

## The inactivation of viruses in cattle and pig slurry by aeration or treatment with calcium hydroxide

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### SUMMARY

Porcine enterovirus type 2 or porcine adenovirus type 3 were seeded into samples of pig slurry, and a bovine enterovirus was seeded into cattle slurry, and samples of the slurry were aerated in the laboratory for 21 days. The viruses were inactivated more rapidly in the aerated slurry than in control slurry which was not aerated. The difference in inactivation rate was greatest for the porcine adenovirus and least for the bovine enterovirus. Inactivation of the porcine enterovirus in aerated distilled water and in aerated, autoclaved pig slurry proceeded at a similar rate as in the same materials which were not aerated. Ten samples of aerated slurry were collected from an aeration tank which received weekly additions of raw pig slurry which was sampled at the same times. Each sample yielded a porcine enterovirus after concentration with the polyelectrolyte PE-60, but in three comparative titrations the viral infectivity titre in concentrates of the raw slurry was at least 1000 times greater than in the aerated slurry. Porcine enterovirus type 2 and porcine adenovirus type 3, which were seeded into pig slurry, and a bovine enterovirus seeded into cattle slurry, were inactivated by treatment of the slurry with calcium hydroxide at pH 11.5. The inactivation rate was highest for the bovine enterovirus and lowest for the porcine adenovirus.

### INTRODUCTION

In a previous paper (Derbyshire & Brown, 1978) we described the isolation of porcine enteroviruses and porcine adenoviruses from samples of pig slurry, and the isolation of bovine enteroviruses from cattle feedlot run-off. Our studies also indicated that, when pig slurry containing porcine enteroviruses was spread on agricultural land, the viruses were able to retain their infectivity for several days, and find their way into surface water supplies by elution from the soil in rain water. It would be desirable to reduce this form of environmental pollution by treatment of the slurry, before its application to the land, in some manner which would achieve viral inactivation.

Primarily because of the offensive odour associated with the spreading of slurry, biological treatment of this material has been investigated, and various aeration

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devices have proved effective in reducing odour (Hawkins, 1978). However, information is lacking on the effect of aeration of slurry on the infectivity of viruses which it might contain, although significant reductions in poliovirus type 1 concentrations were obtained in studies on laboratory models of algal-bacterial sewage treatment systems (Sobsey & Cooper, 1973). In this paper we describe laboratory experiments on the inactivation of three viruses in aerated pig or cattle slurry, together with some field observations on the isolation of viruses from aerated pig slurry. The laboratory phase of the investigation included an experiment in which the inactivation of a porcine enterovirus was studied in aerated distilled water and in autoclaved slurry, in an attempt to provide information on the relative importance of increased oxygen tension, surface inactivation and the microbial flora on viral inactivation in aerated slurry.

Sattar, Ramia & Westwood (1976) described experiments with domestic sewage contaminated with poliovirus which indicated that treatment of the sewage with calcium hydroxide produced highly significant reductions in viral infectivity. We have applied this procedure, on a laboratory scale, to pig and cattle slurry seeded with a porcine enterovirus, a porcine adenovirus or a bovine enterovirus, with promising results which are described in this paper.

## MATERIALS AND METHODS

### *Virological procedures*

The viruses used were the T80 strain of porcine enterovirus type 2 (Betts, 1960), the 6618 strain of porcine adenovirus type 3 (Derbyshire, Clarke & Collins, 1975) and a bovine enterovirus isolated in the authors' laboratory from cattle feedlot run-off (Derbyshire & Brown, 1978). The porcine viruses were cultivated and assayed in primary pig kidney (PK) cell cultures, while the bovine enterovirus was cultivated in embryonic bovine kidney (EBK) cells. Conventional cell culture techniques were used, and the cells were maintained in Eagle's minimum essential medium (EMEM-Gibco, Grand Island, New York, U.S.A.) supplemented with 5% fetal bovine serum (FBS) and penicillin and streptomycin. Viral assays were done by infectivity titration in test tube cultures (Derbyshire & Jessett, 1967), and the results expressed in  $\log_{10}$  median tissue culture infectious doses (TCID<sub>50</sub>) per ml after calculation of 50% end-points by the Kärber formula (Kärber, 1931).

### *Laboratory experiments on the aeration of slurry*

Suitable dilutions of stock cultures of porcine enterovirus type 2 or porcine adenovirus type 3 were seeded into pig slurry, and the bovine enterovirus was seeded into cattle slurry, collected from liquid manure tanks on the University of Guelph farms (Derbyshire & Brown, 1978). After seeding, each sample of slurry was divided into two parts of 1.4 l which were placed in Ehrlenmeyer flasks. The contents of one flask were magnetically stirred and continuously aerated by means of the laboratory compressed-air supply, at room temperature for 21 days, while the second flask was neither stirred nor aerated. Dow Corning Antifoam A was added as required in order to prevent foaming. Immediately after seeding, and at

twice-weekly intervals thereafter, the flasks were sampled and the samples were titrated for infectivity in the appropriate cell cultures after chloroform treatment by the method of Feldman & Wang (1961) to control bacterial contamination. In a further experiment, inactivation of the porcine enterovirus was studied in pig slurry which had been autoclaved at 15 lb/in<sup>2</sup> for 30 min, and in distilled water, using the procedures detailed above.

#### *Field studies on aerated pig slurry*

Samples of raw pig-slurry were collected at weekly intervals for 10 weeks from a slurry tank which received effluent from a swine fattening house, and which had been shown previously to contain porcine enteroviruses fairly consistently (Derbyshire & Brown, 1978). Each sample was collected immediately before the tank was emptied and the contents transferred to a surface-aerated storage tank (Pos & Robinson, 1973) for biological treatment. At the same time intervals, composite samples of the aerated slurry were obtained from different levels of the aeration tank. At the end of the 10-week period the aeration tank was emptied. Each sample of raw or aerated slurry consisted of a volume of about 2 l which was concentrated by adsorption with the polyelectrolyte PE-60 (Wallis *et al.* 1969) as previously described (Derbyshire & Brown, 1978). The final supernatants, after filtration through Millipore 0.45  $\mu$ m filters, were chloroform-treated (Feldman & Wang, 1961) and each was inoculated into four tubes of PK cells. The cultures were examined daily for at least 7 days, when negative cultures were passaged once more in PK cells. Viruses which were isolated were provisionally identified as porcine enteroviruses on the basis of cytopathology and chloroform resistance. In addition, the PE-60 concentrates which were prepared from the first, fifth and ninth pairs of samples of raw and aerated slurry were titrated by infectivity assay in PK cells.

#### *Calcium hydroxide treatment of slurry*

The procedures used were based on those described by Sattar *et al.* (1976). One litre volumes of raw slurry were seeded with suitable dilutions of stock cultures of the appropriate virus. Pig slurry was seeded with porcine enterovirus type 2 or porcine adenovirus type 3, and cattle slurry was seeded with the bovine enterovirus. Immediately after the addition of virus, a sample of the slurry was collected for viral infectivity assay, and the remainder was continuously stirred while sufficient of a suspension of calcium hydroxide was added to increase the pH to 11.5, after which stirring was continued for a further 15 min. The slurry was then transferred to an Imhoff cone and the sludge allowed to settle. In replicate tests, settling periods of 1, 3 and 24 h were employed for each virus. At the end of the settling period a sample of the relatively clear supernatant was collected, the pH adjusted to 7.2 with N-HCl, and retained for infectivity assay. The pH of the sludge was also adjusted to 7.2 and the sludge was centrifuged at 1500 g for 30 min. The deposit was resuspended in 20 ml of 10% FBS and stirred for 15 min in order to elute adsorbed virus. The suspension was again centrifuged and a sample of the supernatant was collected for infectivity assay. Each of the samples was chloroform-treated (Feldman & Wang, 1961) to destroy bacterial contaminants, and titrated for infectivity in the appropriate cell cultures.

## RESULTS

*Laboratory experiments on the aeration of slurry*

The results obtained for the inactivation of the three viruses in pig or cattle slurry are given in Table 1. Inactivation of the porcine enterovirus in aerated pig slurry was complete in 10 days, while infectivity was still detected in the non-aerated slurry after 21 days. A further test on the latter material after 24 days failed to detect infectivity. The rate of inactivation was clearly greater in the aerated slurry. The inactivation rate of the porcine adenovirus was much greater in aerated than in non-aerated pig slurry, and inactivation of the porcine adenovirus was more rapid than that of the porcine enterovirus. While the infectivity titres of the bovine enterovirus in aerated cattle slurry were consistently lower than in the non-aerated slurry, the differences were relatively small. The bovine enterovirus was inactivated more slowly than the porcine adenovirus or porcine enterovirus.

The results obtained for the inactivation of porcine enterovirus type 2 in sterile distilled water and in autoclaved pig slurry are given in Table 2. In neither of these media did inactivation proceed more rapidly in the aerated sample than in the non-aerated material, and in distilled water viral inactivation was somewhat more rapid in the absence of aeration. The viral inactivation rates in the aerated and non-aerated slurry which had been autoclaved before seeding were virtually identical. Inactivation was slower in both distilled water and autoclaved slurry than in raw slurry (Table 1).

*Field studies on aerated pig slurry*

All the samples of raw and aerated slurry which were collected yielded viruses after concentration by the PE-60 adsorption procedure, and each virus which was isolated was identified as a porcine enterovirus on the basis of its chloroform resistance and cytopathology in PK cells. Cytopathic effects usually appeared earlier in the cell cultures inoculated with the concentrates prepared from raw slurry, and the aerated slurry was usually less cytotoxic than the raw slurry samples. Infectivity titration of the concentrates prepared from the first, fifth and ninth samples of raw slurry gave  $\log_{10}$  TCID<sub>50</sub>/ml values of 4.25, 4.0 and 4.75 respectively, while the corresponding titrations of the concentrates of the aerated slurry gave a  $\log_{10}$  TCID<sub>50</sub>/ml value of 1.0 on each of the three samples.

*Calcium hydroxide treatment of slurry*

The results of these experiments are given in Table 3. After treatment of pig slurry containing porcine enterovirus type 2 with calcium hydroxide, no viral infectivity was detected in any of the supernatants, or in the eluted sludge after settling periods of 3 or 24 h. Inactivation of porcine adenovirus type 3 was less rapid, since infectivity was readily detected in both supernatant and eluted sludge after settling for 3 h, but no virus was detected after settling for 24 h. The bovine enterovirus in cattle slurry was inactivated very rapidly by calcium hydroxide

Table 1. *The inactivation of porcine enterovirus type 2, porcine adenovirus type 3 and a bovine enterovirus in aerated and non-aerated pig or cattle slurry*

Source	Treatment	Seeded virus	Days after seeding							
			0	3	7	10	13	17	21	
Pigs	Aerated	Porcine enterovirus	3.5	2.0	1.0	0*	0	0	0	
Pigs	Not aerated	Porcine enterovirus	3.0	2.2	2.5	2.0	1.2	1.0	0.7	
Pigs	Aerated	Porcine adenovirus	3.7	1.0	0	0	0	0	0	
Pigs	Not aerated	Porcine adenovirus	4.0	3.5	3.5	3.2	3.2	1.2	0	
Cattle	Aerated	Bovine enterovirus	3.5	2.5	1.7	1.5	1.0	0	0	
Cattle	Not aerated	Bovine enterovirus	3.5	3.0	2.2	2.0	1.5	0.7	0	

\*0, Viral infectivity not detected.

Table 2. *The inactivation of porcine enterovirus type 2 in aerated and non-aerated sterile distilled water and autoclaved pig slurry*

Medium	Treatment	Seeded virus	Days after seeding								
			0	1	4	7	11	14	19	21	25
Distilled water	Aerated	Porcine enterovirus	4.2	4.0	4.0	3.7	3.7	3.5	3.5	3.0	2.5
Distilled water	Not aerated	Porcine enterovirus	3.7	3.0	2.7	2.5	2.2	2.5	2.0	1.2	1.0
Autoclaved slurry	Aerated	Porcine enterovirus	4.0	3.7	4.0	3.5	3.0	2.7	2.5	1.7	1.2
Autoclaved slurry	Not aerated	Porcine enterovirus	3.5	3.5	3.7	3.2	3.5	3.0	2.5	1.5	1.5

Table 3. *The inactivation of porcine enterovirus type 2, porcine adenovirus type 3 and a bovine enterovirus in pig or cattle slurry treated with calcium hydroxide*

Source of slurry	Seeded virus	Settling time (h)	Infectivity titres (log <sub>10</sub> TCID <sub>50</sub> /ml)		
			Seeded slurry	Super-natant	Eluted sludge
Pigs	Porcine enterovirus	1	4.2	0*	2.5
		3	4.5	0	0
		24	4.5	0	0
Pigs	Porcine adenovirus	1	4.2	4.0	3.2
		3	4.0	3.2	2.7
		24	4.2	0	0
Cattle	Bovine enterovirus	1	3.0	0	0
		3	3.0	0	0
		24	2.7	0	0

\* 0, Viral infectivity not detected.

treatment, since infectivity was not detected either in the supernatant or the eluted sludge after settling for only 1 h.

#### DISCUSSION

The main objective of these studies was to investigate procedures which might be of value for the inactivation of animal viruses in slurry, in order to reduce the environmental pollution hazard associated with the application of this material to agricultural land. From the results which we obtained, both aeration and calcium hydroxide treatment appeared promising.

In the laboratory experiments on aerated slurry, the porcine enterovirus and the porcine adenovirus were both inactivated significantly more rapidly when the slurry was aerated, but for the bovine enterovirus in cattle slurry the difference between the inactivation rates in aerated and non-aerated slurry was relatively small. It is not known whether this resulted from a characteristic of the virus or of the slurry. The experiments on the inactivation of the porcine enterovirus in distilled water and in autoclaved slurry suggested that the rapid inactivation of this virus in aerated, raw pig slurry may have been associated with the activity of aerobic microbial flora of the latter. Since the virus was not inactivated more rapidly in aerated water or aerated, autoclaved slurry than in the same media without aeration, it seems unlikely that either the increased oxygen tension alone, or surface inactivation, contributed significantly to viral inactivation in the aerated raw slurry. Donaldson & Ferris (1976) also found that non-enveloped viruses were not susceptible to surface inactivation. We therefore postulate that viral inactivation in aerated slurry is more likely to be associated with degradation of the viral capsids by microbial activity. This may result from the action of proteolytic bacteria, whose growth may be favoured by aeration, as demonstrated for coxsackievirus and poliovirus by Cliver & Herrmann (1972).

Our field studies on slurry supported our laboratory findings in that viral infectivity titres in the aerated slurry were always much lower than in the raw

slurry. It was not possible to determine under field conditions whether the inactivation which occurred was specifically associated with the aeration procedure, since non-aerated slurry which had been stored for a similar period was not available for comparison. However, taken together, the field and laboratory observations suggest that aeration of slurry may be a worthwhile practice for reducing its viral infectivity and the associated risk of environmental pollution with live viruses.

The results which we obtained on the inactivation of viruses in slurry by treatment with calcium hydroxide suggested that this might prove to be an effective method for the removal and inactivation of viruses, and our findings corresponded with those of Sattar *et al.* (1976) with poliovirus in domestic sewage. It was of interest that this treatment inactivated the bovine enterovirus most rapidly and the porcine adenovirus least rapidly, which was opposite to the effect of aeration of slurry containing the same viruses. The application of the procedure to the treatment of slurry under field conditions would require attention from the engineering aspect at this stage.

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