INACTIVATION OF AVIAN INFLUENZA AND MODEL VIRUS IN ANIMAL BY-PRODUCT COMPOSTS

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1 INTRODUCTION

In case of epizootic viral disease outbreak *e.g.* avian influenza (AI), materials such as carcasses and litter from poultry farms can if not properly sanitised pose a health risk to both humans and animals and be a source of further spread of the pathogens. Thus, a safe sanitisation method adoptable to all sizes, from small to large scale, is preferable. Methods available for disposal of the materials include *e.g.* landfilling, on-site burial, composting, disinfection and incineration.

In-vessel composting of carcasses and litter is together with *e.g.* incineration, out of a risk perspective, the preferable disposal options during an outbreak of AI (Pollard et al., 2008). During the thermophilic phase of aerobic composting temperatures high enough to inactivate most pathogens can be reached, including avian influenza virus (AIV) (Guan et al., 2009; Senne et al., 1994). In addition to this composting may also help to avoid further transmission of AIV since the sanitation can be performed on-site avoiding transport of the materials.

In order to establish guidelines for sanitation treatments in case of an epizootic outbreak validation of treatment processes is required. Viral analysis can be both expensive and time consuming thus the use of bacteriophages as a model organism is a good alternative. Bacteriophages have similar structure as viruses but they are both easier and less expensive to analyse. Various bacteriophages have been suggested as viral indicators (Havelaar et al., 1991) and can be used to monitor the treatment efficiency in terms of viral inactivation.

The aim of the present study was to investigate the inactivation rate (as decimal reduction, D-value) of the highly pathogenic AIV strain H7N1 during thermal treatment in a compost mixture of poultry manure, straw and non-hatched eggs. The AIV strain used has been shown to be relatively heat stable in comparison to other AIV strains (unpublished data). Further the inactivation rate of AIV was compared to that of bacteriophage MS2 (ATCC 15597-B1) during thermal treatment. The inactivation of bacteriophage MS2 during laboratory-scale composting in 1.5 l Dewar flasks was also investigated.

2 MATERIALS AND METHODS

2.1 Compost material and microorganisms

The compost mixture used consisted of poultry manure collected at Lövsta research station, SLU, straw and non-hatched eggs (14 days old embryos) with a final moisture content of 67.1% and 68.2% in the material for thermal treatment at 45°C and laboratory-scale composting respectively. Sanitation was monitored by analysis of viable added microorganisms. The virus strain used were the highly pathogenic avian influenza virus (HPAIV) strain A/turkey/Italy/1387/00 (H7N1) and bacteriophage MS2 (ATCC 15597-B1). The AIV strain was cultivated in 11 days embryonated SPF eggs (SVA production). Propagation of the bacteriophage was performed in nutrient broth (NB, Oxoid AB, Sweden) using the host strain *Salmonella* Typhimurium WG 49 (ATCC 700730).

2.2 Experimental design

2.2.1 Thermal treatment at 45°C

The compost material was inoculated with microorganisms to an initial content of approximately 5.5 tissue culture infectious dose $(TCID)_{50}$ g^{-1} and 7.5 $\log_{10}$ plaque forming units (PFU) g^{-1} of AIV and MS2 respectively and mixed manually. The material was weighed into eppendorf tubes and placed in water bath or block thermostats during 48

hours MS2 and during 24 hours for AIV. Samples were taken in triplicates and the sampling started when the material had reached a temperature 45°C for MS2, for AIV the 0-sample was taken before incubation started

2.2.2 Composting in laboratory-scale reactors

The compost material was inoculated with bacteriophage MS2 to an initial content of 7.5 $\log_{10}$ PFU g^{-1}, mixed manually. The first samples (day 0) were taken and thereafter the compost mixture was filled into two compost reactors. Sampling was performed on day 1, 2, 4 and 7. At each sampling occasion three samples were taken from each reactor and the compost was thoroughly mixed for aeration of the material.

2.3 Enumeration of microorganisms

The AIV was analysed by an end-point titration method through cell culture cytopathic effect using 96-well plates, using MDCK cells and 8 replicates per dilution. Virus titres were calculated according to the Spearmann Kärber formula (Kärber, 1931). The titres were expressed as tissue culture infectious dose $(TCID)_{50}$ values per gram compost.

Enumeration of the bacteriophage was performed according to the standard ISO10705-1 (1995) double-layer agar method (Adams, 1959) using the host strain *S.* Typhimurium WG49 (ATCC 700730). In short samples were serial diluted in saline solution (0.86-0.9% NaCl). Suitable dilutions were mixed with the host strain and soft agar and poured onto agar plates. The plates were incubated at 37°C thereafter plaques were counted with a detection limit of 10 plaque forming units (PFU) g^{-1} compost material.

2.4 Statistical analysis

Linear regression analysis and t-test was performed using Minitab (Minitab® 15.1.20.0., Minitab Inc.). Results obtained from the linear regression analysis were used to calculate D-values (the time required for a 1 $\log_{10}$ reduction in the number of organisms) for the inoculated microorganisms during thermal treatment at 45°C.

3 RESULTS

The results obtained from thermal treatment of bacteriophage MS2 and AIV strain H7N1 at 45°C can be seen in Figure 1. Based on these data the D-value for bacteriophage MS2 was 56.5 h. As for inactivation of the AIV strain H7N1 no virus could be detected after 30 min of thermal treatment (Figure 1b), corresponding to approximately 3.6 $\log_{10}$ reduction within half an hour's time. The rapid inactivation of the AIV results in a D-value of less than 9 min.

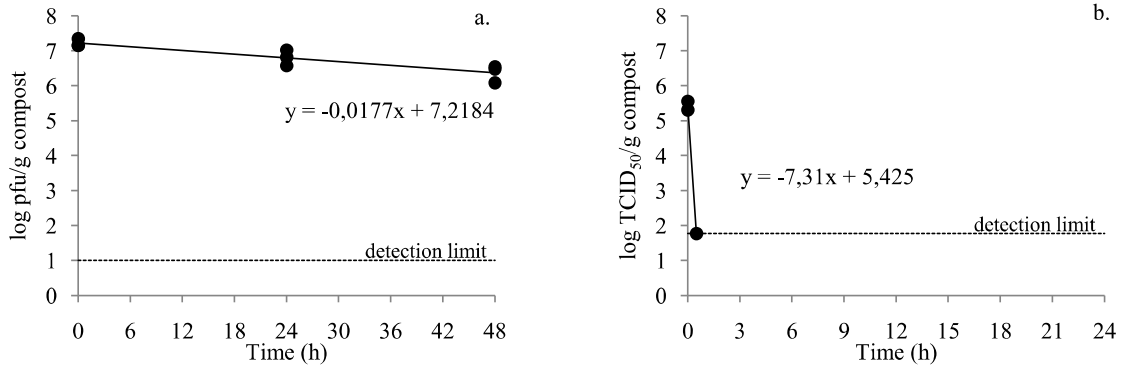

FIGURE 1 **Inactivation of bacteriophage MS2 (a) and AIV strain H7N1 (b) at 45°C in compost material.**

In the laboratory-scale compost reactors the temperature increased steadily during the first day to a temperature >45°C around day 1 (Figure 2 a.). The inactivation rate for bacteriophage MS2 was at its highest between days 2 and 4 as can be seen in Figure 2b. During the compost trial pH increased from 8.5 to 8.8-9 and some changes in moisture content occurred resulting in a MC of 68.3% and 54.4% in reactor 1 and 2 respectively at the end of the trial (day 7).

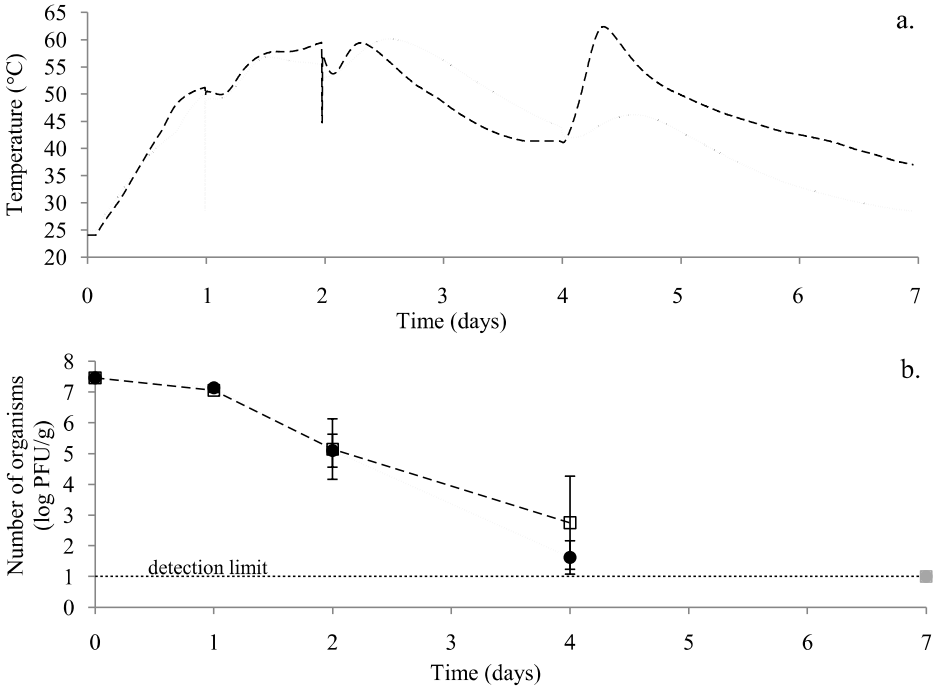

FIGURE 2 **Temperature changes (a) and inactivation of bacteriophage MS2 during composting in laboratory-scale compost reactors. Reactor 1 (●, __) and reactor 2 (□,---). Grey marker represents a sampling occasions were the number of bacteriophages were found to be below the detection limit. Drops in temperature at day 1, 2 and 4 correspond to sampling occasions.**

4 DISCUSSION

The D-value obtained for AIV at 45°C (>9 min) correlate well to data published showing D-values of <0.3-0.9h (Guan et al., 2009). Results from the present study show that the D-value for bacteriophage MS2 is significantly higher than that of AIV strain H7N1. The fact that the D-value for bacteriophage MS2 correspond to a time longer than the time of the performed study results in an uncertain determination of the inactivation rate. However the properties of being both cheaper and easier to analyse makes bacteriophages an attractive alternative to analysis of AIV. Although further studies for more precise determination of the D-value for bacteriophage MS2 is needed for establishing a correlation to the inactivation of AIV. A comparison between AIV and bacteriophage MS2 show that they both are ssRNA virus although AIV is enveloped. Since enveloped viruses are assumed to be more sensitive to heat (Wichuk and McCartney, 2007) the high inactivation rate of AIV in comparison to bacteriophage MS2 is not surprising. As a result of the comparatively high D-value obtained for bacteriophage MS2 it can be argued that it is a too conservative indicator of AIV inactivation and could lead to an underestimation of the sanitation efficiency of the process. Although the use of an indicator with a higher D-value than AIV adds a high degree of security to the sanitation when setting treatment recommendations. Alternatively other bacteriophages could be used as indicators. However, there are few enveloped phages. Pilot trials with the enveloped segmented dsRNA phage Φ6 (Huiskonen et al., 2006) at our laboratory has shown that it is too unstable to use as an indicator (unpublished data).

Compost management is of great importance to ensure that proper sanitation is achieved. In the laboratory-scale compost reactors temperatures above 45°C were reached within one day in both reactors. Following the temperature increase the inactivation rate was at its highest between day 2 and 4, and at the end of the trial the number of bacteriophages had been reduced to below the detection limit corresponding to approximately a 6.5 $\log_{10}$ reduction within 7 days. Compost material is often non-homogenous which can result in an uneven temperature distribution throughout the compost. In addition heat loss to the surrounding might occur due to pore insulation of the reactor. Due to part of the compost keeping a lower temperature than the core there is a risk for increased survival of pathogens during the treatment process. Repeated turning of the compost makes it possible to treat larger parts of the material at high temperatures. Although in the case of composting of materials due to an epizootic

outbreak, special precautions can be necessary and turning of the compost may not be desirable since it can result in formation of airborne particulates spreading the pathogen. Therefore sufficient insulation of the reactor is a necessity to keep high temperatures throughout the whole material. Based on the fact that both of the laboratory-scale compost reactors kept a temperature of above 45°C during more than 55h it is reasonable to assume that a sufficient inactivation of AIV would have occurred, suggesting that composting can fulfil the sanitation requirements in case of an outbreak of AI. The suitability of composting for sanitation of materials from poultry farms in terms of AIV inactivation has previously been shown by Guan et al. (2009) and successful use of composting during AI outbreak has been reported (Spencer et al., 2004).

Further research is needed to be able to establish treatment recommendations for composting of poultry litter in case of an epizootic outbreak. In addition to the present study trials will be carried out in laboratory-scale composting reactors with a compost mixture inoculated with avian influenza strain H7N1.

5 CONCLUSION

Temperatures that can be reached during aerobic composting of hatchery waste are sufficient to inactivate the highly pathogenic AIV strain H7N1. This is assuming that a temperature of at least 45°C is kept throughout the whole material. The bacteriophage MS2 used in the present study was shown to be a conservative indicator for AIV during sanitation through thermal treatment. Results in the present study suggest that a sufficient inactivation of AIV H7N1 can be achieved within 1.7 h at 45C° (corresponding to a 12 $\log_{10}$ reduction).

ACKNOWLEDGEMENTS

This study was financed by EU, Fluresist, Contract No SSPE-CT-2006-44311 and Swehatch.

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