlikely that any link can be made between the acquisition of anticoccidial resistance or antimicrobial resistance and the carryover of resistance genes in this study, as evidenced by the fact that similar results were obtained for the control group, which did not receive any diet supplementation and consequently had no selection pressure for anticoccidial resistance development. Moreover, ionophore anticoccidial agents are known to be ineffective against gram-negative bacteria, as they cannot enter cells because of permeability barriers. Although we cannot exclude the possibility that antimicrobial resistance phenotypes were acquired from gram-positive organisms, it appears more likely that the resistance determinants were derived from other members of the family *Enterobacteriaceae*, as the exchange of plasmids among this large group of bacteria is very common. The *Salmonella* strain used in this study is not a multiresistant strain; in fact, it is sensitive to most antimicrobial agents tested and was isolated from poultry during an episode of septicemia. Whether the results of this study could be extrapolated to an already multiresistant strain (such as the one responsible for epidemics in the 1990s) is difficult to say, but it would be interesting to investigate by replicating this study with such a strain.

With regard to excretion levels, the findings of this study can probably be extrapolated to other *Salmonella* serotypes. In fact, a comparison of in vitro and in vivo colonization levels among various *Salmonella* serotypes was done recently by Aabo et al. (1). In their study, invasion by 15 selected *Salmonella* serotypes in an intestinal loop model in vivo and in epithelial cell cultures in vitro was compared to that by a reference strain (*Salmonella* serotype Typhimurium 4/74 *invH21::TnpH21*). They concluded that different *Salmonella* serotypes do not differ fundamentally in their behavior from the reference strain, except for *Salmonella* serotype Enteritidis PT4 (1.5  $\log_{10}$  CFU higher in cell cultures) and *Salmonella* serotype Tennessee (0.7  $\log_{10}$  CFU lower in cell cultures). In addition, although multiresistant DT104 has been considered more invasive than other serotype Typhimurium phage types, Threlfall et al. (28) found no significant difference in blood or fecal detection ratios.

The findings of this study are suggestive of many interesting and still unknown interactions that occur among bacteria within the very dynamic intestinal environment every time a pathogen such as *Salmonella* is ingested. Thus, in addition to selective pressure, other, unknown host-pathogen interactions play a major role in important phenomena, such as the acquisition of antimicrobial resistance. Even more intriguing is the finding of other phenotypic changes in, for example, colony morphology, phage sensitivity, and loss of motility. Given that in this study such changes were more or less fortuitous findings, it can be expected that in reality their incidence far exceeds what was observed in this study. This situation could have

consequences for the estimation of the likelihood of acquiring antimicrobial resistance and for epidemiological surveys, as isolates having lost the typical morphology, motility, or phage type of a typical *Salmonella* colony are not likely to be further characterized. Finally, given the importance of the subject and the potential implications of the results, we encourage the replication of this study by other researchers.

## APPENDIX

Access to the building which housed the animal accommodation units (a new purposely built building that had never housed any animal previously) was strictly controlled, and only personnel involved in the study and whose feces were screened to confirm the absence of *Salmonella* were allowed entry. The animal units were built inside the building on each side of a central corridor (Fig. A1). Before entering the central access corridor, all personnel took a shower and put on appropriate protective clothing. Disposable protective overalls, shoe covers, gloves, and hats were donned on entering the service area of each animal unit and were left there before leaving each animal unit, and new disposable items were donned on entering another animal unit. Each animal unit consisted of a single floor pen and a separate individual service area which, through a door, opened onto the central common corridor. The appropriate use of filters and air pressure control for the duration of this study ensured that airborne transfer of infectious material between pens did not occur. Air pressure was higher in the service area than in both the central corridor and the pen housing the animals, thus ensuring that the risk of infectious material leaving the animal units was minimal and also that the risk of airborne contamination from the corridor to the animal units was minimal.

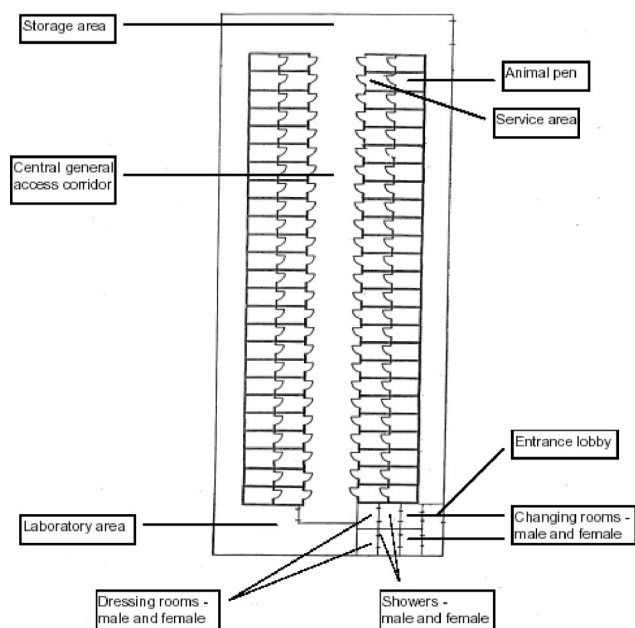


FIG. A1. Floor plan of building housing animal units.

## ACKNOWLEDGMENTS

We thank C. Wray and R. Davis for providing *Salmonella* serotype Typhimurium isolate DT104. We warmly thank Jean Pierre Lafont (INRA, Santé Animale, Nouzilly, Tours, France) for providing invaluable advice on the experimental setting and on the interpretation of results. We also acknowledge the excellent technical support that we received from the staff of the laboratories involved in this study. We are grateful to J. Sherington (Pfizer Animal Health) for statistical advice and analysis. This study complied with relevant guidelines regarding the use of animals for research purposes and was conducted in accordance with VICH-GCP standards.

## REFERENCES

- Aabo, S., J. P. Christensen, M. S. Chadfield, B. Carstensen, J. E. Olsen, and M. Bisgaard. 2002. Quantitative comparison of intestinal invasion of zoonotic serotypes of *Salmonella enterica* in poultry. *Avian Pathol.* **21**:41–47.
- Bolder, N. M., J. A. Wagenaar, F. F. Putirulan, K. T. Veldman, and M. Sommer. 1999. The effect of flavophospholipol (Flavomycin) and salinomycin sodium (Sadox) on the excretion of *Clostridium perfringens*, *Salmonella enteritidis*, and *Campylobacter jejuni* in broilers after experimental infection. *Poult. Sci.* **78**:1681–1689.
- Butaye, P., L. A. Devriese, and F. Haesebrouck. 2003. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. *Clin. Microbiol. Rev.* **16**:175–188.
- Cohen, M. 1992. Epidemiology of drug resistance: implications for a post-antimicrobial era. *Science* **257**:1050–1055.
- Corkill, J. E., C. A. Hart, A. G. McLennan, and S. Aspinall. 1991. Characterization of a  $\beta$ -lactamase produced by *Pseudomonas paucimobilis*. *J. Gen. Microbiol.* **137**:1425–1429.
- Gast, R. K. 1994. Understanding *Salmonella enteritidis* in laying chickens: the contributions of experimental infections. *Int. J. Food Microbiol.* **21**:107–116.
- Graham, M. S., E. M. Molyneux, A. L. Walsh, J. S. Cheesbrough, M. E. Molyneux, and C. A. Hart. 2000. Nontyphoidal *Salmonella* infections of children in tropical Africa. *Pediatr. Infect. Dis. J.* **19**:1189–1196.
- Guillot, J.-F., E. Chaslus-Dancla, and J.-P. Lafont. 1977. Spontaneous implantation of antibiotic-resistant *Enterobacteriaceae* in the digestive tract of chickens in the absence of selective pressure. *Antimicrob. Agents Chemother.* **12**:697–702.
- Hassan, J. O., S. B. Porter, and R. Curtiss. 1993. Effect of infective dose on humoral immune responses and colonization in chickens experimentally infected with *Salmonella typhimurium*. *Avian Dis.* **37**:19–26.
- Heir, E., B.-A. Lindstedt, I. Nygård, T. Vardund, V. Hasseltvedt, and G. Kapperud. 2002. Molecular epidemiology of *Salmonella* Typhimurium isolates from human sporadic and outbreak cases. *Epidemiol. Infect.* **128**:373–382.
- Helmut, R. 2000. Antibiotic resistance in *Salmonella*, p. 80–106. In C. Wray and A. Wray (ed.), *Salmonella* in domestic animals. CABI Publishing, New York, N.Y.
- Humphrey, T. 2001. *Salmonella* Typhimurium definitive type 104. A multi-resistant *Salmonella*. *Int. J. Food Microbiol.* **67**:173–186.
- Lafont, J.-P., A. Bree, and M. Plat. 1984. Bacterial conjugation in the digestive tracts of gnotoxenic chickens. *Appl. Environ. Microbiol.* **47**:639–642.
- Lepage, P., J. Bogaerts, C. Van Goethem, M. Ntahorutaba, F. Nsengumuremy, D. G. Hitimana, J. Vandepitte, J.-P. Butzler, and J. Levy. 1987. Community acquired bacteraemia in African children. *Lancet* **1**:1458–1461.
- Levy, S. B. 1997. Antibiotic resistance: an ecological imbalance. *Ciba Found. Symp.* **207**:1–14.
- Linton, K. J., H. N. Cooper, I. S. Hunter, and P. F. Leadlay. 1994. An ABC transporter system for *Streptomyces longisporoflavus* confers resistance to the polyether-ionophore antibiotic tetracosin. *Mol. Microbiol.* **11**:777–785.
- McDougald, L. R. 1982. Chemotherapy of coccidiosis, p. 373–427. In P. L. Long (ed.) *The biology of the coccidia*. University Park Press, Baltimore, Md.
- Methner, U., P. A. Barrow, A. Berndt, and G. Steinbach. 1999. Combination of vaccination and competitive exclusion to prevent *Salmonella* colonization in chickens: experimental studies. *Int. J. Food Microbiol.* **49**:35–42.
- Molbak, K., D. L. Baggesen, F. M. Aarestrup, J. M. Ebbesen, J. Engberg, K. Frydendahl, P. Gerner-Smith, A. M. Petersen, and H. C. Wegener. 1999. An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT 104. *N. Engl. J. Med.* **341**:1420–1425.
- Ribot, E. M., R. K. Wierzbza, F. J. Angulo, and T. J. Barrett. 2002. *Salmonella enterica* serotype Typhimurium DT 104 isolated from humans, United States, 1985, 1990 and 1995. *Emerg. Infect. Dis.* **8**:387–391.
- Sansonetti, P., J.-P. Lafont, A. Jaffé-Brachet, J.-F. Guillot, and E. Chaslus-Dancla. 1980. Parameters controlling interbacterial plasmid spreading in a gnotoxenic chicken gut system: influence of plasmid and bacterial mutations. *Antimicrob. Agents Chemother.* **17**:327–333.
- Schneitz, C., and G. Mead. 2000. Competitive exclusion, p. 301–322. In C. Wray and A. Wray (ed.), *Salmonella* in domestic animals. CABI Publishing, New York, N.Y.

23. **Shaw, K. J., F. J. Sabatelli, L. Naples, P. Mann, R. S. Hare, and G. H. Miller.** 1998. The application of molecular techniques for the study of aminoglycoside resistance. *Methods Mol. Med.* **15**:555–577.
24. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev.* **57**:138–163.
25. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**:2233–2239.
26. **Threlfall, E. J.** 2000. Epidemic *Salmonella typhimurium* DT 104—a truly international multiresistant clone. *J. Antimicrob. Chemother.* **46**:7–10.
27. **Threlfall, E. J.** 2002. Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food- and water-borne infections. *FEMS Microbiol. Rev.* **26**:141–148.
28. **Threlfall, E. J., L. R. Ward, and B. Rowe.** 1998. Multiresistant *Salmonella typhimurium* DT104 and *Salmonella* bacteraemia. *Lancet* **352**:287–288.
29. **Witte, W.** 1998. Medical consequences of antibiotic use in agriculture. *Science* **279**:996–998.