Improved control of multiple-antibiotic-resistance-related microbial risk in swine manure wastes by autothermal thermophilic aerobic digestion
Il. Han, Shankar Congeevaram and Joonhong Park

ABSTRACT
In this study, we microbiologically evaluated antibiotic resistance and pathogenicity in livestock (swine) manure as well as its biologically stabilized products. One of new livestock manure stabilization techniques is ATAD (Autothermal Thermophilic Aerobic Digestion). Because of its high operation temperature (60–65°C), it has been speculated to have effective microbial risk control in livestock manure. This hypothesis was tested by evaluating microbial risk in ATAD-treated swine manure. Antibiotic resistance, multiple antibiotic resistance (MAR), and pathogenicity were microbiologically examined for swine manure as well as its conventionally stabilized (anaerobically fermented) and ATAD-stabilized products. In the swine manure and its conventionally stabilized product, antibiotic resistant (tetracycline-, kanamycine-, ampicillin-, and rifampicin-resistant) bacteria and the pathogen indicator bacteria were detected. Furthermore, approximately 2–5% of the Staphylococcus and Salmonella colonies from their selective culture media were found to exhibit a MAR-phenotypes, suggesting a serious level of microbe induced health risk. In contrast, after the swine manure was stabilized with a pilot-scale ATAD treatment for 3 days at 60–65°C, antibiotic resistant bacteria, pathogen indicator bacteria, and MAR-exhibiting pathogens were all undetected. These findings support the improved control of microbial risk in livestock wastes by ATAD treatment.

Key words | antibiotic resistance, livestock waste stabilization, multiple antibiotic resistance, organo-fertilizer, pathogenic microorganisms

INTRODUCTION
As the cases of concentrated animal feeding operations have grown in agricultural industry, the volume of livestock manure waste produced has increased (US EPA 2003). Animals are fed with feeds containing large quantities of antibiotics. Due to the recalcitrant nature of antibiotics, significant amount of residues generally linger in the manure wastes without degradation (Daughton & Ternes 1999; Carlson & Fangman 2000; Christian et al. 2003). It is highly likely that microbial exposure to such condition leads to the appearance of antibiotic-resistant microorganisms (Bax et al. 2000; Walsh 2003; Josephson 2006; Wright 2007). In nature, antibiotic-resistance genes from the antibiotic-resistant microorganisms can be often transferred to human pathogenic microorganisms (Bushman 2002; Summers 2006). If such human pathogens can exhibit resistance against antibiotic treatments, it is serious concern to public health. Furthermore, if human pathogens gain resistance against multiple antibiotics, they can be devastating because infections caused by MAR-(multiple-antibiotic-resistant) pathogens are difficult to treat and are often fatal (Lewis 1995).
In animal agriculture, anaerobic lagoon or slurry fermentation methods are commonly used for stabilizing livestock manure wastes, and the stabilization products are often used as organic fertilizers (UN Division of Sustainable Development 2000; US EPA 2003). Because many antibiotics are not biologically degraded in such anaerobic fermentation conditions (Daughton & Ternes 1999), the anaerobic lagoons can act as reservoirs of various veterinary antibiotics, and in turn, a portion of microbial communities in the lagoons can develop strong resistance to the veterinary antibiotics. The accidental seepage and runoff of lagoon wastewater and the farm application of lagoon fermentation products as fertilizers may lead to the contamination of surface and groundwater with antibiotics as well as harmful microorganisms, thus posing a severe threat to ecology as well as public health (CheeSanford et al. 2001; Kolpin et al. 2002).

Aerobic digestion processes may be an alternative in controlling microbial risk of livestock manure wastes. An advantage of using aerobic digestion is that 1) they are generally faster than anaerobic fermentation processes; 2) because of their thermodynamically favourable nature, an aerobic process generally generates more heat than anaerobic processes. Notably, when autothermal thermophilic aerobic digestion (ATAD) processes were used in stabilizing biosolids of high organic concentrations, a significant amount of heat was generated, resulting in an increase in temperature up to 60–65°C (Staton et al. 2001; Dahab & Surampalli 2002; Zanbransk et al. 2003). The high temperature developed from ATAD treatment had disinfective effects when ATAD was used in stabilizing wasted sludge from a municipal wastewater treatment plant (Dahab & Surampalli 2002; Zanbransk et al. 2003). Because the disinfective response to high temperature was found to be dependent upon bacterial populations (Dahab & Surampalli 2002), it was unclear whether the conclusion from the studies for the wasted sludge would be directly applicable in controlling microbial risk in livestock manure wastes. Thus, the disinfective effects of ATAD in livestock manure waste have yet to be microbiologically examined. In this study, manure waste from chlortetracycline-fed pigs was treated with anaerobic lagoon fermentation and ATAD process, respectively and their corresponding microbial risks were evaluated by examining their pathogenicity and antibiotic-resistance.

MATERIALS AND METHODS

Swine manure and its stabilized products

Liquid samples were collected from Daun Swine Farm (Seosan, Chungcheongnam-do, South Korea). The feed used in the farm contained chlortetracycline, 0.2% (w/w). For an example of conventional stabilization, swine manure was fermented in a field anaerobic lagoon for 6 months. For ATAD stabilization, the swine manure was aerated for 3 days with agitation in a pilot scale batch reactor in the same field. Samples were collected from untreated swine manure (“Original”), the lagoon fermentation treatment (“Fermented”), and the ATAD treatment (“ATAD”). The samples were stored under 4°C prior to microbiological evaluation.

Antibiotic agents

For antibiotic agents, tetracycline hydrochloride (min. 95%), kanamycin monosulfate (750 μg/mg), ampicillin and rifampicin (∼90%, HPLC-grade, powder) were purchased from Sigma-Aldrich. These antimicrobial agents have various bactericidal and bacteriostatic actions: tetracycline (Tet) and kanamycin (Kan) inhibit protein synthesis (Sambrook & Russel 2000; Walsh 2003); ampicillin (Amp) disturbs cell walls synthesis; and rifampicin (Rif) sensitively inhibits RNA/DNA synthesis (Sambrook & Russel 2000).

Total culturable microorganisms

R2A agar (Difco) was used for viable counts of heterogeneous bacteria. Its low concentration of yeast extract, casein hydrolysate, peptone and glucose allows a wide spectrum of bacteria to grow without fast growing species suppressing slow ones. After serial dilution of the test sample with a sterilized potassium phosphate buffer (0.1 M, pH 6.8), 0.1 ml of the diluted sample was spread on each R2A plate. The spread plates were incubated at 24°C for 3 days, after which viable colonies were counted.

Antibiotic resistance detection

ARMs (Antibiotic Resistant Microorganisms) were selectively detected using the R2A solid media amended with
each antibiotic agent (100 μg/ml). The number of antibiotic resistant colonies formed on the antibiotic-amended R2A media were counted as described above.

Pathogenicity detection

Total *Escherichia coli* counts were analysed because it is a measure for possible pathogenicity in water. For the total *E. coli* counts, solid mTEC spread plates were used (Greenberg et al. 1992). To analyse specific human pathogenic bacteria indicators, *Staphylococcus* and *Salmonella* in the swine manure samples were cultivated using Mannitol Salt Agar (BBL) and Desoxycholate Citrate Agar (BBL), respectively. *Staphylococcus* is a gram positive pathogen causing nosocomial infection, while *Salmonella* a gram negative pathogen causing an intestinal infection (Murray et al. 1999).

Detection of MAR-phenotypes in pathogenic microbes

The MAR-phenotypes among viable pathogen colonies were analysed by examining whether the colonies could survive in the presence of a mixture of antibiotics. Among *Staphylococcus* and *Salmonella* colonies formed on the corresponding selective plates, 80 viable colonies were transferred onto the multiple antibiotics amended (MAA) plates. The MAA plates were then incubated under 24°C for 3 days and colonies formed on each plate medium were counted. The antibiotic combination used for these experiments were either Tet + Ka + Rif or Tet + Ka + Rif (100 μg/ml of each antibiotic).

RESULTS AND DISCUSSION

Reduction of pathogen indicators

In the “Fermented” samples, the gram positive indicator, *Staphylococcus*, increased approximately three times compared to those for the “Original” samples, while the colonies of the gram negative indicator, *Salmonella*, were comparable to those for the “Original” samples (Figure 1). These indicated that the lagoon fermentation did not significantly reduce the colonies of the pathogenic bacteria indicators. In the ATAD-treated swine manure waste, however, the colonies of the tested pathogen indicators were undetected. Because the comparison between ATAD and lagoon fermentation was performed under fairly consistent conditions, the results strongly suggested that the ATAD process could effectively reduce pathogenicity risk while the lagoon fermentation process could not. Similar observations were reported in other previous studies with ATAD-treated municipal sludge (Dahab & Surampalli, 2002; Zabranska et al. 2003).

Reduction of antibiotic resistant bacteria

Significant numbers of ARMs (10^5–10^8 CFU/gVSS) were colonized on antibiotic-amended plates when analysing the “Original” and “Fermented” samples (Figure 2).

![Figure 1](https://iwaponline.com/wst/article-pdf/59/2/267/436726/267.pdf)
In the “Fermented” sample, the viable counts of tetracycline-resistant and kanamycin-resistant colonies increased ten times compared to the original swine sample (Figure 2A) while their relative abundances slightly decreased (Figure 2B). This indicates that lagoon fermentation was ineffective in removing ARMs. In contrast, antibiotic resistant colonies were undetected in the “ATAD” sample. These results indicate that the ATAD treatment could effectively remove ARMs in swine wastewater.

In the “Original” and “Fermented” samples, the cultivated microorganisms exhibited resistance to the four different antibiotics (Figure 2). This led us to speculate that some of the bacteria in the samples may have MAR-phenotypes. Because a significant amount of chlortetracycline (0.2% of feed) was fed to pigs in the farm, the presence of chlortetracycline residue may have been involved in selecting MAR-exhibiting microbial populations. In addition, because chlortetracycline is a protein synthesis inhibitor, it is possible that chlortetracycline exposure led to the selection of microbial populations that has resistance to other protein synthesis inhibitors such as tetracycline and kanamycin. This speculation was supported by the fact that the numbers of microorganisms resistant to protein synthesis inhibitors (tetracycline and kanamycin) were greater than those for ampicillin (cell wall inhibitor) and rifampicin (RNA/DNA polymerase inhibitor) (Figure 2).

**Examination of MAR-phenotype in pathogens**

In the “Original” and “Fermented” samples, among the detected *Staphylococcus* and *Salmonella* colonies (80 colonies), 2–4% (two or three colonies) could grow on MAA plates. This proved the existence of MAR-pathogens in the samples (Table 1). In the ATAD-treated samples, however, pathogen colonies were undetected. These results confirmed the validity of ATAD application in control of microbial risk in swine wastewater.

**Table 1** Detection of MAR-phenotypes in the cultured pathogen indicator bacteria

<table>
<thead>
<tr>
<th>Pathogenicity</th>
<th>Antibiotics</th>
<th>Original</th>
<th>Fermented</th>
<th>ATAD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus</em></td>
<td>Tet + Ka + Amp</td>
<td>3%&lt;sup&gt;†&lt;/sup&gt;</td>
<td>2%</td>
<td>ND&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Tet + Ka + Rif</td>
<td>5%</td>
<td>5%</td>
<td>ND</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>Tet + Ka + Amp</td>
<td>4%</td>
<td>4%</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Tet + Ka + Rif</td>
<td>4%</td>
<td>3%</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>†</sup>Percentage of MAR colonies among 80 pathogenic colonies.

<sup>‡</sup>ND indicate “non-detected.”

**CONCLUSION**

In this study, the applicability of ATAD in controlling microbial risk in livestock waste was microbiologically evaluated. After the manure waste from chlortetracycline fed pigs was treated by anaerobic lagoon fermentation and ATAD processes, respectively, pathogenicity, antibiotic resistance, and MAR-exhibiting pathogenicity were compared between the conventional (anaerobic lagoon fermentation) stabilization treatment and the ATAD treatment. The major findings from this work are:

1. The anaerobic lagoon fermentation did not significantly reduce the colonies of the pathogenic bacteria indicators.
In contrast, the ATAD treatment resulted in no detection of *Staphylococcus* and *Salmonella* colonies. These results support the hypothesis that the ATAD process could improve the control of pathogenicity risk, compared to the conventional stabilization approach.

2. The lagoon fermentation was ineffective in removing ARMs. In contrast, the ATAD treatment resulted in no detection of antibiotic resistant colonies. These results provide the first evidence that the ATAD treatment could effectively remove ARMs in livestock wastes.

3. Our results also revealed the presence of MAR-exhibiting pathogens in the swine manure, and that the high level of microbial risk was not removed by the anaerobic lagoon fermentation. In the ATAD-treated samples, however, pathogen colonies were undetected. These results confirmed the validity of ATAD application in control microbial risk in swine wastewater.

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