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The inactivation of a bovine enterovirus and a bovine parvovirus in cattle manure by anaerobic digestion, heat treatment, gamma irradiation, ensilage and composting

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SUMMARY

A bovine enterovirus and a bovine parvovirus seeded into liquid cattle manure were rapidly inactivated by anaerobic digestion under thermophilic conditions (55 °C), but the same viruses survived for up to 13 and 8 days respectively under mesophilic conditions (35 °C). The enterovirus was inactivated in digested liquid manure heated to 70 °C for 30 min, but the parvovirus was not inactivated by this treatment. The enterovirus, seeded into single cell protein (the solids recovered by centrifugation of digested liquid manure), was inactivated by a gamma irradiation dose of 1·0 Mrad, but the parvovirus survived this dose. When single cell protein seeded with bovine enterovirus or bovine parvovirus was ensiled with cracked corn, the enterovirus was inactivated after a period of 30 days, while the parvovirus survived for 30 days in one of two experiments. Neither the enterovirus nor the parvovirus survived composting for 28 days in a thermophilic aerobic environment when seeded into the solid fraction of cattle manure. It was concluded that, of the procedures tested, only anaerobic digestion under thermophilic conditions appeared to be a reliable method of viral inactivation to ensure the safety of single cell protein for refeeding to livestock. Composting appeared to be a suitable method for the disinfection of manure for use as a soil conditioner.

INTRODUCTION

There is increasing interest among livestock producers in the use of anaerobic digestion for the processing of animal manure, particularly in intensive production systems in which large volumes of liquid manure or slurry are produced. Successful full-scale examples of the technology have been demonstrated on both intensive beef cattle and swine farms in Canada. The advantages over alternative methods such as aerobic treatment of liquid cattle manure include the production of methane gas as a by-product, and the production of single cell protein (SCP), which is the solid matter recovered by centrifugation of the digested liquid manure. The SCP can be mixed with conventional rations for re-feeding to livestock as a protein

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and phosphorus supplement. A concern over the re-feeding of SCP is that it may contain pathogenic micro-organisms, including viruses, originating from the raw manure (Jones, 1980).

In this study, we investigated the inactivation of a bovine enterovirus and a bovine parvovirus during the anaerobic digestion, under both thermophilic and mesophilic conditions, of liquid cattle manure, as well as the effect of heating at 70 °C for 30 min on the infectivity of the same viruses in digested liquid manure, and of gamma irradiation and ensilage of SCP derived from anaerobic digestion. The effect of composting cattle manure on the infectivity of these viruses was also studied. The viruses were selected as representatives of the most resistant bovine enteric viruses. Since our objective was to determine whether a particular procedure would completely inactivate the viruses, the latter were seeded into the various test materials at concentrations which were comparable to the anticipated levels of contamination in the field, and residual infectivities were determined by a sensitive qualitative virus isolation procedure rather than by more elaborate titration methods. The study reported in this paper was part of a larger project which included bacteriological and parasitological investigations.

MATERIALS AND METHODS

Virological procedures

The bovine enterovirus was isolated by Derbyshire & Brown (1978) and an enteric isolate of bovine parvovirus was kindly supplied by Dr J. A. Lynch, Ontario Ministry of Agriculture and Food, Guelph, Ontario. A stock of the enterovirus was prepared by cultivation in Madin–Darby bovine kidney (MDBK) cells, and titrated for infectivity in the same cells. The titre of the stock virus was 10^6 median cell culture infectious doses ($CCID_{50}$) per ml. The bovine parvovirus was cultivated and titrated in embryonic bovine spleen (EBS) cells, and the titre of the stock virus was $10^{5 \cdot 5}$ $CCID_{50}$ per ml. These virus stocks were used to seed the various materials described below.

For the determination of residual infectivity in samples from the anaerobic digestion and heat treatment experiments, each sample was frozen and thawed three times, centrifuged at low speed, and the supernatant filtered through a 0·45 μm Millipore membrane filter which had been pre-treated with fetal bovine serum. The samples of single cell protein cake used in the irradiation experiments, the ensiled samples and the composted samples were suspended (10 %, w/v) in cell culture medium before freezing and thawing, centrifugation and filtration as above. Each filtrate was inoculated onto four monolayer cell cultures of MDBK cells for the isolation of the enterovirus, and onto EBS cells for parvovirus isolation. The cultures were examined daily for cytopathic effects for 7 days, and all negative cultures were passed a second time in the same cells. The EBS cultures were tested for haemagglutinating activity before they were discarded. The isolated viruses were presumptively identified on the basis of chloroform sensitivity, filtration through 0·05 μm membrane filters, cytopathic effect and haemagglutination reaction.

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Table 1. *The inactivation of a bovine enterovirus during the anaerobic digestion of liquid cattle manure*

Digester		Residual infectivity after inoculation											
					Days								
Temperature (°C)	Retention time (days)	Before inoculation	30 min	4 h	1	2	3	5	8	11	13
55	10	–	–	–	–	–	–	–	–	–	–
55	40	–	–	–	–	–	–	–	–	–	–
35	10	–	+	+	(+)	(+)	(+)	–	(+)	(+)	–
35	40	–	+	+	(+)	–	–	(+)	–	–	(+)

–, No virus was isolated. +, Bovine enterovirus isolated on first passage in MDBK cells. (+), Bovine enterovirus isolated on second passage in MDBK cells.

Anaerobic digestion

The laboratory scale digesters used were 25 l sealed stainless steel vessels with top-mounted stirrers. The digesters were fed once daily with sufficient liquid cattle manure to produce hydraulic retention times (total volume/volume added daily) of 10 days and 40 days. Before the addition of the required volume of manure (one tenth or one fortieth of the digester volume) an identical volume of the digester contents was removed. Two digesters were operated under thermophilic (55 °C) and two under mesophilic (35 °C) conditions, with hydraulic retention times of 10 days or 40 days. In two separate experiments, each digester was inoculated with 50 ml of stock bovine enterovirus or bovine parvovirus. The digesters were sampled pre-inoculation and at intervals after inoculation with virus as indicated in Tables 1 and 2. Each sample was tested for residual viral infectivity as described above.

Heat treatment

Flasks containing 1 l volumes of liquid cattle manure which had been anaerobically digested under mesophilic conditions were seeded with 2·0 ml of stock bovine enterovirus or bovine parvovirus. The flasks, together with an uninoculated control, were incubated in a water bath at 70 °C, and sampled after incubation for 5 min and 30 min for residual viral infectivity assay. The contents of the flasks were continuously stirred during the experiments, each of which was repeated once.

Gamma irradiation

Approximately 1 kg amounts of single cell protein cake prepared by centrifugation of anaerobically digested liquid cattle manure were thoroughly mixed with 10 ml of stock bovine enterovirus or bovine parvovirus. The inoculated materials, and an uninoculated control, were spread in 1 cm layers in plastic petri dishes, and each was sealed with a second dish. The materials were then irradiated by exposure to a cobalt-60 gamma irradiation source (IR-37 Irradiator, Atomic Energy of Canada) at University Hospital, London, Ontario at a rate of 2·29 Krad per min to provide dosages of 0·4 and 1·0 Mrad. Similar inoculated and uninoculated control samples were not irradiated, and all were tested for residual viral infectivity. The experiment was repeated once.

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Table 2. *The inactivation of a bovine parvovirus during the anaerobic digestion of liquid cattle manure*

Digester			Residual infectivity after inoculation												
				Hours			Days								
Temperature (°C)	Retention time (days)	Before inoculation	30 min	2	4	7	1	2	3	4	5	8	10	15	20
55	10	−	+	+	+	+	(+)	−	−	−	−	−	−	−	−
55	40	−	+	+	(+)	(+)	−	−	−	−	−	−	−	−	−
35	10	−	+	+	+	+	+	+	+	+	(+)	(+)	−	−	−
35	40	−	+	+	+	+	+	+	+	−	−	−	−	−	−

−, No virus isolated. +, Bovine parvovirus isolated on first passage in EBS cells. (+), Bovine parvovirus isolated on second passage in EBS cells.

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Ensilage

Single cell protein cake was mixed with bovine enterovirus or bovine parvovirus as in the irradiation experiment. Each of the inoculated materials, together with an uninoculated control, was then mixed with sufficient cracked corn to produce a final bulk moisture content of approximately 55 %. Each mixture was then placed in a double wrapped polyethylene bag from which the air was removed by vacuum. The sealed bags were incubated at 30 °C for 30 days. Samples were collected for virus isolation before and after incubation. The pH of the samples was determined as a measure of the effectiveness of the ensilage process. The experiment was repeated once.

Composting

Cattle manure solids were obtained from the low-speed centrifuge used by a local beef feedlot operator to recover the solids for a full-scale composting operation. The screened solids were returned to the laboratory where they were subdivided into approximately 600 g lots, and each lot was inoculated with 10 ml of stock bovine enterovirus or bovine parvovirus. The inoculated solids were placed in a 1 l glass beaker fitted with a fine screen approximately 2 cm from the bottom of the beaker. Water was injected by syringe into the cavity below the screen as required throughout the study to maintain a relatively constant moisture content in the composting solids. The solids were placed in an oven in which the temperature was controlled to simulate the internal temperature rise of a compost pile. The operating temperature of 30 °C on day 1 was increased to 45 °C on day 2 and maintained at 60 °C from day 3 to the termination of the experiment on day 28. The solids were turned daily to promote aeration and to prevent crusting on the sides of the beaker. The same procedure was followed for an uninoculated control. Volatile organic matter was determined at the beginning and end of each trial by standard waste analytical procedures (American Public Health Association, 1981). Samples of the solids from the inoculated and control beakers were tested for infectivity at the beginning and end of the composting trials. The experiment was repeated once.

RESULTS

Viral inactivation by anaerobic digestion

The results of the experiment with the bovine enterovirus are given in Table 1. The virus was rapidly inactivated in the thermophilic anaerobic digesters since residual infectivity was not detected after treatment for only 30 min. Under mesophilic conditions, residual infectivity was detected consistently for up to 3 days in the 10 day retention time digester, with further sporadic isolations on second passage only throughout the sampling period from both of the mesophilic digesters. The bovine parvovirus was also fairly rapidly inactivated under thermophilic conditions (Table 2), with no residual infectivity detected by 2 days. Under mesophilic conditions, the parvovirus was completely inactivated by 10 days.

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Table 3. *The inactivation of a bovine enterovirus and a bovine parvovirus in digested liquid cattle manure heated at 70 °C*

		Residual infectivity after inoculation			
		Control slurry		Infected slurry	
Virus	Experiment number	5 min	30 min	5 min	30min
Bovine enterovirus	1	−	−	+	−
	2	−	−	−	−
Bovine parvovirus	1	−	−	+	+
	2	−	−	+	+

−, No virus isolated. +, Virus isolated on first passage in MDBK or EBS cells.

Table 4. *The inactivation of a bovine enterovirus and a bovine parvovirus in single cell protein cake by gamma irradiation*

		Residual infectivity after irradiation dose		
Inoculated virus	Experiment number	None	0·4 Mrad	1·0 Mrad
None (control)	1	−	−	−
	2	−	−	−
Bovine enterovirus	1	+	+	−
	2	+	+	−
Bovine parvovirus	1	+	+	+
	2	+	+	+

−, No virus isolated. +, Virus isolated on first passage in MDBK or EBS cells.

Viral inactivation by heat treatment

The results of these experiments are given in Table 3. No virus was isolated from the control samples. In the first experiment residual bovine enterovirus infectivity was present after 5 min but not after 30 min. In neither experiment was the bovine parvovirus inactivated by heating for 30 min.

Viral inactivation by gamma irradiation

The results of these tests are given in Table 4. No virus was isolated from the uninoculated controls in either experiment. The infectivity of the bovine enterovirus survived an irradiation dose of 0·4 Mrad but was inactivated by 1·0 Mrad in both experiments. The bovine parvovirus was not inactivated by either of the irradiation doses used.

Viral inactivation by ensilage

The results of the ensilage tests are given in Table 5. The pH values of the final samples ranged from 4·4 to 6·1. No virus was isolated from the control samples, and the bovine enterovirus was inactivated by the ensilage process in both experiments. Bovine parvovirus infectivity was demonstrated in the final sample in experiment 1 but not in experiment 2.

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Table 5. *The inactivation of a bovine enterovirus and a bovine parvovirus during ensilage of single cell protein cake for 30 days*

Inoculated virus	Experiment number	pH		Residual infectivity	
		Initial	Final	Initial	Final
None (control)	1	7·8	5·7	−	−
	2	7·8	4·6	−	−
Bovine enterovirus	1	7·8	5·9	+	−
	2	7·8	4·4	+	−
Bovine parvovirus	1	7·9	6·1	+	+
	2	7·9	5·9	+	−

−, No virus isolated. +, Virus isolated on first passage in MDBK or EBS cells.

Table 6. *The inactivation of a bovine enterovirus and a bovine parvovirus in separated manure solids by simulated composting*

Inoculated virus	Experiment number	Residual infectivity	
		Initial	Final
None (control)	1	−	−
	2	−	−
Bovine enterovirus	1	+	−
	2	+	−
Bovine parvovirus	1	+	−
	2	+	−

−, No virus isolated. +, Virus isolated on first passage in MDBK or EBS cells.

Viral inactivation by composting

The results of the composting trial are summarized in Table 6. No infectivity was found in the control samples at any time. Both the bovine enterovirus and the bovine parvovirus were isolated from the inoculated samples at the beginning of the trials; after 28 days of composting, no residual infectivity was detected in either of the two experiments. The initial volatile organic matter content of the solids was 52–53 % of the total dry matter, whereas at the end of the composting trials the volatile content was reduced to 36–40 % of the dry matter.

DISCUSSION

Anaerobic digestion under mesophilic conditions (35 °C) was an inconsistent method for the inactivation of both of the viruses which we tested. While there are no directly comparable studies in the literature, Srivastava & Lund (1980) found that a bovine parvovirus remained infectious for 81 days at 20 °C, and inctivation of a porcine enterovirus required 5 days at 35 °C (Meyer *et al.* 1971). The continued isolation of the enterovirus from mesophilic digests over a period of several days only on second passage in cell culture suggests the persistence of a small, relatively stable fraction, perhaps associated with viral aggregation or

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adsorption to particulate solids in the manure as discussed by Stagg (1982). On the other hand, inactivation of both viruses by anaerobic digestion under thermophilic (55 °C) conditions was rapid, and this finding suggests that this should be a safe procedure for the treatment of cattle manure. Unfortunately, thermophilic digestion is more costly to operate than mesophilic digestion because of the energy required to maintain the higher operating temperature.

Because of the apparent persistence of a small stable fraction of the enterovirus, no conclusions were reached with respect to the effect of retention time on the inactivation of the enterovirus. The bovine parvovirus did appear to be inactivated more rapidly in digesters operating at 40 day retention periods than in the 10 day retention times at both 35 and 55 °C, although additional testing should be conducted to confirm this. Sanders *et al.* (1979) reported that poliovirus type 1 was inactivated more rapidly at 35 °C when the retention time was longer. They noted that retention time had no effect on the loss of poliovirus infectivity at thermophilic temperatures.

While the bovine enterovirus was inactivated in digested manure by heating at 70 °C for 30 min, this treatment failed to inactivate the bovine parvovirus. This finding corresponds with the demonstration of high resistance of the bovine parvovirus to thermal inactivation by Srivastava & Lund (1980). Parvovirus inactivation would clearly require heat treatment at a higher temperature or for a longer period than we used, with consequently higher operating costs. We found that the parvovirus appeared to be more resistant than the enterovirus to inactivation by gamma irradiation, since it survived an irradiation dose of 1·0 Mrad. This finding is in general accord with the conclusions of others that doses of gamma irradiation of 3·0 Mrad (Simon *et al.* 1983) or 4·0 Mrad (Thomas, Ouwerkerk & McKercher, 1982) are necessary for the safe disinfection of animal viruses in effluents. We did not study higher doses of irradiation because the capital and operating costs of an appropriate on-farm facility for gamma irradiation would be high relative to the costs of the other processes which we have investigated.

Ensilage showed promise as a viral inactivation procedure, although the bovine parvovirus remained infectious after 30 days in one experiment. Gilbert *et al.* (1983) reported the inactivation of bovine virus diarrhoea virus in less than 2 h, and the inactivation of a porcine enterovirus after 3 days at 30 °C and after 8 days at 20 °C in an ensilage process. When material has been properly ensiled, the pH should fall to between 4·0 and 4·5 due to the production of lactic acid by fermentation. However, most of the pH values which we obtained were above this range, suggesting that optimum conditions for ensilage were not achieved. The highest pH value was found in the material in which the bovine parvovirus survived for 30 days, and the survival of the virus may be due to the ensilage conditions being less than optimum. Despite this, ensilage appeared to be at least as effective a viral inactivation procedure as heat treatment or gamma irradiation under the conditions which we used.

In these studies, composting was a successful method for inactivating both viruses. The prolonged retention at 60 °C was sufficient to inactivate even the heat-resistant parvovirus. These results are in general agreement with reports in the literature, which indicated that poliovirus types 1 and 2 were inactivated by a maximum of 7 days (Wiley & Westerberg, 1969; Gaby, 1975), and the

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heat-resistant bacteriophage f2 was inactivated by 17 days (Burge, Cramer & Epstein, 1978). Because the enterovirus and parvovirus were inactivated within 2 days of inoculation into the thermophilic (55 °C) anaerobic digesters, it is probable that a composting time shorter than 28 days could be used to destroy viral infectivity. The reduction in the volatile organic content of the manure solids by the end of the composting trials indicated that aerobic stabilization had occurred in the laboratory simulators.

Anaerobic digestion of raw manure under thermophilic conditions would not require the use of an additional inactivation procedure on the single cell protein produced, and this would appear to be the optimum method for the production of SCP which can be safely utilized for refeeding to livestock. However, in field experiments, SCP derived from mesophilic digestion has been refed to the animals which produced the manure without apparent adverse effects (Monteith *et al.* 1982). For treating manure prior to its disposal on the land instead of refeeding it to livestock, aeration of the liquid (Derbyshire & Brown, 1979; Albrecht & Strauch, 1980; Lund & Nissen, 1983) or composting of the solids may be satisfactory procedures for reducing the risk of environmental pollution with live viruses.

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