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# Impact of medicated feed along with clay mineral supplementation on *Escherichia coli* resistance to antimicrobial agents in pigs after weaning in field conditions

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ABSTRACT

The aim of this study was to examine changes in antimicrobial resistance (AMR) phenotype and virulence and AMR gene profiles in *Escherichia coli* from pigs receiving in-feed antimicrobial medication following weaning and the effect of feed supplementation with a clay mineral, clinoptilolite, on this dynamic. Eighty *E. coli* strains isolated from fecal samples of pigs receiving a diet containing chlortetracycline and penicillin, with or without 2% clinoptilolite, were examined for antimicrobial resistance to 15 antimicrobial agents. Overall, an increased resistance to 10 antimicrobials was observed with time. Supplementation with clinoptilolite was associated with an early increase but later decrease in $bla_{CMY-2}$, in isolates, as shown by DNA probe. Concurrently, a later increase in the frequency of $bla_{CMY-2}$ and the virulence genes *iucD* and *tsh* was observed in the control pig isolates, being significantly greater than in the supplemented pigs at day 28. Our results suggest that, in the long term, supplementation with clinoptilolite could decrease the prevalence of *E. coli* carrying certain antimicrobial resistance and virulence genes.



## 1. Introduction

Antimicrobial use in animal production has been monitored over the past two decades because of potential adverse effects on animal and human health related to antimicrobial resistance. Administration of antimicrobials in animal production began early after their initial discovery, primarily for treatment of diseases, but also for promoting growth and for disease prevention. The latter is particularly important because animals are commonly housed at high densities that can facilitate the spread of disease. Nevertheless, there are disadvantages associated with antimicrobial use in animal production. For instance, administration of antimicrobials may select, or co-select for the presence of AMR genes in the commensal or pathogenic bacterial populations in animals and lead to a breakdown in the treatment of associated diseases or transfer of resistance pathogenic bacteria to humans by contaminated meat. AMR genes may also be transferred to human pathogens and lead to problems in the treatment of disease in human patients (Johnston, 2001; Wegener, 2003). In light of these disadvantages, animal feeds maybe supplemented with other feed additives as alternatives for or a complement to, the use of antimicrobials. For example, the clay mineral clinoptilolite has been used in an attempt to improve performance and health but also as an alternative to the use of antimicrobials or together with antimicrobials for prevention of diarrhea in weaned piglets (Papaioannou et al., 2004). However, little is known of the mechanism of action of clinoptilolite. It has been demonstrated that clay minerals induce bacterial mutation and promote genetic variability of bacteria (Yoshida et al., 2004), chromosomal and plasmid DNA bound to clay minerals being more resistant to degradation by DNase I than free DNA (Romanowski et al., 1991). Some in vitro studies have shown that clay minerals promote direct horizontal transfer of AMR genes in different bacterial species (Lotareva and Prozorov, 2000; Rodriguez-Beltran et al., 2013). These data suggest that clay minerals may modulate the prevalence of AMR and virulence genes of bacteria in the animal gut.

In weaning pigs, antimicrobials are used in feed primarily as a medication to prevent disease and thus reduce mortality and morbidity (Cromwell, 2002). Among the most frequently used in-feed antimicrobials are penicillin and tetracycline, often in combination (Akwar et al., 2008). Use of in-feed antimicrobials in pigs has been associated with increased resistance of fecal *Escherichia coli* within and between classes of antimicrobials (Akwar et al., 2008; Kim et al., 2005). The main objective of the present study was to examine the dynamic of AMR phenotype and virulence profiles in *E.coli* and AMR gene profiles in these isolates from pigs receiving a diet containing chlortetracycline and penicillin in therapeutic doses following weaning on a commercial

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farm and to investigate the effect of simultaneous feeding of the clay mineral, clinoptilolite, on this dynamic.

## 2. Materials and methods

### 2.1. Antimicrobial medication regime

Pigs from a commercial crossbred genetic line were weaned at 21 days of age and transferred to pens in the nursery barn. Pigs received a standard commercial diet, three different rations (1, 2 and 3) being given continuously in feeders during the 28 days of the trial. The feed contained the following antimicrobials: chlortetracycline (Aureomycin® 220 G) in therapeutic doses as prescribed for protection of respiratory problems and penicillin G (Pen-P 110) at a metaphylactic dose as prescribed to prevent *Streptococcus suis* infections. Antimicrobials were used in feed as follows: chlortetracycline (1100 g/t) from day 0 to day 7; chlortetracycline (660 g/t), penicillin G (198.6 g/t) and IVOMEC from day 8 to day 14; and chlortetracycline (660 g/t) and penicillin G (198.6 g/t) from day 15 to day 28. On days 3 and 5 after weaning, all pigs received a circovirus (Circumvent® PCV) and mycoplasma (Myco Silencer® Once) vaccine, respectively.

### 2.2. Trial design, collection and preparation of samples

This study was conducted on 168 pigs. At day 0 (first day of placement in the nursery), the pigs were randomly divided into 2 groups: control (C) and supplemented (S). Feeding and housing conditions were identical for the two groups, except for the addition of 2% clinoptilolite to the feed of the supplemented group. The control group received a standard commercial feed (basal diet) and the supplemented group received the basal commercial diet with 2% clinoptilolite. Both groups were housed in the same room, each group allocated into 3 different pens, 28 pigs being placed in each pen, so that the average pen weights were similar.

Twelve pigs were tagged in each group to permit individual follow-up. Samples were collected at days 0, 2, 7, 14, and 28 after weaning, either directly from the rectum of the tagged pigs using a cotton swab or by pooling feces from five sites on the floor of the pens as described by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (CIPARS, 2008). On each sampling day, 8 samples were collected per group and were shipped on ice to the laboratory for further analysis. The method used for sample preparation was a modification of the CIPARS protocol (CIPARS, 2008). Briefly, floor fecal samples were diluted to 1/10 (weight-for-weight) in buffered peptone water and 200 μl of diluted fecal samples and rectal swabs were inoculated into 5 ml Luria Bertani broth (LB – Difco, USA) and incubated overnight at 37 °C. DNA templates were prepared from the processed samples by boiled cell lysis for examination by PCR, as described previously by Maluta et al. (Maluta et al., 2014).

Processed fecal samples (a total of 80 samples consisting of 60 floor samples and 20 rectal samples) were serially diluted in peptone water to $10^{-5}$, from which a last dilution was made in peptone water containing 1% tween 80 to obtain a $10^{-7}$ diluted subsample (approximately $10^{2}$ CFU/ml) (Sharpe and Peterkin, 1988). A volume of 2 ml of this final dilution was filtered through a hydrophobic grid membrane filter (HGMF) using a Spreadfilter (Filtaflex, Almonte, Ontario, Canada). Filters were placed onto MacConkey agar plates and incubated overnight at 37 °C to obtain HGMF master filters bearing predominantly *E. coli* lactose-positive colonies.

### 2.3. Bacterial isolation and identification

A total of 80 presumptive *E.coli* isolates were randomly selected from HGMF master filters to represent the different sampling days (except for day 2) and the two groups (n = 40 for each of the groups supplemented or not with clinoptilolite). Isolates were identified as *E. coli* by biochemical tests (Simmons Citrate Agar, mobility and indole) and by PCR for the presence of *uidA E.coli* housekeeping gene, which encodes Beta-glucuronidase (Table 1 in the supplemental material).

### 2.4. Antimicrobial susceptibility testing

The selected *E. coli* strains (n = 80) were examined for susceptibility to the same 15 antimicrobial agents examined in the CIPARS surveillance program in Canada (CIPARS, 2008) using the disk-diffusion (Kirby–Bauer) assay. Bacterial strains grown overnight on blood agar were mixed in tubes containing 10 ml of sterile water to reach a turbidity of 0.5 McFarland Standard. The contents of the tubes were placed onto Mueller–Hinton agar plates using a sterile swab. The plates were incubated at 37 °C for 24 h, and the diameters of the zones of complete inhibition were measured. The strains were recorded as susceptible, intermediate, or resistant according to the zone diameter interpretative standards recommended by Clinical and Laboratory Standards Institute (CLSI) in 2010 (CLSI, 2010) for most of the antimicrobials and in 2008 for ceftiofur (CLSI, 2008). The following antimicrobial disks were used; amikacin (30 μg), kanamycin (30 μg), gentamicin (10 μg), streptomycin (10 μg), ceftriaxone (30 μg), ceftiofur (30 μg), cefoxitin (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), trimethoprim–sulfamethoxazole (23.75 μg), sulfisoxazole (250 μg), ampicillin (10 μg), amoxicillin/Clavulanic acid (30 μg), chloramphenicol (30 μg), and tetracycline (30 μg).

### 2.5. PCR for determination of virulence and AMR genes

Boiled cell lysates from the samples and the 80 selected *E. coli* strains were tested by multiplex PCR to determine the presence of the virulence genes which define the *E. coli* pathotypes commonly found in animals: Enterotoxigenic *E. coli* (*eltB*, *estA*, *estB*), Enteropathogenic *E. coli* (*eae*), Shiga toxin-producing *E. coli* (*stxA*, *stx2A*) and Extraintestinal pathogenic *E. coli* (*cnf*, *papC*, *iucD*, *and tsh*). PCR procedures for detection of these genes were performed according to the protocol of the Reference Laboratory for *Escherichia coli* (EcL- Faculté de Médecine Vétérinaire de L'Université de Montréal) available in the animal pathogenic zoonotic *Escherichia coli* website (http://apzec.ca/en/Protocols). The presence of the AMR genes $bla_{TEM}$, $bla_{CMY-2}$, *tetA*, and *aadA1* was determined by PCR using primers and conditions as shown in Table 1 in the supplemental material.

### 2.6. Phylogenetic analysis

Phylogenetic grouping was carried out for the 80 selected *E. coli* strains using a multiplex PCR-based assay as described by Clermont et al. (Clermont et al., 2000). Based on the presence or absence of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TSPE4.C2), strains were classified into four main *E.coli* phylogenetic groups (A, B1, B2, or D).

### 2.7. Detection of AMR and virulence genes in an extended collection of E. coli isolates

A HGMF Interpreter (Filtaflex, Almonte, Ontario, Canada) was used to count presumptive *E. coli* colonies on HGMF master filters, which were then replicated using a HGMF Replicator (Filtaflex, Almonte, Ontario, Canada), placed onto MacConkey agar plates, and incubated overnight at 37 °C to obtain HGMF replicates. The positive and negative control strains used in the hybridization process were also filtered and replicated as described above. To generate DNA hybridization probes to detect each of the virulence or antimicrobial resistance genes, templates were prepared from overnight LB cultures of the appropriate control strains. Digoxigenin (DIG) alkaline phosphatase labeled probes were generated by using the specific primers (Table 1 in the supplemental material and (http://apzec.ca/en/Protocols) and a PCR DIG Probe Synthesis Kit (Roche Diagnostics) following instructions. The bacterial colonies on the HGMF replicates were pre-treated as described