RESEARCH REPORT

PORK SAFETY

Title: The effect of antibiotics used to control pleuropneumonia on antibiotic resistance in human foodborne pathogens – **NPB# 99-067**

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I. Stated objectives from the original proposal

Objective 1. To investigate the effect of antibiotics used to control pleuropneumonia caused by APP in swine on the antibiotic susceptibility of fecal isolates of *E. coli*, *Salmonella spp* and *Campylobacter spp*.

Hypothesis: The use of therapeutic antibiotics in growing swine leads to increased antibiotic resistance and decreased antibiotic susceptibility in *E. coli, Salmonella spp.*, *C. coli* and *C. jejuni* isolated from feces.

Objective 2. To compare the consequences of pleuropneumonia prevention in swine herds using vaccination versus antibiotics on the prevalence of antibiotic resistance and antibiotic susceptibility of human foodborne pathogens in feces, and on the incidence and severity of the disease.

Hypothesis: Prevention of pleuropneumonia in swine using a vaccine leads to a reduction of clinical disease, an improved growth rate and decreased antibiotic resistance in the enteric flora.

Progress towards meeting objectives

Objective 1 is advancing as expected, two trials will be completed by February 2000 and the calculation of Minimum Inhibitory Concentration for *E. coli*, *Salmonella spp* and *Campylobacter spp* isolates will begin in 2000.

Objective number 2 has not been addressed because the incidence of clinical APP on the study farm has declined to undetectable levels- therefore another farm has been lined up to achieve objective 2, including a vaccine group in the trial design. This trial will begin in January or February of 2000.

The National Food Safety and Toxicology Center and the Research Excellence Fund (Center for Animal Production Enhancement), Michigan State University have awarded matching funds for this project. To complete the study over multiple farms funding is being sought from the USDA National Research Inititative (Epidemiology of Food Safety) call for proposals due on January 15, 2000. The preliminary data from this pilot study will support that proposal.

Status of project in regards to stated timeline

The scheduled timeline suggested completion of all the pilot farm trials by January 2000. This timetable was to be achieved by carrying out different replications concurrently. This protocol was abandoned to reduce confusion to the farm workers regarding the different groups, to minimize cross contamination and improve the quality control of the laboratory procedures and data collection. The third replication of the trial on farm one will begin in December and is due to finish in April, within the timeline of this grant proposal. Data from this and the other trials will be analyzed as it is collected. The objectives of the pilot study will be completed in the time frame of the grant award from the NPPC.

Modifications of project from original proposal
The modifications have been:

- Elimination of the vaccine group on the pilot farm due to lack of clinical APP disease. Another farm will be studied in 2000, with confirmed chronic APP. This study will include the economic and production analysis of the two control strategies in addition to the antibiotic resistance data.
- Methodology for Salmonella and Campylobacter isolation and identification (see below).
- Only one in feed antibiotic has been studied (ASP-250) due to the unexpected and interesting nature of the results of the first trial (see results), that warranted repetition of the same experiment. Tilmicosin will be included in the new farm, particularly as this antibiotic is often used in APP outbreaks and in endemic herds. In view of the high percentage of tetracycline resistant *E. coli* found in the studies so far and the poor response to Chlortetracycline in the control of APP reported at farm two, this treatment will be excluded fro the study.

Preliminary results
The pilot study was carried out in a 250-sow farrow to finish operation, which fulfilled all the trial requirements. The farm lacked pigs showing clinical signs of APP, therefore the vaccine group was omitted from the first trials. Fecal samples from sows, nursery piglets, growers and finishers were collected to determine the *E. coli* colony forming units per gram (cfu/g) of feces, to allow correct dilution rates during the study. Initial sampling of all pigs revealed major individual variation in cfu/g between pigs, and between the pigs at different ages. Four ten-fold dilutions from 10^{-4} to 10^{-7} allowed enumeration of all plates except for samples collected from finishing pigs and sows. The total coliform count diminished with age, therefore required dilution from a range between 10^{-3} to 10^{-6} to accurately count the colonies.
The culture and identification of *Salmonella* and *Campylobacter* has been optimized. For Salmonella isolation samples are enriched in tetrathionate broth incubated overnight at 42^{o}C in a shaking water bath followed by selective plating on Brilliant Green Agar. Suspect Salmonella colonies are tested by inoculation of Triple Sugar Iron Agar, urea and lysine decarboxylase broth.
Campylobacter isolation relies on a Campylobacter Blood Free Medium (Oxoid) with a selective supplement (CAT- cefoperazone, teicoplanin and anphotericin B) incubated for at least 48 hours in 5% CO_2. Presumptive identification OF Campylobacter relies on a rapid oxidase test and gram staining. Definite identification of the species relies on an API Campy (Biomerieux) identification kit.
Different methods of enumeration of coliforms were explored. A visit to researchers in the University of Guelph, Ontario and Agriculture Canada allowed a study of the use of hydrophobic membrane grids for the analysis of *E. coli* populations. This has started a valuable collaboration with Dr. Scott McEwen and Dr. Friendship (Dept. of Population

Medicine) regarding antibiotic use in swine farms and development of antibiotic resistance. The Hydrophobic membrane grid is an elegant technique, but better suited for processing a limited number of samples. Another method for counting coliforms investigated was by using automatic spiral platting and computerized counting, but the technology was expensive and not designed for a large throughput of samples or differentiation of coliforms on the spiral plates.

During the summer (June 1999), collections for the pilot study began with all samples completed for the first replication. The second replication using the same antibiotic was started in September 1999 and is on going. During the treatment period pigs in the cold nursery were treated in the feed with ASP 250 at 250g/ton for three weeks, whilst the control group received an identical ration without the in feed antibiotic. Treatment was allocated at random. To avoid cross contamination a solid partition and an aisle separated pigs in the two groups and farm personnel wore disposable plastic boots when entering the pens. This farm uses split-sex feeding, therefore experimental groups were separated by gender. There is no evidence of differences between males and females in regards to development of antibiotic resistant flora. Fecal samples (30 per group) were collected at random from pigs at weaning, before treatment (in the hot nursery), after treatment and before slaughter. Samples from 6 pigs from each of 5 pens in either treatment were diluted 1/10 in phosphate buffered saline and homogenized by vortexing for 2 minutes or until the feces were totally suspended. Samples from each pen were pooled and analyzed separately.

The percent resistance values were derived either from individual random samples collected from pigs in a pen or by pooling the 6 fecal samples from that pen. Analysis of the complete data-set shows that both methods of detecting percent resistance are well correlated. On the other hand, correlation of the % resistance of the pooled samples versus the sample mean for each antibiotic or collection period shows a good correlation for ampicillin and sulfa, but is poorly correlated regarding resistance to tetracycline. From a practical stand-point the advantages of pooling are considerable allowing a reduced number of agar plates and processing time. Poor correlation for tetracycline resistance between the two methods could be due to the elevated counts encountered. In the pilot farm, a very high proportion (79.9% average for all samples) of isolates were resistant to tetracycline (32□g/ml) independently from treatment group or age.

The treatment group had a reduced percentage of ampicillin and sulfamethoxazole resistant coliforms in the fecal samples and the difference was statistically significant (see graphs). On the other hand resistance to tetracycline decreased more dramatically in the group without antibiotics. These findings for ampicillin resistance persisted in fecal samples collected from pigs prior to slaughter, two months after the different treatments were applied. These results are intriguing, but require further analysis and replication, before any firm conclusions can be reached. Duplicating these results on other farms will also be required, and this work will begin in January/February of 2000.

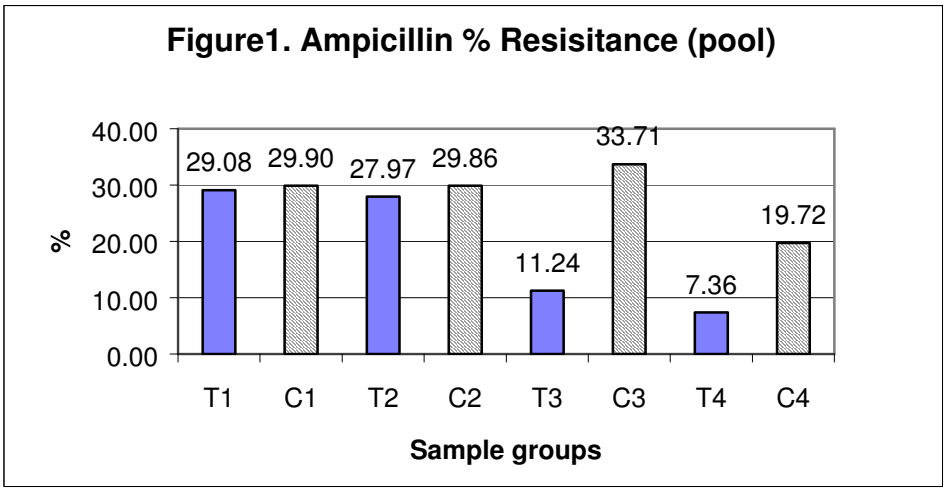
Individual *E. coli* colonies (5 per sample) were also tooth-picked onto MacConkey agar with ampicillin, sulfamethoxazole or tetracycline to determine individual isolate susceptibility to those antibiotics. Strains of *E. coli* isolated from piglets at weaning and 3 weeks later were <u>all</u> resistant to tetracycline and sulfonamides. The tests were repeated on Mueller Hinton Agar with all 3 antibiotics with identical results. Isolates from samples before treatment indicated that a quarter of all *E. coli* isolated were resistant to ampicillin and there was no difference between both groups (treatment vs control). After treatment some tetracycline and sulfa susceptible strains were isolated. The unmedicated group had less *E. coli* isolates exhibiting triple antibiotic resistance than the treated group. These results do not support our hypothesis, but these findings

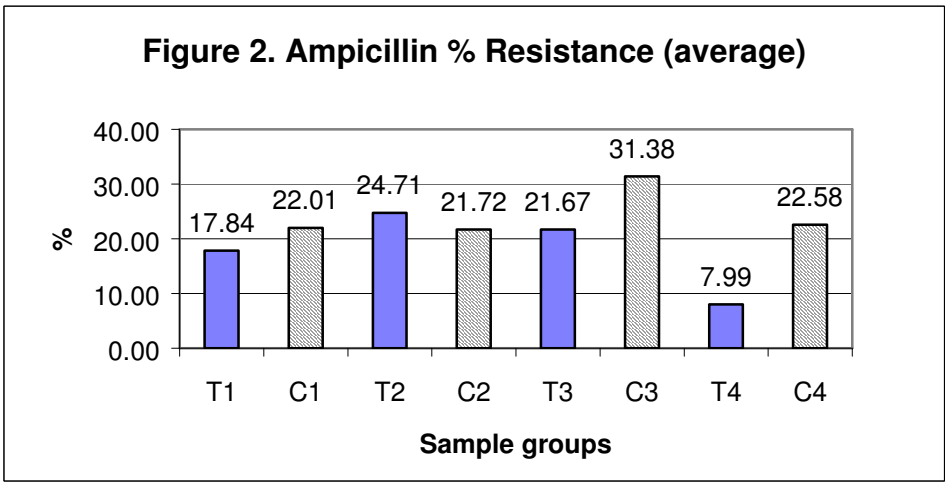
support the data from the analysis of the resistant coliform population in feces (see above).

Salmonella and Campylobacter isolation from individual samples has been carried with a small number of isolates both from the treated and control pigs. More Campylobacter strains were isolated in young pigs, whilst Salmonella was isolated at similar rates from all ages. Further work characterizing these isolates is on going. All Isolates have been collected and stored frozen for further work including calculation of the Minimum Inhibitory Concentration using broth microdilution and the Sensititre System. A semi-automated Sensititre Apparatus has recently being purchased by the Food Safety and Toxicology Center and will be utilized for this purpose. Isolates collected from trial 1 include 3,400 *E. coli*, 20 *Salmonella* (untyped) and 15 *Campylobacter spp* isolates.

Data from this pilot study will be used to support the grant proposal to the USDA National Research Initiative 2000, to expand the investigation to multiple farms. Presentations of preliminary data will be carried out in December to the Bad Bug Club a discussion and journal club with faculty and Graduate students from the Microbiology, Large Animal Clinical Sciences, Food Science and Toxicology Departments at Michigan State University. In February a presentation to Michigan Pork Producers will be given as part of the Michigan Pork Symposium. Other national meetings will be targeted for presentation in the coming year, including the Al Leman Conference and the Conference of Research Workers in Animal Diseases.

Figures 1-6. Percentage of antibiotic resistant E. coli- calculated as follows: Number of E. coli on MacConkey Agar/ Number of E. coli on MacConkey+ antibioticsX 100. Three different antibiotic used- ampicillin (32□g/ml), sulphamethoxazole (800□g/ml) and tetracycline (32□g/ml).
Data is calculated from the individual samples and averaged for each collection group or from the average of the pools from each group.
Treatment group (T1-4) includes the samples from pigs treated with the in feed antibiotic (represented by lighter bars on the chart).
T1- weaning, T2- before medication, T3- after medication, T4- Pre-slaughter.
Control group (C1-4) includes the samples from pigs not medicated during the three week period.
C1- weaning, C2- before medication, C3- after medication, C4- Pre-slaughter.





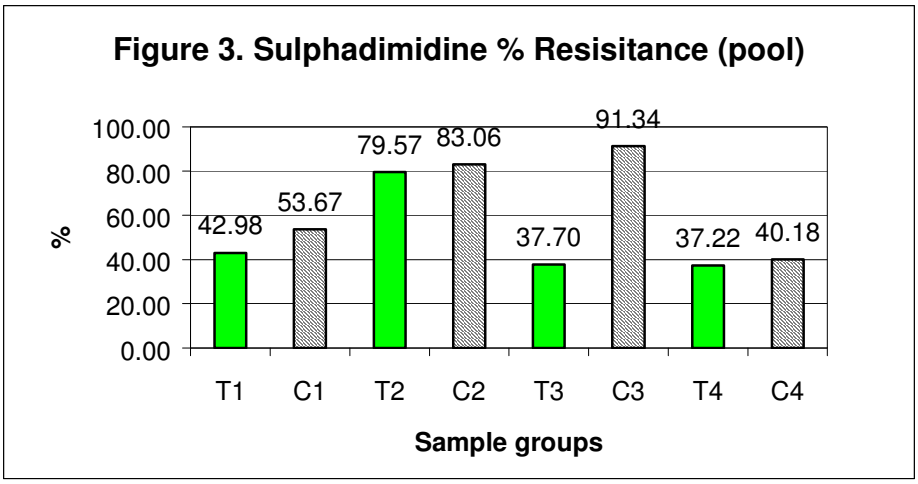


Figure 3. Sulphadimidine % Resisitance (pool)
100.00
80.00
60.00
40.00
20.00
0.00
%
42.98
53.67
79.57
83.06
37.70
91.34
37.22
40.18
T1
C1
T2
C2
T3
C3
T4
C4
Sample groups

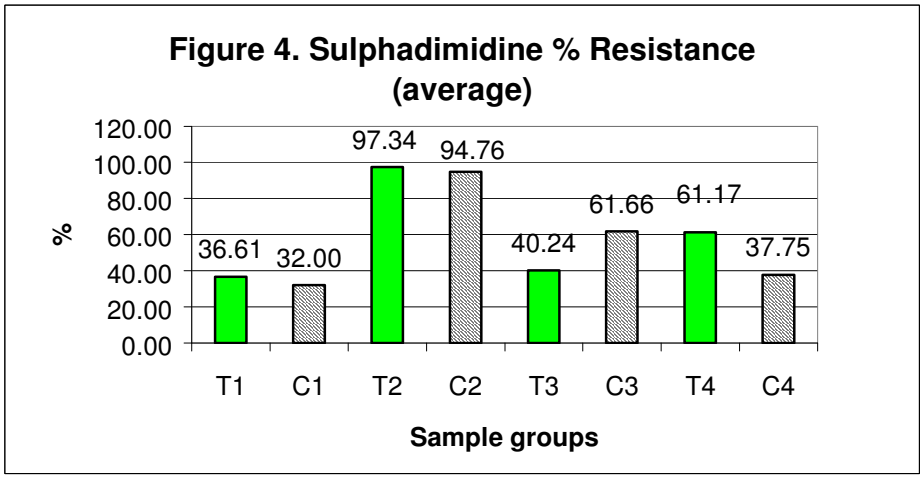


Figure 4. Sulphadimidine % Resistance (average)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
%
36.61
32.00
97.34
94.76
40.24
61.66
61.17
37.75
T1
C1
T2
C2
T3
C3
T4
C4
Sample groups

Figure 5. Tetracycline % Resistance (pool)

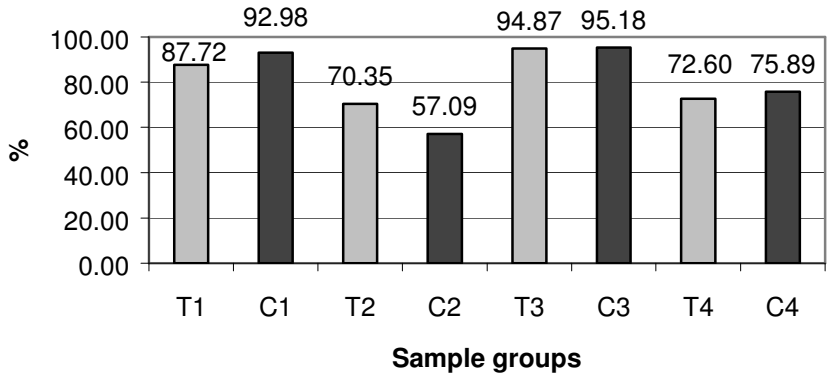


Figure 6. Tetracycline % Resistance (average)