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**Survival of faecal indicator bacteria in treated pig manure stored in clay-covered
heaps in Vietnam**

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Abstract

Small-scale pig farmers in Vietnam typically store manure in heaps covered by a layer of clay. However, the hygienic quality of manure applied to agricultural soil is uncertain as storage time is determined by the need for soil fertilizer. This study aimed to assess how the storage of pig manure added straw, lime stone or urea in heaps covered by clay affected the survival of faecal bacteria. *E. coli* was reduced from 4 log₁₀ CFU/g to below the detection limit within two weeks with 2% w/w urea (CO(NH₂)₂) whereas other amendments were associated with limited *E. coli* reductions. *Enterococcus* spp. were not reduced in any heaps indicating possible re-growth and that enterococci should be used as hygiene indicators with caution. Follow-up research should document the practical conditions under which the farmer practice of storing manure with a clay cover can be utilized to obtain a bactericidal effect of ammonia and a safe product with high fertilizer value.

Key words: Pig manure, urea, ammonia, *E. coli*, survival, Vietnam.

1. Introduction

The pig population in Viet Nam is expected to reach more than 33 million by 2011 (Huynh et al. 2007) with small-scale pig farming accounting for about 80% of pig production (Lapar et al. 2003). Current management practices of pig manure in Vietnam as well as other developing countries, i.e. collection, storage, transportation, treatment and application, show that pig manure is mainly used as fertilizer in agricultural fields (e.g. rice, maize) and gardens (root and leafy vegetables) or is discharged into aquatic recipients, including fish ponds (Guan and Holley 2003;Unc and Goss 2004;Vu et al. 2007). Little knowledge is available about environmental hygiene and veterinary public health hazards of such practices, e.g. occupational risks and food safety, including type and transmission of main pathogens.

Vietnamese farmers typically add rice straw, plant waste, ash or lime stone to manure to absorb liquid and to make it easier to handle and process, i.e. in small manure heaps typically covered with a layer of clay that prevents dogs and other animals to access and destroy the heaps (Vu, Tran, & Dang 2007). Composting and storage periods vary between 3-4 months depending on crop growth periods and needs for fertilizer, but with little if any awareness of hygiene impacts.

The use of a layer of clay on manure heaps implies reduced aeration, which is likely to reduce the temperature development in the manure because the aerobic and heat-generating composting process is inhibited. As temperature and exposure time are key factors determining pathogen survival (Guan and Holley 2003;Nicholson et al. 2005), the hygienic safety of manure treated by current management practices is uncertain. On the other hand, the combined use of clay cover and addition of lime stone and possible urea may increase pH and ammonia concentrations to levels that could significantly inactivate pathogens so the manure can safely be disposed off or used as fertilizer (Vinnerås, 2007). The objective of this study was to assess how the storage of pig manure added straw, lime stone, or urea in heaps covered by clay as practiced by

Vietnamese farmers affect hygienic quality as determined by the survival of the faecal indicator bacteria *E. coli* and *Enterococcus* spp..

2. Materials and methods

2.1. Treatment and storage of pig manure

The study was conducted from January to March, 2008, at a small-scale farm located in peri-urban Hanoi. This period is the coldest in northern Vietnam with night temperatures down to 8-15°C. Survival of faecal bacterial indicators and pathogens are usually longer at such low temperatures compared to the higher temperatures found during the rest of year. Three heaps were made by mixing 500 kg of fresh solid pig manure with 50 kg rice straw only, 50 kg rice straw + 2% lime stone (CaCO_3), or 50 kg rice straw + 2% w/w urea ($\text{CO}(\text{NH}_2)_2$), respectively; and the heaps were then covered by a 3-cm thick mud layer (Table 1). The addition of rice straw or other organic straw material as well as lime stone is a normal practice of Vietnamese farmers. Lime stone is traditionally added to the manure when making the heap partly to absorb liquid (i.e. urine and water) but mainly because farmers use lime stone as fertilizer in the rice field (Vu et al. 2007). Vietnamese farmers do not have any tradition of using slaked lime and the associated increased pH levels as a sanitizer of organic wastes. The urea was added to test effect on survival of faecal bacterial indicators at elevated ammonia levels, which is produced when urea hydrolyses in the manure. All material in each heap was mixed well with a shovel before covered by mud. Six glass thermometers were placed in six different locations inside each heap to measure possible differences in development of temperature. Those locations involved dividing each of the composting heaps into three vertical parts (top, center, and bottom). Samples and temperature measurements were collected at the right and left inside of the heap for all three parts of the heap. The bottom radius of the heaps was about 2 m and they had a height of about 1.3 m.

2.2. Sample collection and enumeration of fecal bacterial indicators

Samples were collected at days 0, 14, 28 and 45 during storage of the manure. At these four sampling times, sub-samples (100 g/sample) were collected from each of the six locations in the heap. The samples were collected by carefully digging a sterile hard plastic tube into the different locations in the heap. The mud crust was initially removed from the area in which the plastic tube was to be inserted. Immediately after collecting the sample, mud crust was placed to cover the hole made thereby minimizing the entry of air into the heap. Also, it was noted that the hole made during sampling most often quickly disappeared due to replacement of heap material. The samples were transported to the laboratory in an insulated box with cooling elements and then analyzed the same day for numbers of *E. coli* and *Enterococcus* spp. as well as total coliforms. A 10-g sub-sample was weighed and added 90 ml peptone (Oxoid, Cambridge, England) diluents containing 0.9% NaCl to make a stock solution (10^{-1}). This solution was serially diluted from 10^{-1} to 10^{-8} , and 100 μ l samples were subsequently taken from each dilution and spread on Selective *E. coli*/coliform Chromogenic medium agar plates (Oxoid) and Slanetz & Batley agar plates (Oxoid) for enterococci enumeration. Each dilution was analyzed in duplicate and agar plates were incubated at 44 °C for 24 h before bacterial colonies were enumerated. The number of CFU (cell forming units)/g stored manure of each indicator bacteria were calculated based on enumerations in two sequential dilutions.

2.3. pH and moisture content measurements

The pH of manure samples was determined by pH meter (Precisa model pH 900, Zürich, Switzerland) one hr after the samples were mixed with distilled water (1:5 v: v). Samples were stored in sterile glass vials with closed lids. The moist manure samples were weighed and dried in an oven at 100-105 °C for 6-8 hrs. The weight of the initial

moist sample was subtracted the remaining dry weight and the moisture content was then expressed as a percentage of the wet weight.

2.4. Statistical analysis

Bacterial counts were logarithmically transformed (base 10) after adding 1 and these were then analyzed using multiple linear regressions with location of manure sample in the heap, method of treatment and storage time as categorical variables. Model assumptions, normality and homogeneity of variance, were assessed by plots of residuals, i.e. histogram and residual versus fitted plots, respectively. Temperature, moisture content and pH were also added as explanatory factors in the model. This was done one at a time due to the small sample size. P-values of <0.05 were considered significant.

3. Results and discussions

3.1. Bacterial elimination in manure heap added straw and urea

The addition of 2% urea to the manure was associated with a fast and significant reduction in numbers of *E. coli* and total coliforms (Figs. 1 and 2). None of the bacterial counts varied significantly among locations within the heap on day 0. The pH was slightly alkaline (mean value of 8.5) and increased over time ($p<0.001$). It was higher in the middle and bottom section of the heap than in the top layer ($p<0.01$). No apparent temperature increase was seen with a maximum measured temperature of 33 °C (Table 1). At day 0, the mean $\log_{10}$ value concentrations of *E. coli* and total coliforms were 4.22 and 5.11 (CFU/g), respectively. Two weeks later numbers of *E. coli* and total coliforms were below the detection limit of <5 (CFU/g) manure. At day 45, total coliforms were found at low concentration 0.72 $\log_{10}$ CFU/g which is likely due to re-growth of some of the non-faecal bacterial species represented in the group of total coliform bacteria, e.g. *Citrobacter* spp. or *Arizona* spp..

The fast reduction of *E. coli* and Gram-negative rods in the presence of ammonia in animal manure is supported by earlier studies where *Salmonella* was eliminated within two weeks at 14 °C in cow manure added 2% w/w urea (Ottoson et al. 2008).

The concentration of *Enterococcus* spp. in this treatment did not vary significantly by location within the heap and varied during the experiment ($p < 0.05$) with an initial concentration of 3.97 log₁₀ CFU/g at day 0 and a similar concentration at day 45, i.e. 3.62 log₁₀ CFU/g (Fig. 3). The genus *Enterococcus* consists of several species of which some, e.g. *E. faecium* and *E. faecalis*, are strictly of faecal origin. However, some species, e.g. *E. casseliflavus* and *E. mundtii*, are not of faecal origin and may be found in the environment, e.g. as plant-associated species (Petersen and Dalsgaard 2003). It is unknown to what extent the added rice straw may have contributed with such non-faecal *Enterococcus* spp. as initial analyses of rice straw for enterococci were not done. The apparent lack of reduction in numbers of enterococci may be explained by survival and multiplication of non-faecal *Enterococcus* spp., despite a reduction of *Enterococcus* spp. of strictly faecal origin. Preliminary studies of *Enterococcus* spp. survival in stored human urine did show elimination of *E. faecium* and *E. faecalis*, but clonal re-growth of *E. gallinarum* (unpublished data). Our findings are supported by (Ottoson, Nordin, von Rosen, & Vinnerås 2008) that also found only limited reductions in numbers of enterococci in cow manure containing 2% w/w urea stored at 14 °C. Here it was suggested that Gram-positive cocci with their lower cell-permeability as well as surface-to-volume ratio are less susceptible to ammonia than Gram-negative rods. Further studies should investigate possible re-growth of certain *Enterococcus* spp. by determination of species and genotypes involved as well as the explanatory factors associated with a possible reduced susceptibility of *Enterococcus* spp. to ammonia. In conclusion, *E. coli* seems to be a more appropriate bacterial indicator as compared with *Enterococcus* spp. for monitoring hygienic quality of manure treated with urea.

3.2. Bacterial elimination in manure heaps added straw or straw + lime stone

The addition of straw only significantly reduced numbers of *E. coli* only (Fig. 1). Counts of *E. coli* differed between these two treatments ($p < 0.05$) and varied over time ($p < 0.001$), while there was no effect of location. The interaction between the two treatments and time was not significant (location was dropped from this model). Concentration of *E. coli* in the heap added straw only was reduced from $4.20 \log_{10}$ CFU/g on day 0 to $1.63 \log_{10}$ CFU/g after 45-days of storage. The mean temperature in this manure heap was 34°C with a maximum temperature of 47°C registered at days 2, 3 and 4 and pH was neutral (7.5) with little changes measured (Table 1). These findings indicate that *E. coli* and therefore possible also other Gram-negative rods, e.g. *Salmonella*, are reduced significantly (2-log units) in clay-covered heaps with pig manure amended rice straw following six weeks of storage. However, it should be noted that a similar reduction in *E. coli* numbers was not seen in manure heaps added straw together with lime (Fig. 1). It is difficult to explain this apparent lack of *E. coli* reduction in the heap with lime, but it may partly be explained by little if any temperature development in this heap (mean temperature of 29°C). It should also be noted that the addition of lime stone had limited effect on pH which was only slightly alkaline (pH=8.4)

Numbers of total coliforms were not reduced during storage of manure added straw only and lime stone/straw with CFU/g ranging from 5-6 $\log_{10}$ (Fig. 2). Interestingly, however, total coliform counts were significantly higher at the bottom of the heaps than in the top and the middle part ($p < 0.001$). *Enterococcus* spp. counts did not differ significantly among locations in the heaps but differed between the two treatments ($p < 0.05$) and varied among time points ($p < 0.001$). *Enterococcus* spp. counts were higher in the treatment with lime than in the treatment with only straw at days 15, 30 and 45. An increase in CFU/g was seen at day 15 ($p < 0.001$) followed by a decline until day 45 (Fig. 3).

3.3. Influence of temperature, pH, and moisture content

Temperature varied significantly over time ($p < 0.001$) and differed between the three different manure heaps ($p < 0.001$) after adjusting for variation over time and location in the heaps. Temperature did not vary significantly among the three locations within the heaps. Also pH varied over time ($p < 0.001$) and between the three types of manure heaps ($p < 0.001$) and variation among locations within heaps was not significant. Moisture content only varied significantly over time, i.e. a gradual decline ($p < 0.001$). pH was negatively associated ($p < 0.01$) with total coliforms counts after adjusting for main effects of the composition of the heap (straw only versus straw/lime) and time. Similarly, pH was negatively associated with enterococci counts ($p < 0.05$) after adjusting for main effects of all three compositions of manure heaps and sampling times.

The practice of almost all small-scale Vietnamese pig farmers in Northern Vietnam is to cover manure heaps with clay. The readily availability of urea at low cost offers a unique opportunity to utilize ammonia to inactivate pathogens and produce a hygienic safe fertilizer with reduced storage time allowing the farmers to apply the manure when needed in the field. Ammonia (NH_3) is a small, uncharged molecule that can cross the membrane of bacteria and damage the cell either by causing rapid alkalization of the cytoplasm or through a decrease in intracellular K^+ concentration (Kadam and Boone 1996; Park and Diez- Gonzalez 2003). A negative effect on Gram-negative bacteria has been reported at ammonia concentrations $> 30 \text{ mM}$ (Park and Diez- Gonzalez 2003). A higher pH will result in higher concentrations of uncharged ammonia. Thus, a higher pathogen die-off from urea treatment can be achieved by a combined effect of ammonia and carbonate by the addition Na_2CO_3 or lime stone (CaCO_3) at pH 9.5. However, it should be noted that in the current experiments, mean pH values were only 8.4 and 8.5 in manure heaps added rice straw/lime stone and rice

straw/urea, respectively (Table 1). Since ammonia concentrations were not measured in the heaps, we do not know if the reported ammonia threshold value of 30 mM ammonia needed to produce a hygienic safe fertilizer were obtained. In addition to reducing bacterial faecal indicators and pathogens, ammonia is also known to cause similar reductions of viruses and helminth parasites (Nordin et al. 2009; Vinnerås et al. 2008). Further studies, including cost-benefit analyses, should be done to evaluate whether the mixing of different concentrations of urea with pig manure are applicable to common operational practices of rural Vietnamese farmers as well as meeting the soil requirements for nitrogen. In particular, it is of interest to determine the level of ammonia gas emission in manure heaps covered by clay as is the normal practice of Vietnamese farmers. It could be that the clay cover together with the practice to apply manure by hand reduces ammonia emission to acceptable low levels at which the fertilizer value of the added nitrogen is maintained. Further, the addition of urea may enable the farmers to obtain a more rapid hygienization of the stored manure that will allow them to apply safe manure fertilizers when nutrients are demanded by crops.

4. Conclusion

Further studies should determine under which conditions the common practice of Vietnamese farmers to cover their manure heaps with clay can be utilized to obtain a safe and high-value fertilizer by adding urea and utilize the bactericidal effect of the ammonia generated. Documentation should be generated on the percentage of urea, amount of straw material, pH and other conditions needed to obtain ammonia concentrations sufficient to inactivate both bacterial, viral and parasite pathogens. Considerations should be given to cost-effectiveness and applicability to common operational practices of rural Vietnamese farmers.

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306 Table 1: Characteristics and composition of heaps with pig manure.

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308 Figure 1. Temporal reduction of *E. coli* in heaps with stored pig manure following
309 different treatment methods.

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311 Figure. 2. Temporal reduction of total coliforms in stored pig manure.

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313 Figure. 3. Temporal reduction of *Enterococcus* spp. in stored pig manure.

Table 1: Characteristics and composition of heaps with pig manure.

Characteristics/ Composition of manure heap	500 kg manure, 50 kg rice straw	500 kg manure, 50 kg rice straw, 2% lime stone (CaCO ₃)	500 kg manure, 50 kg rice straw, 2% w/w urea
Duration of storage (days)	45	45	45
Moisture (%)	67.9 ± 4.6	67.6 ± 3.8	67.7 ± 5.7
pH	7.5 ± 1.2	8.4 ± 0.6	8.5 ± 0.3
Temperature (°C)	34.3 ± 7.7	23.9 ± 2.2	26.4 ± 2.5
Maximum temperature (°C)	47	29	33
Time with highest measured temperature	Days 2, 3, 4	Days 10-18	Days 6, 7, 8