AMMONIA TREATMENT OF HATCHERY WASTE FOR ELIMINATION OF AVIAN INFLUENZA VIRUS

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SUMMARY

The inactivating capacity of ammonia to the avian influenza virus (H5N3) was investigated, using influenza virus spiked hatchery waste and ammonia to final concentrations of 0.25, 0.5 and 0.75% (w/w), at a temperature of 15ºC. As control hatchery waste with only deionised water was used. To evaluate the possible use of bacteriophages as indicator organisms, the hatchery waste was challenged with Enterobacteria phages MS2 and ϕX174 to monitor the inactivation. The results indicate that avian influenza virus H5N3 is readily inactivated at all ammonia concentrations tested. Bacteriophage ϕX174 did not show any significant decay at any of the ammonia concentrations, whereas bacteriophage MS2 was inactivated at a slower rate than influenza virus. Ammonia is a good alternative for inactivation of avian influenza virus in hatchery waste, and bacteriophage MS2 can be used as a conservative indicator.

Keywords: avian influenza virus; ammonia sanitation; inactivation; hatchery waste

INTRODUCTION

Hatchery waste (HW) is produced by the hatchery industry and may contain pathogenic microorganisms, e.g. viruses. According to the regulation (EC) 1774/2002, which lays down health rules for animal by-products not intended for human consumption, the HW needs sanitation treatment. The most common method to do this in Sweden today is by liming. However, liming is technically complicated and can lead to unsuitable working conditions, e.g. due to the high pH (>12), lime dust and formation of sediments. In the case of an epizootic disease outbreak, a suitable environmentally safe sanitation method would be desirable, instead of e.g. sodium hydroxide or formaldehyde treatments now recommended for use in Sweden (1). Ammonia sanitation of biowaste can be performed by using ammonia (aq) or urea (s), which has been studied regarding faeces (2). Free uncharged ammonia (NH_3 (aq)) is the active substance and is present at pH values >8 with a pKa of 9.3. The equlibrium of NH_3 and its ionised form NH_4^+ depends on both pH and temperature and is moved towards NH_3 when either of them is increased. Studies have indicated that NH_3 acts by cleavage of viral RNA; this has been shown regarding poliovirus, a picornavirus which possess single stranded (ss) RNA, at a temperature range of 10 to 40ºC (3). As influenza virus also possesses ssRNA it could be expected that it would be sensitive to ammonia. The ammonia treatment contributes further to the agricultural fertiliser value of the biowaste, as ammonia persists during the treatment, and therefore may be used as a plant nutrient.

OBJECTIVE

The objective of this study was to investigate, in laboratory scale, the potential of using ammonia as a chemical treatment method for disinfection of HW. A second objective was to evaluate usage of bacteriophages as monitoring models for virus inactivation.

MATERIAL AND METHODS

The material used for the disinfection studies was untreated hatchery waste (HW) obtained from Lantmännen SweHatch AB, Kristianstad, Sweden. The HW consisted of egg shells, egg yolk and chicken embryos, and was stored in aliquots at 4–6 °C for 2 weeks before experimental start.

Disinfection of the HW was monitored by analysis of viable added microorganisms. The viruses used were avian influenza A virus (AIV), strain H5N3, isolated at Department of Virology, SVA. The bacteriophages used were Enterobacteria phage MS2 (ATCC 15597-B1) and Enterobacteria phage ϕX174. Cell culture medium for AIV was Eagle's minimal essential media (4) (EMEM) containing trypsin (Worthington Biochemical Corporation, Lakewood, NJ, USA) at a concentration of 2.5 µg/ml, and cell line was Madin Darby Canine Kidney (MDCK) cells (ATCC CCL-34).

HW in 1-g portions was spiked 10-fold with the respective microorganism to an initial concentration of 5–7 $\log_{10}$ per gram. Ammonia (Rectapur, PROLABO, Stockholm, Sweden) was added to final concentrations of 0.25, 0.5 or 0.75%(w/w) to the spiked HW, by thoroughly vortexing for complete homogenisation. As controls, spiked HW with only deionised water was analysed, to determine the inactivating effect of the HW and temperature alone. For comparison, pure virus suspension was kept at the actual temperature and time periods. All mixtures were treated at 15±0.5 ºC, the temperature and pH was monitored during the treatment. Sampling was performed 4–5 times for each ammonia inactivation trial. At each sampling two 1-g samples of HW were taken, diluted 10-fold in cell culture media, and extracted by vigorous shaking. After centrifugation at 3000 g for 10 min, the supernatant was gel filtrated through Sephadex G-25 columns (GE Healthcare, Uppsala, Sweden) in cell culture media, to remove ammonia and other cytotoxic low molecular weight substances.

To assess the detection limit before virus analysis, a viral interference assay was performed: HW with the highest ammonia concentration was extracted as above, and subjected to gel filtration. AIV was titrated in the resulting effluent, and the virus titre was compared to virus titres obtained using cell culture medium only as titration media.

Virus was analysed by an end-point titration method through cell culture cytopathic effect using eight 50-µl replicates per dilution, and the virus titres were calculated according to the Spearmann Kärber formula (5) and expressed as tissue culture infectious dose $(TCID)_{50}$ values per gram HW. The virus reduction factors where no virus was found were calculated according to (6). The double agar layer method (7) was used to determine the number of the bacteriophages. For MS2, the *Salmonella enterica* strain WG49 (ATCC 700730) was used as the host bacterium, and for ϕX174 the *Escherichia coli* strain C (ATCC 13706™) was used. The bacteriophage titres were expressed as plaque forming units (PFU) per gram HW.

RESULTS

The pH of the HW with 0, 0.25, 0.5 and 0.75%(w/w) ammonia added was 7.5, 8.5, 9.0 and 9.0–9.5, respectively, and was not altered up to 18 hours of treatment. The results from the viral interference assay for AIV showed that the undiluted, gel filtrated samples could be used for virus analysis. The titres from the analysis of the microorganisms are shown in Table 1.

Table 1.

Microorganism/ NH_3 %	0	3	6	Time 8	Hours 12	18	29	72
AIV 0	5.4	5	4.7	4.5	3.8	[a]	[a]	[a]
AIV 0.25	5.4	3.6	3.4	3.0	<0.9	[a]	[a]	[a]
AIV 0.5	5.4	2.9	2.5	<0.9		[a]	[a]	[a]
AIV 0.75	5.4	2.9	<0.9			[a]	[a]	[a]
MS2 0	5.9	[a]	5.8	[a]	[a]	5.8	5.5	5.4
MS2 0.25	5.9	[a]	5.5	[a]	[a]	4.6	3.6	<0.9
MS2 0.5	5.9	[a]	4.8	[a]	[a]	2.9	2.0	<0.9
MS2 0.75	5.9	[a]	3.6	[a]	[a]	2.5	<0.9	<0.9
ϕX174 0	7.0	[a]	7.0	[a]	[a]	7.0	7.0	7.0
ϕX174 0.25	7.0	[a]	6.9	[a]	[a]	7.1	7.0	7.0
ϕX174 0.5	7.0	[a]	7.0	[a]	[a]	6.9	6.9	6.9
ϕX174 0.75	7.1	[a]	7.1	[a]	[a]	6.8	7.1	6.8

Mean titres in $\log_{10}$ tissue culture infectious dose (TCID) $_{50}$ (AIV) or plaque forming units (PFU) (bacteriophages) per gram HW. Detection limit was 0.9 $\log_{10}$
[a] = not tested

As can be seen in Table 1, AIV was inactivated to below detection limit after 6–12 hours at 0,25–0,75% NH_3 concentrations, a virus reduction of >4.5 $\log_{10}$. Concerning MS2, complete reduction of >5.0 $\log_{10}$ was evident after 29 hours at 0.75% NH_3 and after 72 hours for 0.25–0.5% NH_3. ϕX174 was not significantly inactivated at any ammonia concentration. The controls kept in cell culture media were inactivated about 1 $\log_{10}$ after 12 hours for AIV, and about 0.5 $\log_{10}$ for MS2 after 72 hours. ϕX174 control in cell culture media was inactivated by about 1 $\log_{10}$ after 72 hours.

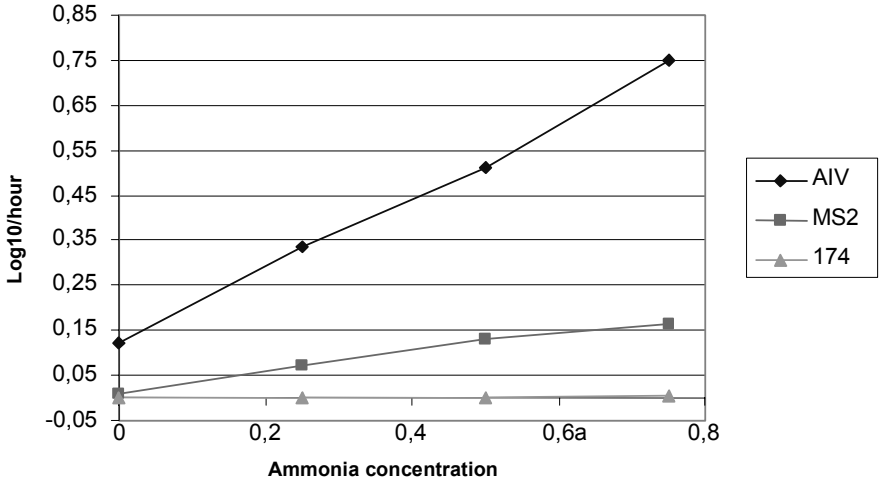

Figure 1. Inactivation rates in $\log_{10}$ per hour of avian influenza virus H5N3 (AIV), Enterobacteria phage MS2 (MS2) and Enterobacteria phage ϕX174 (174) at different ammonia concentrations at 15°C.

As can be seen in figure 1, the inactivation rates of AIV are higher than for MS2 and ϕX174 at all ammonia concentrations, indicating that the bacteriophages investigated are more stable.

DISCUSSION

Studies regarding Enterobacteria phage f2, a phage of the same virus family and genus as Enterobacteria phage MS2, showed that f2 is about 4.5 times more resistant to ammonia inactivation as poliovirus, an enterovirus of the virus family Picornaviridae, both possessing ssRNA (8). In our study the ssRNA phage MS2 was 4–5 times more resistant to ammonia inactivation than AIV. Influenza virus is also more sensitive to environmental factors due to its lipid envelope, and is inactivated at pH >9.5, a lower pH than for enteroviruses such as poliovirus which is inactivated at pH ≥11 (9).

The bacteriophage ϕX174 was not inactivated significantly at any of the ammonia concentrations used, during the whole time span of the trial, 72h. ϕX174 possess circular ssDNA that could make it more resistant to NH_3 inactivation, as it is otherwise quite similar to bacteriophage MS2, generally DNA is more stable compared to RNA.

AIV in the controls of HW with deionised water at pH 7.5 was reduced by 1.6 $\log_{10}$ after 12 hours, due to intrinsic properties of the hatchery waste. Results reported in earlier studies on swine influenza A virus show that at 5°C and 20°C, the survival in cattle slurry is 9 and 2 weeks, respectively, a survival comparable to picornavirus at 20°C (10). Avian influenza virus (H7N2) in chicken manure was reported to be inactivated in less than one week at 15–20°C (11). Thus both temperature and properties of different vehicles might influence influenza virus survival.

It can be concluded that avian influenza virus (H5N3) is readily inactivated by ammonia treatment. After 12 hours in the lowest ammonia concentration used, more than the requested 3

log_{10} reduction was achieved. The MS2 phage showed a lower reduction rate than AIV; therefore it can be used as a conservative indicator for inactivation of AIV, as one log10 reduction in MS2 corresponds to at least three log_{10} reduction of AIV.

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