RESEARCH REPORT
PORK SAFETY

Title: Use of sodium chlorate to eliminate gram-negative pathogens in live hogs: Dose titration study to determine safe tissue residues, **NPB #05-043**

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Abstract

An experimental chlorate-based product (ECP) has been developed which selectively kills or eliminates *Salmonella* species and *E. coli* O157:H7 in the gastrointestinal tracts of live swine. Several studies by Robin Anderson of USDA ARS Food and Feed Safety Research Laboratory in College Station, TX have clearly demonstrated the efficacy of ECP at eliminating these pathogens from grower and finisher hogs. Prior to the successful commercial use of ECP as a feed or water additive, the identity and magnitude of chlorate-based residues remaining in edible tissues of treated swine must be determined. Such data will be useful in the safety assessment of ECP as a possible pre-harvest food safety tool.

In this study three sets of two pigs, each consisting of a barrow and a gilt, were orally dosed with a total of 20, 40, or 60 mg of sodium [^{36}Cl]chlorate per kg body weight via the drinking water. Urine and feces were collected throughout the 30-h study. Twenty-four h after the last exposure to [^{36}Cl]chlorate, each pig was harvested and both edible and inedible tissues were collected. Urine and tissue samples were analyzed for total radioactive residues and for chlorate metabolites. Greater than 80% of the radioactivity was eliminated in the urine regardless of dose, indicating that most of the ECP was absorbed from the gastrointestinal tract. Fecal elimination of radioactivity averaged 1.1% of the dosed radiochlorine across all doses. Parent chlorate always represented greater than 97.4% of the urinary radiochlorine with the remaining radiochlorine being excreted as chloride ion (the form of chlorine found in table salt). Chlorate represented 39 to 77% (7 to 110 ppm on a concentration basis) of fecal radioactivity, depending upon the dose. These results indicate that ECP delivered via the drinking water is delivered to the lower gastrointestinal tract in an inefficient manner. ECP concentrations in edible tissues ranged from 0.01 to 0.49 ppm, with residues in liver and skeletal muscle generally being lower than those in kidney and adipose tissue. In edible tissues, chlorate concentrations fell well below provisional safe tissue concentrations estimated by the US FDA Center for Veterinary Medicine. Chlorate residues were concentrated in thyroid tissues (7.7 to 25.4 ppm) relative to edible tissues, however. No evidence for the presence of chlorite was observed in excreta or in tissues and the only chlorate metabolite present was chloride, a naturally occurring nutrient. Results of this study suggest that further development of chlorate as a pre-harvest food safety tool in swine merits serious consideration.

Introduction

A significant problem in the swine industry is the post-harvest contamination of animal carcasses with gram-negative bacteria that may subsequently cause human illness. Although several post-harvest intervention strategies have been developed that reduce the number of these pathogens on animal carcasses, none are entirely satisfactory because of cost, consumer or packer acceptance, or lack of efficacy. A fundamental problem with post-harvest intervention strategies is that they are directed towards the remediation of carcasses already contaminated with pathogens.

Anderson et al. (2000), of the Food and Feed Safety Research Unit (USDA-ARS, College Station, TX) recognized that sodium chlorate ($NaClO_3^-$) has the potential to serve as an effective pre-harvest food safety tool in live animals. Gram-negative pathogens such as *E. coli* O157:H7 and various *Salmonella* species contain specific nitrate reductase enzymes that, under normal circumstances, allow the bacteria to convert nitrate to nitrite (Stewart, 1988). Anderson et al. realized that chlorate may also serve as a substrate for these enzymes and that when chlorate is used by the enzyme, it is converted to the bacterial toxin chlorite (ClO_2^-; van Wijk and Hutchinson; 1995). The vast majority of bacteria present in swine gastrointestinal tracts, however, do not possess nitrate reductase making it an attractive target for development of a pathogen-specific food safety tool. Knowing that chlorite is cytotoxic to bacteria, Anderson et al. hypothesized that when sufficient levels of chlorate are present in the alimentary tract, pathogens will generate "suicidal" levels of intracellular chlorite and will die; those organisms that do not express nitrate metabolizing enzymes were proposed to be unaffected by chlorate.

Studies in both ruminants and non-ruminants have validated this hypothesis. For example, chlorate significantly reduced *E. coli O157:H7* populations in gastrointestinal tracts of cattle and sheep (Callaway et al., 2002; Callaway et al., 2003), but had little effect on bacterial counts of total culturable anaerobes in ruminal fluid (Anderson et al., 2000). Market-age broilers given access to a chlorate-containing product during the 48 hours prior to slaughter had significant reductions (40-99%) in crop and cecal *Salmonella* populations (Byrd et al., 2003).

In swine, treatment with chlorate was highly effective at reducing populations of both *E. coli* O157:H7 (Anderson et al., 2001a) and *Salmonella* serotype Typhimurium (Anderson et al., 2001b; 2004). Gastrointestinal levels of E. Coli O157:H7 decreased 1.03 to 2.9 log units (a 62 to 99.9% reduction, depending on tissue) when sodium chlorate was administered to experimentally infected pigs (Anderson et al., 2001a) and euthanized 8 hours after the last chlorate administration. In weaned pigs artificially infected with *Salmonella* Typhimurium (Anderson et al., 2001b), a huge difference in pathogen numbers existed between control animals and chlorate treated animals. For example, pigs treated with chlorate contained only about 3 colony-forming units (CFU) of *Salmonella* Typhimurium per gram of cecal contents, whereas control animals contained approximately 1,400 CFUs of the pathogen. Commensurate with these results are those of Anderson et al.

(2004) who demonstrated that chlorate eliminates *Salmonella* Typhimurium in finishing hogs. The numbers of CFUs in cecal contents and the incidence of animals testing positive for *Salmonella* were both decreased after treatment with chlorate. Independently, Burkey et al. have shown that sodium chlorate feeding reduces fecal shedding of *Salmonella enterica* in swine (Burkey et al., 2004), and that sodium chlorate did not adversely affect animal performance (Burkey et al., 2003).

Collectively, these data suggest that a chlorate-containing product could have several commercial applications with the pre-slaughter elimination or reduction of both *E. coli* and various *Salmonella* species being of primary importance. Burdens of pathogenic bacteria in live swine could be reduced in a cost effective and convenient (via feed or water) manner and the use of such a chlorate-containing product could significantly lessen the probability that swine carcasses are contaminated with pathogens at slaughter.

Studies investigating the efficacy of ECP in live hogs have progressed to the degree that knowledge of chlorate residues in swine is critical to further development of a chlorate-based product. Because sodium chlorate is not naturally-occurring and will ultimately be added to swine feed or water, the US FDA CVM must approve its use. To date, no studies have been conducted investigating the magnitude of residues or degree of chlorate metabolism in hogs. The purpose of this study was to provide data either supporting or refuting the safe use of sodium chlorate in growing hogs.

Objectives

- To determine chlorate concentrations remaining in edible tissues of swine after oral administration of three levels of sodium chlorate. Doses selected will be at, above, and below doses previously shown to have efficacy in swine.
- To determine the metabolism of chlorate in swine
- To determine the absorption and elimination of chlorate in tissues and excreta of swine after oral administration

Materials and Methods

Chemicals

Non-labeled chemicals and reagents were obtained from well known vendors. Radiolabeled sodium chlorate ($Na^{36}ClO_3$) was purchased from Ricerca Biosciences (Concord, OH). Stock sodium [^{36}Cl]chlorate was purified on Sephadex G-10 to a radiochemical purity of 99.9% essentially as described by Ruiz-Cristin et al. (1989). Radiochemical purity of the purified [^{36}Cl]chlorate peak was assessed by ion chromatography as described by Smith et al.(2005a).

Dose Preparation.

Purified [^{36}Cl]chlorate was diluted with non-radioactive sodium chlorate to a specific activity of 399 ± 1 dpm/μg. Specific activity was determined as described by Smith et al. (2005a). Three dosing solutions (1 L each) containing 7.5, 15, and 22.5 mM sodium [^{36}Cl]chlorate, respectively, were prepared in aqueous solutions of 2.5 mM sodium nitrate. Sodium nitrate has been shown to increase the efficacy of chlorate in reducing

pathogen numbers in live animals (Jung et al., 2003), presumably by inducing respiratory nitrate reductase in nitrate respiring bacteria. Each dosing solution was transferred to duplicate 1-L plastic water bottles (Kaytee®; Kaytee Products, Chicago, IL) so that water bottles contained 498 ± 0.5 g of dosing solution. Sipper tubes, supplied with the water bottles, were attached and the bottles were stored frozen until dosing.

Animals and Animal Dosing

Three crossbred barrows (9.2 ± 0.4 kg) and gilts (8.2 ± 0.8 kg) were purchased from the North Dakota State University swine herd. Animals were ear tagged and housed by gender in concrete-floored pens during an 18 to 25 d acclimation period. Pigs were provided with ad-libitum access to a swine starter ration (21.6% crude protein, 3.3% fat, 2.6% fiber; 3204 kcal/kg metabolizable energy; 77.7% total digestible nutrients; North Dakota State University Feed Mill), which they received for the duration of the study. During the acclimation period, pigs were trained to metabolism crates (Pekas, 1968).

Low, medium, and high chlorate doses were each administered to a single barrow or gilt in each of two periods (i.e., within period, a low, medium, and high dose was administered to three swine). The pigs drank the chlorate containing water as it thawed such that the total dose was delivered to pigs within 6.1 ± 0.8 h instead of the 24 h as originally planned. Nevertheless, a 24-h withdrawal period was maintained, and pigs were harvested at 24.0 ± 0.1 h.

Collection of Excreta

Urine and feces excreted in the 0-12, 12-24, and 24-30 h time periods were pooled within excreta type for each animal, were weighed, and frozen. At collection, urine and feces were collected as quantitatively as possible.

Animal harvest and tissue collection

Pigs were harvested at the appropriate time by captive-bolt stunning followed by exsanguination into a weighed basin. Pigs were washed and eviscerated. Edible tissues (adipose tissue, skeletal muscle, liver, and kidney) and non-edible tissues (bile, blood, bone, brain, diaphragm, gastrointestinal contents, gastrointestinal tract, heart, lung, skin, spleen, thyroid gland, and remainder of the carcass) were collected. The gastrointestinal tract, from the esophagus to the anus was removed (with pancreatic tissues attached); the gastrointestinal contents were removed, and the gastrointestinal tissue and contents were each weighed, the gastrointestinal contents, sub sampled, and both contents and tissue were frozen. Pigs were boned, bones were weighed, and the scapula removed as the bone sample. The total muscle was weighed and a sub sample removed from the *longissimus dorsi*.

Partially thawed tissues of masses sufficient to pass through a grinder with greater than 50% recovery (brain, diaphragm, GI-tract, heart, kidney, liver, lung, and skeletal muscle) were ground; the spleen and thyroid gland were homogenized on dry ice as described by Benville and Tindle (1970). Adipose tissue was ground with a mortar and pestle after the addition of liquid N_2. Skin was prepared for total residue analysis by placing

10 ± 0.1 g aliquots into a glass container, adding 90 mL of 1 N NaOH, weighing, and incubating at 46 °C for approximately 60 h. Bone was prepared by dissolving approximately one-half of the scapula in 350 mL of concentrated NaOH over 72 h at 90 °C. The solubilized bone solution tended to gel upon cooling, therefore bone solutions were re-heated prior to analysis by LSC (described below).

Analytical Methods

Liquid scintillation counting techniques, determination of background radiochlorine, and speciation of total radioactive residues were conducted essentially as described by Smith et al. (2005b) with the following exceptions. Urine and tissue sample sets were run with both blanks and blanks fortified with known amounts of [^{36}Cl]chloride and [^{36}Cl]chlorate to determine recovery.

Results

Objective 1. *Determine chlorate concentrations remaining in edible tissues of swine after oral administration of three levels of sodium chlorate.*

Table 1 shows the residues of parent chlorate in edible tissues of swine. Residues were lowest in liver and greatest in adipose tissue (which had a fat content of only 62% on a wet basis). Nevertheless, chlorate residues in edible tissues of growing swine were always less than 25% of the FDA-estimated safe tissue concentration. Because residues were always less than the estimated safe tissue concentration, even for the high dosed animals, the experimental chlorate product has an excellent residue profile for a feed additive. Also shown in Table 1 is the chlorate concentration of thyroid glands. Thyroid gland chlorate concentrations were always greater than 3 ppm and represented greater than 33% of the total radioactive residue in thyroid (the remaining residue was chloride ion). Thyroid chlorate residues will likely have little to no impact on a potential ECP product because thyroid is considered to be an inedible tissue.

Table 1. Chlorate residues in edible tissues and thyroids of swine.

Tissue	Dose[a] Low	Medium	High	% of FDA STC[b]
	ppm	*ppm*	*ppm*	*%*
Liver	0.01	0.02	0.04	<2
Skeletal muscle	0.07	0.07	0.18	<25
Kidney	0.18	0.20	0.19	<5
Adipose tissue	0.19	0.13	0.49	<15
Thyroid	8.4	7.7	25.4	Not edible

[a]Low, medium, and high doses were 20, 40, and 60 mg/kg , respectively

[b]Residue values are expressed as a percentage of the Food and Drug Administration estimated safe tissue concentration for chlorate

Objective 2. *Determine the metabolism of chlorate in swine*.

Parent chlorate was nearly quantitatively excreted in the urine of dosed hogs. Figure 2 shows the cumulative urinary excretion of parent chlorate and its metabolite chloride ion in urine. Chloride ion always represented less than 4% of the total raciochlorine excreted in urine. No chlorite (ClO_2^-) was measured in urine, feces, or any of the tissues analyzed. In tissues, choride ion represented the major portion of the total radioactive residue. For example, in liver, chloride comprised >98% of the total radioactive residue. In kidney, skeletal

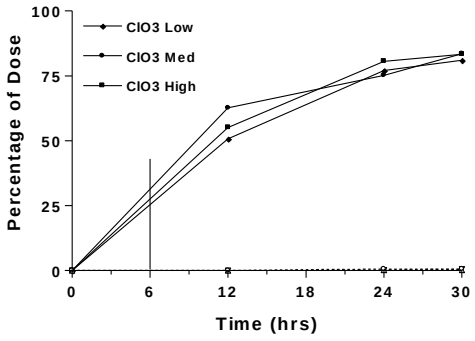

Figure 1. The cumulative urinary excretion of chlorate (solid lines) and chloride (hatched lines, barely visible on the "X" axis) in orally dosed swine. The vertical line at 6 hours represents the termination of chlorate exposure.

muscle, and adipose tissue, chloride represented greater than 92.5, 86.0, and 84.8 percent of the total radioactive residue, respectively. In each tissue, no chlorite was present at the detection limits (approximately 19 ppb). Thus, the only metabolite of chlorate detected in this study was chloride, a naturally occurring nutrient common to all life forms.

The fact that chloride was the only metabolite present in edible tissues is important for two reasons. First, it is a naturally occurring nutrient which, from a food-safety perspective, is inconsequential. The portion of an animal feed additive that is metabolized to a naturally occurring metabolite or nutrient is discounted from evaluation as a residue of food safety concern. Chloride is naturally present in swine tissues, so it would pose no health risks to consumers of chlorate treated swine. Second, the absence of chlorite in edible tissues of treated animals simplifies the regulatory evaluation of residues because FDA considers chlorite a potentially toxic metabolite of chlorate.

Objective 3. *To determine the absorption and elimination of chlorate in tissues and excreta of swine after oral administration*

The apparent absorption of chlorate is shown in Table 2. Apparent absorption, defined as the summation of radioactivity present in edible and non-edible tissues (excluding gastrointestinal contents) and in urine, was about 87%. This value is likely an underestimate of true absorption for two reasons: 1) nearly 10% of the dose was recovered in cage washes which contained primarily urinary radiochlorine; and 2) chloride ion is extensively recycled via excretion into the stomach as HCl and subsequent resorption in the lower GI tract as Cl^-. Relatively small quantities of radiochlorine were excreted into feces (0.1 to 2.3% of the dose).

Figure 2 shows the radiochemical composition of residues excreted into feces during the 6 hour period immediately prior to slaughter. Chlorate comprised from 39 to 77% of the total radioactivity in fecal residues of individual animals. The corresponding chlorate concentrations ranged from 7 to 110 ppm in feces of individual swine.

Discussion

Data generated in this study clearly demonstrate that chlorate was rapidly absorbed and excreted in the urine of swine. In these pigs, 83.1 ± 2.6% of the radiochlorine present in the initial dose was excreted in the urine during the 30-h study period with 56.2 ± 8.5% of the dosed radiochlorine excreted during the first 12 h of the study. Overall, 67% of the total radiochlorine excreted in the urine was excreted during the first 12 h of the study. The rapid absorption and elimination of chlorate clearly indicates that oral delivery of chlorate via the drinking water is an inefficient means to deliver chlorate to the lower gastrointestinal tract. Presumably, a more efficient delivery of chlorate to the lower gastrointestinal tract would increase the efficacy of killing pathogens. Nevertheless, even with the inefficient delivery of chlorate to the lower gastrointestinal tract, numerous studies have demonstrated chlorate's efficacy against *E. coli* and *Salmonella enterica* in swine (Anderson et al. 2001a,b; 2004) dosed in a manner similar to the procedure used in this study.

Table 2. Apparent absorption and total recovery of radioactivity in [^{36}Cl]chlorate in growing swine. Data are expressed as percentages of the total radiochlorine administered

Item	Low Dose	Medium Dose	High Dose
Edible tissues	1.15	0.95	0.90
Inedible tissues	4.20	3.10	2.80
Urine	81.60	83.70	83.85
Apparent absorption	**86.95**	**87.75**	**87.55**
Gastrointestinal contents	0.50	0.35	0.35
Feces	0.85	2.30	0.10
Cage wash	7.90	7.25	7.80
Total Recovery	**96.20**	**97.65**	**96.30**

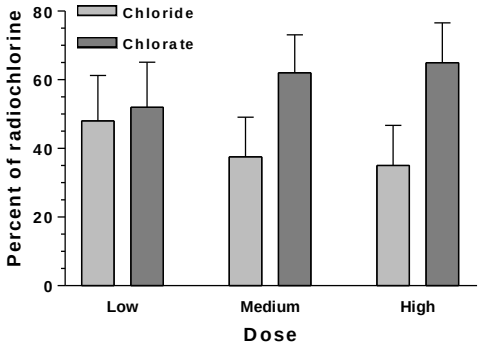

Figure 2. Radiochemical composition of residues 24-30 hour feces of growing swine. Chlorate concentrations ranged from 7 to 110 ppm.

Chlorate concentrations of 1.25 mM (equivalent to 160 ppm) in bovine ruminal fluid were sufficient to cause 3-log unit reductions of *E. coli* O157:H7 and *Salmonella* Typhimurium (Anderson et al., 2000). In this study, feces excreted during the 6-h period immediately prior to slaughter contained 7 to 110 ppm of chlorate residue. Thus, chlorate concentrations in these swine were below chlorate concentrations previously shown to be active against relevant pathogens *in vitro*. Total radioactive residues in gastrointestinal contents at slaughter were only 1 to 4 ppm. It is not known if chlorate is active against gram-negative pathogens at levels below this, but the low gastrointestinal residues at 24 h might help to explain why chlorate reduced swine cecal *Salmonella* Typhimurium concentrations about 3 log units 16 h after the last exposure to chlorate, but had no effect 24 h after the last chlorate dose (2001b). Anderson et al. (2001b) suggested that the absence of a chlorate effect at 24-h was a function the kinetics of chlorate in live swine. The current study serves to emphasize Anderson et al's point that there is a "need to develop practical administration procedures that optimize delivery and maintenance of effective concentrations of chlorate to the lower gut".

Even with the high degree of chlorate absorption, chlorate residues in edible tissues of growing swine were always less than 25% of the FDA estimated safe tissue concentration for chlorate. Tissue residues this low strongly suggest that chlorate is an excellent candidate for development into a safe and effective feed or water additive for use in swine.

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Lay Interpretation.

Each year thousands of US consumers become ill because they have eaten food products that are contaminated with pathogenic bacteria such as *Salmonella* or *E. coli* O157:H7. Intense efforts have been made during the last decade to eliminate pathogens which contaminate food animals. To date, no single strategy to eliminate pathogens from food animal products has been widely accepted. A new pre-harvest food safety strategy utilizing an experimental chlorate product (ECP) has been developed that greatly reduces, or even eliminates gram-negative pathogens from swine. Use of ECP has not been approved by regulatory organizations because it is not known whether residues present in edible tissues of treated animals represent a health risk to humans. The purpose of this study was to determine the magnitude and identity of ECP residues in edible tissues of growing swine after oral administration. For all of the doses tested, chlorate residues in liver, kidney, muscle, and fat always fell below 25% of the estimated FDA-estimated safe tissue concentration. The only metabolite of ECP present in tissues was chloride ion, a naturally-occuring nutrient. The study also clearly demonstrated that most of the ECP delivered in drinking water was absorbed from the gastrointestinal tract and was eliminated very rapidly in urine. Thus, delivery of the ECP to its intended site of action, the lower gastrointestinal tract, was very inefficient. Development of an ECP formulation that would prevent absorption from the upper GI tract would likely increase efficacy against pathogens in the lower gastrointestinal tract and would likely decrease tissue residues even further. Further research on ECP is warranted because ECP could have a significant impact on lowering the incidence of harmful bacteria on pork carcasses, and because tissue residues of ECP fall below levels that regulatory agencies have estimated to be safe.