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Control of *Salmonella enteritidis* Infections in Poultry by Polymyxin B and Trimethoprim

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Antimicrobial compounds were screened in vitro in Trypticase soy broth for antimicrobial activity against a virulent strain of *Salmonella enteritidis*. Of the several compounds tested, polymyxin B showed the strongest inhibition in vitro, preventing growth at a concentration of ≤10 μg/ml. Polymyxin B administered in the drinking water was effective in vivo for preventing infections in 1-day-old chickens but did not remove established infections in 1-week-old chickens. It was found that trimethoprim, which was not active in vitro, prevented colonization and removed existing infections in 1-day-old chickens when it was administered together with polymyxin B sulfate. Enrichment cultures in which selenite-cystine and tetrathionate broth media were used showed that chickens given a combination of 100 μg of polymyxin B sulfate per ml and 250 μg of trimethoprim per ml 24 h prior to oral inoculation with 10^8 to 10^9 CFU were negative for *S. enteritidis* after 7 days. Established infections (10^5 to 10^6 CFU/g of feces) in 1-week-old chickens were eliminated by treatment with the polymyxin-trimethoprim system. This antimicrobial agent treatment may be useful for preventing colonization in poultry and for eliminating *S. enteritidis* from infected flocks.

Over the past eight years *Salmonella enteritidis* has been recognized as a leading cause of salmonellosis in humans in the United States and Europe (5, 7, 13, 14). In 1989 there were at least 49 foodborne outbreaks and 13 deaths attributed to *S. enteritidis* in the United States (2, 7). The Centers for Disease Control expects that more than 100 outbreaks will occur in 1990 (2). Epidemiological investigations have implicated raw shell eggs and foods containing unpasteurized liquid egg products as the food vehicles in the majority of the outbreaks in the United States and Europe (5–7, 13, 14).

For several years the control of *Salmonella* spp. in poultry has been a high priority of the U.S. Department of Health and Human Services and the Agricultural Research Service (3). Much of the effort has focused on exclusion or reduction in the numbers of salmonellae by introducing competitive microfloras and by hygienic processing of feed (3). Control of salmonellae can also be achieved by administration of antimicrobial agents in the drinking water (10, 11, 16) and by combined therapy involving antimicrobial agents and competitive floras (11, 12). William Smith and Tucker (16) reduced the number of *Salmonella typhimurium* cells in experimentally infected chickens by using a combination of trimethoprim and sulfadiazine. Trimethoprim and sulfamethoxazole have been recommended for treatment of salmonellosis in humans (9). Seuna and Nurmi (11) showed that combined therapy involving bacterial cultures and certain antimicrobial agents, including oxytetracycline, neomyxin, polymyxin, and trimethoprim, significantly reduced the infection rate in chickens by *Salmonella infantis*, while the bacterial cultures alone only had a slight anti-salmonella effect. Antibiotic treatment followed by feeding of bacterial cultures helped prevent reappearance of infections (12).

In this study we tested the efficacy of various antimicrobial compounds against a pathogenic *S. enteritidis* strain. Several compounds showed activity in vitro, and a combination of polymyxin and trimethoprim was effective in vivo in controlling *S. enteritidis* in young chickens.

MATERIALS AND METHODS

Organism. A virulent strain of *S. enteritidis* serotype O:1, 9,12;H:g,m, strain E40, phage type 8, was provided by M. P. Doyle. This strain was involved in an egg-associated outbreak of salmonellosis and was recovered from an ovary of a hen in an implicated flock by workers at the State of New York Department of Health. It was stored at −70°C in Trypticase soy broth (TSB) (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.) containing 25% glycerol. The purity of the strain was checked periodically by streaking the culture onto Trypticase soy agar (TSB containing 2% Bacto agar [Difco Laboratories, Detroit, Mich.]) and determining the serological characteristics.

In vitro testing of antimicrobial activity. Most of the compounds tested were selected because they showed some antimicrobial activity against other organisms tested in our laboratory. The antimicrobial agents which we tested were obtained from Sigma Chemical Co., St. Louis, Mo. (Table 1). The compounds were assayed for antimicrobial activity singly and in combination with one another. They were filter sterilized and then added to tubes containing TSB (final volume, 10.0 ml). Each tube was then inoculated with one loopful (ca. 10^5 to 10^6 CFU) of an actively growing culture of *S. enteritidis* E40 in TSB. The tubes were incubated statically at 37°C, and the optical density of each tube was measured periodically at 660 nm with a Spec 20 spectrophotometer (Milton Roy Co., Rochester, N.Y.). The optical density readings obtained were then compared with the optical density of a positive control tube without antimicrobial compounds, and the percent of inhibition was calculated.

In vivo inhibition of *S. enteritidis* in chickens. Leghorn X New Hampshire chickens were obtained from the University of Wisconsin-Madison Poultry Science Department within 24 h of hatching. The birds were placed into cages and given

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TABLE 1. Inhibition of *S. enteritidis* by various compounds in TSB

Compound or combination[a]	Concn (μg/ml)	% Inhibition[b]
Butylparaben	100	28
EDTA	380 (1 mM)	13
Butylparaben and EDTA	100 and 380	85
Butylparaben and tripolyphosphate	100 and 1,000	44
Monocaprin glyceride	100	4
Monocaprin glyceride and EDTA	100 and 380	44
Polymyxin B	1	2
Polymyxin B	3	93
Polymyxin B	10	100
Polymyxin B and EDTA	3 and 380	97
p-Aminobenzoic acid	1,000	57
p-Aminobenzoic acid and butylated hydroxyanisole	1,000 and 100	43
Butylated hydroxyanisole and EDTA	100 and 380	51
Glycine	10,000	40
Polymyxin E	10	8
Polymyxin E and butylparaben	10 and 10	95
Polymyxin B and trimethoprim	1 and 10	4
Trimethoprim	25	10

[a] The following compounds were tested but did not produce significant inhibition of *S. enteritidis*: ascorbic acid (1,000 μg/ml), pantothenic acid (1,000 μg/ml), *p*-aminobenzoic acid (1,000 μg/ml), methylparaben (100 μg/ml), ethyl-*p*-aminobenzoate (100 μg/ml), butyl-*p*-aminobenzoate (100 μg/ml), phytic acid (200 μg/ml), sorbic acid (1,000 μg/ml), sodium sulfite (1,000 μg/ml), bacitracin (100 μg/ml), tripolyphosphate (1,000 μg/ml), and sodium acetate (1,000 μg/ml). These compounds were tested singly and in combination with lysozyme (100 μg/ml) and lysozyme (100 μg/ml) plus EDTA (380 μg/ml).

[b] Absorbance values were determined at several time points until stationary phase was reached (optical density at 660 nm, 0.8 to 1.0).

food and water ad libitum. When we attempted to inhibit colonization, antimicrobial agents were administered in the drinking water, which was available when the birds were placed into their cages. When removal of existing infections was evaluated, the antimicrobial agents were given in the drinking water 2 to 3 days after inoculation with *S. enteritidis*. The temperature of the incubation room was initially 38°C and was decreased 1°C per day to a final temperature of 25°C (in experiments which lasted 14 days or more).

Chickens that were 1 day old were challenged orally with *S. enteritidis* by feeding 0.5-ml aliquots of a 24-h-old culture to the birds with an 18-gauge feeding needle. The cultures were grown in TSB statically at 37°C for 18 to 24 h prior to challenge and had an optical density at 660 nm of 0.7 to 0.8 (10^8 to 10^9 CFU/ml). All challenged birds became colonized.

To determine the *S. enteritidis* levels in the birds, fecal samples were obtained by gently compressing the lower abdomen of each bird and collecting the sample in a sterile, preweighed tube. *S. enteritidis* counts were obtained by plating serially diluted samples of freshly collected feces onto bismuth sulfite agar and xylose lysine deoxycholate agar (Difco). Dilutions were done with 65 mM phosphate-buffered saline (pH 7.4), and 0.1-ml portions were plated onto the two media. The plates were incubated aerobically at 37°C for 24 to 48 h. Characteristic reactions exhibited by the *S. enteritidis* colonies on these media were evaluated after 24 to 48 h and compared with the reactions of positive controls. The numbers of CFU per gram of feces were determined by averaging the results obtained from the bismuth sulfite agar and xylose lysine deoxycholate agar plates. When the fecal samples collected from the birds had less than 10^2 CFU/g, selective enrichment cultures in which selenite-cystine broth and tetrathionate broth (Difco) were used were prepared by using the method proposed by the U.S. Department of Agriculture for voluntary monitoring of poultry flocks (8). Enrichment cultures were grown at 37°C for 24 h and were then streaked onto bismuth sulfite agar and xylose lysine deoxycholate agar plates for *S. enteritidis* detection and scored plus or minus. Nonselective enrichment cultures in TSB followed by selective enrichment cultures in selenite-cystine broth and tetrathionate broth gave results similar to those obtained with selective enrichment cultures alone, indicating that no injured salmonellae were present.

Periodically during each in vivo experiment the serotypes of random *Salmonella* isolates from the bismuth sulfite agar and xylose lysine deoxycholate agar plates were compared with the parental *S. enteritidis* serotype (O:1,9,12;H:g,m) by using a slide agglutination assay. The monovalent antisera used in the assay were obtained from Difco, and the procedure was done by using the manufacturer's protocol.

RESULTS

In vitro inhibition of *S. enteritidis*. Several compounds tested singly and in combination inhibited *S. enteritidis* E40 in vitro (Table 1). Of the compounds screened, the most effective were the polymyxins. The MIC for pure polymyxin B sulfate was 1 to 3 μg/ml. Other effective inhibitory compounds included butylparaben, *p*-aminobenzoic acid, and EDTA, singly and in combination with each other (Table 1). Many compounds were ineffective (Table 1). On the basis of these in vitro results, we tested the ability of inhibitory compounds to control *S. enteritidis* in 1-day-old chickens.

In vivo inhibition of *S. enteritidis*. Antimicrobial compounds were evaluated for their ability to prevent infections or to remove existing infections in chickens. The compounds which we tested were dissolved in the drinking water and included polymyxin B sulfate, butylparaben, glycine, polymyxin E, and trimethoprim. Our results indicated that polymyxin B sulfate was effective in preventing infections when it was used at a concentration of 50 μg/ml or higher (Fig. 1). These initial studies were done with a total of 15 test birds and 4 control birds. There were three birds in each test group (i.e., three birds in the group which received 10 μg of polymyxin B sulfate per ml, three birds in the group which received 50 μg of polymyxin B sulfate per ml, etc.). The counts of *S. enteritidis* (CFU/g of feces) did not vary by more than 0.5 log unit; this variance was consistent throughout the experiment. Polymyxin B sulfate by itself was ineffective in removing established infections in 3- to 4-day-old chickens. Butylparaben and glycine did not prevent infections in vivo in 1-day-old chickens.

S. enteritidis was not detected in birds that were given only polymyxin B sulfate (100 μg/ml) or polymyxin E (100 μg/ml) after 6 and 2 days, respectively. After these periods of time, the numbers of *S. enteritidis* CFU in these birds increased markedly despite the continued administration of polymyxins (Fig. 1). The *S. enteritidis* cells recovered from the birds treated with polymyxin B sulfate were not resistant to the antimicrobial agent (10 μg/ml) on Trypticase soy agar.

Although polymyxin B sulfate was ineffective by itself, we unexpectedly found that a combination of polymyxin B sulfate (50 to 100 μg/ml) and trimethoprim (250 μg/ml) consistently prevented infection (Fig. 2). In this experiment a total of 33 birds were used; 2 birds received 10 μg of

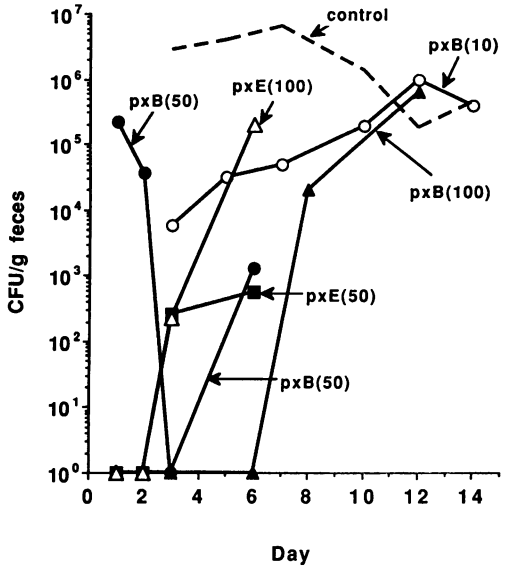

FIG. 1. Inhibition of *S. enteritidis* colonization of 1-day-old chickens by polymyxins B and E. Abbreviations: pxB, polymyxin B sulfate; pxE, polymyxin E. The numbers in parentheses are concentrations (in micrograms per milliliter).

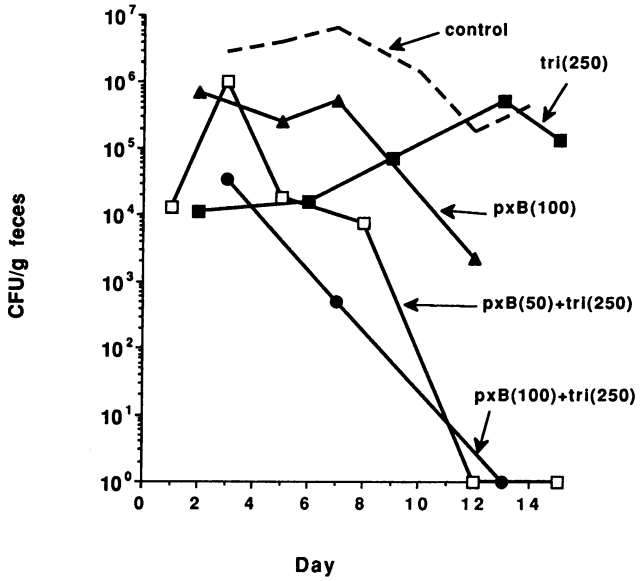

FIG. 3. Removal of *S. enteritidis* existing infections by polymyxin B and trimethoprim. Abbreviations: pxB, polymyxin B sulfate; tri, trimethoprim. The numbers in parentheses are concentrations (in micrograms per milliliter).

polymyxin B sulfate per ml plus 250 μg of trimethoprim per ml, 2 birds received 50 μg of polymyxin B sulfate per ml plus 250 μg of trimethoprim per ml, 10 birds received 100 μg of polymyxin B sulfate per ml plus 250 μg of trimethoprim per ml, 10 birds received 250 μg of trimethoprim per ml, and there were 9 control birds. The variance in the *S. enteritidis* counts (CFU per gram) in this experiment was similar to that shown in Fig. 1 and was not more than 0.5 log unit. The enhancement by trimethoprim was surprising since trimethoprim alone only weakly inhibited *S. enteritidis* in vitro. When the combination was used, *S. enteritidis* was undetectable in the fecal samples by using enrichment cultures after 7 days. Recurrence of infection did not occur during continued treatment for 14 days with the antimicrobial agents.

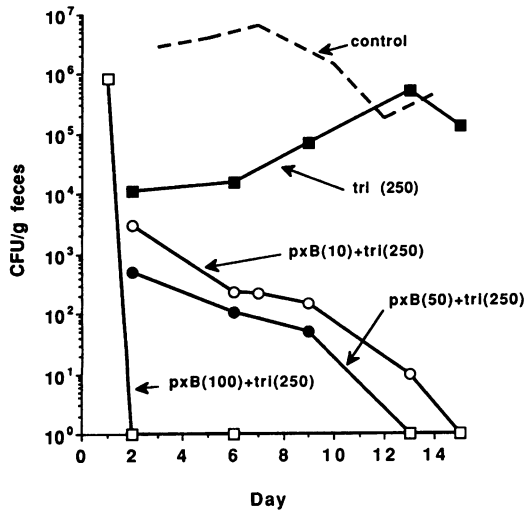

FIG. 2. Inhibition of *S. enteritidis* colonization of 1-day-old chickens by polymyxin B and trimethoprim. Abbreviations: pxB, polymyxin B sulfate; tri, trimethoprim. The numbers in parentheses are concentrations (in micrograms per milliliter).

Removal of existing *S. enteritidis* infections. We performed experiments to determine whether polymyxin B-trimethoprim could remove established *S. enteritidis* infections. Chickens that were 1 day old were infected by feeding 10^8 to 10^9 CFU of *S. enteritidis* E40 to each bird and providing the birds with tap water that did not contain antimicrobial agents. Antimicrobial agent-containing water was then offered 2 to 3 days after inoculation of the birds, when they were excreting ca. 10^6 *S. enteritidis* CFU per g of feces.

The combination of polymyxin B sulfate and trimethoprim at concentrations of 100 and 250 μg/ml, respectively, removed infections after 12 days (Fig. 3). A total of 10 birds were used for this experimental procedure. The birds given 100 μg of polymyxin B sulfate per ml plus 250 μg of trimethoprim per ml were still negative for *S. enteritidis* after 15 days, as determined by using selenite-cystine and tetrathionate enrichment cultures. The control group, the group that received 100 μg of polymyxin B sulfate per ml, and the group that received 250 μg of trimethoprim per ml contained 13, 13, and 10 birds, respectively. The variance in *S. enteritidis* counts was within the same limits described above for Fig. 1 and 2. In repeated experiments we found that reinfection occurred in birds (>80%) when the antibiotics were no longer administered. The cage environment probably contributed to reinfection since it contained large numbers of infectious *S. enteritidis*; in addition, positive control chickens that harbored salmonellae were in nearby cages.

DISCUSSION

In this study we found that polymyxin B sulfate is strongly bactericidal for *S. enteritidis* in vitro. Polymyxin B sulfate has been shown to inhibit *Salmonella* sp. and other members of the family *Enterobacteriaceae* at very low concentrations (1, 15) and to protect mice against endotoxic shock during

infections by gram-negative pathogens (4). Although in this study polymyxin was inhibitory in vitro at very low concentrations, in vivo control of *S. enteritidis* in 1-day-old chickens required much higher levels of the antimicrobial agent. Furthermore, polymyxin prevented infection only for a limited time. Since polymyxins are poorly absorbed across the intestinal barriers of animals (17), the lack of effectiveness in vivo was probably not due to absorption into the tissues of the birds. It is possible that the antimicrobial agent bound to food particles present, which sequestered it from the target organism, or that it was inactivated by peptidases present in the guts of the birds. Poor feeding and water consumption by the chickens may also have reduced the effective concentration of polymyxin in the intestines.

We found that trimethoprim enhanced the in vivo activity of polymyxin. The use of the two antimicrobial agents effectively prevented colonization and eliminated existing *S. enteritidis* infections. Other studies have demonstrated a synergistic antimicrobial effect between polymyxin B sulfate and trimethoprim in treatment of salmonellosis in humans (9). It is not clear why trimethoprim enhanced the anti-*S. enteritidis* activity. This antibiotic was poorly inhibitory in vitro against *S. enteritidis*, although it did reduce the size and alter the morphology of *S. enteritidis* colonies on agar media. In vivo, the antimicrobial agent may have altered the microbial flora in a way that did not favor *S. enteritidis* colonization. Trimethoprim acts on the folic acid biosynthetic pathway by inhibiting dihydrofolate reductase (9), decreasing nucleotide synthesis. Its mode of action suggests that nucleotide biosynthesis may limit *S. enteritidis* development in chicken intestines. The associated intestinal flora in nontreated birds may help alleviate limitation by providing precursors or cofactors for nucleotide synthesis, such as *p*-aminobenzoic acid. Alternatively, trimethoprim may inhibit the infectious process of *S. enteritidis*.

When the antimicrobial agents were removed from the drinking water, some chickens became reinfected with *S. enteritidis*, probably because *S. enteritidis* was present at high infectious doses in the environment. This indicates that other exclusionary methods are probably necessary for control, including good hygiene, nutrition, and possibly use of bacterial cultures to prevent reinfection of chickens. Further work is needed to assess the efficacy of a combination of therapies.

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