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Isolation of animal viruses from farm livestock waste, soil and water

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SUMMARY

Ten porcine enteroviruses, 2 porcine adenoviruses and 1 coronavirus were isolated directly from 32 samples of slurry collected from a pig fattening house. Concentration of the same samples by adsorption with the polyelectrolyte PE-60 yielded 24 porcine enteroviruses and 3 porcine adenoviruses. A porcine enterovirus was isolated, following PE-60 concentration, from 1 of 6 slurry samples from a sow farrowing house. No virus was isolated from 12 samples of slurry from dairy cows nor from 6 slurry samples from a calf-rearing unit. A porcine enterovirus was isolated from soil samples, after concentration with PE-60, collected 1, 2 and 8 days after pig slurry was spread on hay stubble. Two porcine enteroviruses were isolated by membrane filtration from 26 samples of surface run-off from land on which pig slurry was routinely spread, and 2 bovine enteroviruses were isolated from cattle feedlot run-off after adsorption to layers of talc and celite followed by hydroextraction. A porcine enterovirus was also isolated from 1 of 33 samples of surface water collected on farms on which pig slurry was routinely spread on the land, but no virus was isolated from 36 samples of ground water from the same farms. The surface water and ground water samples were concentrated by talc-celite adsorption and hydroextraction.

INTRODUCTION

The widespread collection and disposal on agricultural land of livestock wastes in the form of liquid manure or slurry presents a greater microbial pollution hazard than the traditional composting of manure, which provides a better opportunity for the thermal inactivation of microbial agents. Several workers have investigated this problem from the bacteriological aspect. An early contribution was by Rankin & Taylor (1969), who isolated *Salmonella dublin* and *Escherichia coli* from samples of cattle slurry. More extensive studies were reported by Jones & Matthews (1975) who found salmonellas in 11 % and leptospires in 30 % of 187 samples of slurry from cattle, and by Jones *et al.* (1976), who found salmonellas in pig slurry from 12 farms and enteropathogenic *E. coli* from 13 farms out of 54 which were sampled. *Salmonella infantis* was isolated from cattle feedlot litter and run-off by Miner, Fina & Piatt (1967), while Hrubant, Daugherty & Rhodes (1972) found a range of enterobacteria in cattle feedlot wastes. In this report, the occurrence of smaller

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numbers of the same organisms in run-off and drainage ditches at the feedlot was also described. Studies have also been reported on the survival of pathogenic bacteria on land which had been sprayed or irrigated with liquid manure. When slurry contaminated with *S. typhimurium* was sprayed on pasture, the organisms persisted for 18 days (Taylor & Burrows, 1971), and the survival of *S. dublin* in soil cores for 5 months after the spreading of cattle slurry was described by Findlay (1971). Jack & Hepper (1969) reported an outbreak of *S. typhimurium* infection in cattle grazing pasture which had been irrigated 3 weeks previously with slurry containing this organism, and the same workers isolated *S. typhimurium* from a stream into which seepage from the slurry tank drained.

Virological studies comparable with the above have not been reported, although the presence of human viruses in domestic sewage, sewage effluents, rivers and streams is well recognized (Berg, 1971), and farm livestock are known to excrete a variety of viruses in their faeces. Malherbe, Strickland-Cholmley & Geyer (1967) isolated enteroviruses, adenoviruses and reoviruses from intestinal washings from cattle and sheep in an abattoir, and the ability of foot-and-mouth disease virus to survive in liquid manure was described by Rozov & Andryunin (1972), Bauer & Eissner (1972) and by Polyakov & Adryunin (1974). These workers found that the survival of the virus was temperature-dependent, varying from 25 days in summer to 180 days in winter. In the present paper we describe attempts to isolate viruses from pig and cattle slurry, from soil samples from land on which pig slurry had been spread, and from samples of surface run-off, surface water and ground water collected on farms where pig slurry was routinely spread on agricultural land. We also examined samples of surface run-off collected at cattle feedlots. Some of our findings were summarized in a previous paper (Derbyshire, 1976).

MATERIALS AND METHODS

Isolation of viruses from slurry

Slurry samples were collected from 4 sites on the University of Guelph farms. One site was a liquid manure tank which received effluent from a swine fattening house. The tank was routinely emptied each week, and 32 samples of slurry were collected at weekly intervals, usually on the day after the tank was emptied. Six samples of waste draining from a sow farrowing house were collected at the same piggery. Samples of cattle slurry were collected at 2 sites on a dairy farm. Twelve samples were obtained from a liquid manure tank which received effluent from a large barn of milking cows, and 6 samples were collected from a tank which received liquid manure from a unit which contained weaned dairy calves. Each of these tanks was routinely emptied at intervals of 6 to 8 weeks. Slurry samples of about 2 litres were collected in buckets and transported to the laboratory in plastic containers. Each sample was concentrated by adsorption on the insoluble polyelectrolyte PE-60 (Wallis *et al.* 1969) as follows. The slurry was first centrifuged at 5860 *g* for 20 min, and the supernatant was passed through a serum-treated Millipore AP-20 filter. The pH of 1 litre of filtrate was adjusted to 4·5, and 1 ml of 10 % PE-60 (Monsanto Co., St Louis, Missouri, U.S.A.), suspended as described

by Wallis *et al.* (1969) was added and stirred for 2 h with a magnetic stirrer. The suspension was then filtered through an untreated Millipore AP-20 filter pad, the PE-60 was collected from the pad, suspended in 5 ml Eagle's minimum essential medium (EMEM – Gibco, Grand Island, N.Y., U.S.A.), the pH adjusted to 8·0 and the suspension centrifuged at 650 *g* for 10 min. The supernatant was then filtered through a serum-treated 0·45 μm Millipore filter, and this filtrate was used to inoculate cell cultures. In addition, about 10 ml of the original clarified raw slurry supernatant was filtered through a serum-treated 0·45 μm Millipore filter. Each of the above 0·45 μm filtrates was inoculated into 4 tubes of monolayer cell cultures in volumes of 0·1 ml. Primary pig kidney (PK) cells were used for inoculation with the filtrates of pig slurry, and the filtrates of cattle slurry were inoculated into tube cultures of primary embryonic bovine kidney (EBK) cells. Conventional cell culture techniques were used, and the cells were maintained in EMEM supplemented with 5 % fetal bovine serum (FBS) and with penicillin and streptomycin. The cultures were inoculated at 37 °C and examined daily for cytopathic effects. When the latter were detected, the infected cultures were examined electron microscopically by the negative staining technique (McFerran, Clarke & Curran, 1971). If the cytopathology and morphology suggested the isolation of an enterovirus, further identification was based on chloroform sensitivity (Feldman & Wang, 1961), pH sensitivity (Tyrrell & Chanock, 1963) and size (Hsiung, 1965) determinations. Negative cultures were passed once in the same cell system. Three of the positive samples of pig slurry from the swine fattening house were titrated for infectivity in PK cells as described by Derbyshire & Jessett (1967).

Isolation of viruses from soil samples

Pig slurry was spread in July on hay stubble at the rate of 15 tons per acre. A sample of the slurry was subjected to the PE-60 concentration and virus isolation procedure detailed above. After spreading of the slurry, samples of surface vegetation and soil to a depth of 2·5 cm were collected 1, 2, 3, 8, 15 and 22 days after spreading. The volume of each sample collected was about 1 litre, and the material was suspended in distilled water and then centrifuged to remove gross particles. The supernatant was then clarified and concentrated by adsorption with PE-60 as described above for slurry samples, and each concentrate was inoculated on PK cell cultures. Each sample was passaged at least once more in PK cells, and any virus which was isolated was provisionally identified as before on the basis of cytopathology, morphology and size, and chloroform and pH resistance.

Isolation of viruses from surface run-off

Collection sites were established on 13 pig farms in Southern Ontario. Each site was in a location where pig slurry was routinely spread on sloping ground. At the foot of the slope in each instance, a collection device, consisting of plastic sheeting providing drainage into a 4 litre plastic container buried beneath the soil surface, was installed. Each site was inspected routinely at weekly intervals, and as soon as possible after heavy rainfall in the area. At each visit, water samples of less than 2 litres were discarded, and greater volumes were transported to the laboratory

where they were concentrated by a membrane filter adsorption technique based on that described by Berg, Dahling & Berman (1971). The water was first buffered with 0·5 % dibasic sodium phosphate and the pH adjusted to 7·0 with citric acid. The sample was then clarified by pre-filtration through a serum-treated Millipore AP-20 filter, and then passed through an untreated 0·45 μm Millipore filter, using 4 or 5 filters to process each sample. The filters were then soaked in 10 ml of 3 % beef extract and sonicated for 15 min for elution of any adsorbed virus. The disintegrated filter material was removed by centrifugation and the supernatant passaged twice in PK cell cultures as described above. The presence of cytopathogenic agents was always confirmed by further passages in PK cells, and by reisolation of the agent from the original concentrate. Agents which were isolated were provisionally identified on the basis of cytopathology, morphology, and size, and chloroform and pH resistance as before.

Samples of surface run-off were also collected, after heavy rain, at 4 cattle feedlots in Southern Ontario. The samples, which varied in volume from 2 litres to 4 litres, were concentrated by adsorption with layers of talc and celite by a method based on that described by Sattar & Westwood (1974), which we had shown in preliminary studies to give recoveries of bovine and porcine enteroviruses seeded into water of 10 % to 50 %. In this procedure, the pH of the water was adjusted to 6·0, and the sample was then passed under gravity through a layer consisting of a mixture of 15 g talc and 5 g celite between sheets of Whatman no. 114 filter paper in a modified Buchner funnel at 4 °C. Then, 50 ml of 10 % FBS in 0·85 % saline was passed through the talc-celite layer in order to elute adsorbed virus, and the eluate was further concentrated by hydroextraction at 4 °C with polyvinylpyrollidone (Bucca, Casey & Winn, 1960) to a volume of 5 ml. This concentrate was then passaged twice in EBK cell cultures, as described above for cattle slurry, and viruses isolated were identified as before.

Isolation of viruses from surface water and ground water

Twelve of the pig farms on which surface run-off was collected were also used for the collection of surface water or ground water. On 11 of these farms, surface water samples were obtained from ponds or streams which received run-off from the sites on which pig slurry was spread, and on each of the 12 farms ground water was available from a well which provided the supply of farm water. Three samples of surface water and ground water were collected from each farm. Each sample consisted of 20 litres of water, which were concentrated by talc-celite adsorption followed by hydroextraction with polyvinylpyrollidone as described above. Each final concentrate was passaged twice in PK cell cultures in the same way that the concentrates obtained from the surface run-off samples were processed.

RESULTS

Isolation of viruses from pig and cattle slurry

The results of this study are given in Table 1, from which it will be seen that the major source of positive samples was slurry collected from the swine fattening

Table 1. *Isolation of viruses from pig and cattle slurry*

Source of slurry	Number of samples	Viruses isolated	
		Directly	After concentration
Swine fattening house	32	10 enteroviruses 2 adenoviruses 1 coronavirus	24 enteroviruses 3 adenoviruses
Sow farrowing house	6	0	1 enterovirus
Dairy cattle barn	12	0	0
Calf rearing unit	6	0	0

house, which yielded 28 viruses, of which 24 were porcine enteroviruses, from 32 samples, 13 of which contained sufficient virus to be isolated without concentration. Titration of 3 samples gave titres of 4·7, 5·0 and 5·5 $\log_{10}$ median tissue culture infectious doses (TCID50) per litre of clarified slurry. One porcine enterovirus was isolated from the sow slurry, but no isolations were made from the cattle slurry. The enteroviruses were identified on the basis of their characteristic cytopathic effects in PK cells, size and resistance to chloroform and pH 4·0. The identification of the adenoviruses and the coronavirus was based on their morphology; no serological typing of the viruses was attempted.

Isolation of viruses from soil samples

A porcine enterovirus was isolated, after concentration with PE-60, from a sample of the slurry which was spread on the stubble. The soil samples which were collected 1, 2 and 8 days after the slurry was spread each yielded a porcine enterovirus after concentration with PE-60, while the soil samples obtained 3, 15 and 22 days after spreading were negative. Heavy rainfall was recorded in the area 7 and 16 days after the slurry was spread.

Isolation of viruses from surface run-off

A total of 26 samples of surface run-off were obtained from the sites on which pig slurry was routinely spread. Four samples were collected in July, 2 in August, 10 in September, 7 in October and 3 in November. The collection of a satisfactory sample was dependent upon the occurrence of local rainfall. Each of 6 of the collection sites yielded only a single sample of greater than 2 litres, 3 sites yielded 2 samples each, 3 samples were obtained from each of 3 sites and five samples were obtained from 1 site. Two confirmed isolations (Table 2) of porcine enterovirus were made, each from a different location, and both collected at the end of July. During the testing of an additional 2 samples, from 2 different sites, cytopathic effects were seen in the second passage of the concentrates in PK cells, but these were not confirmed when attempts were made to reisolate a virus from the concentrate of the original sample, and these latter isolations are regarded as equivocal.

Three samples of cattle feedlot run-off were obtained in June, 1 in October and a further 3 in November. One collection site yielded 3 samples, 1 yielded 2 samples and a single sample was obtained from each of the other 2 sites. Two bovine

Table 2. *Isolation of viruses from surface run-off, surface water and ground water*

Sample	Number of samples	Virus isolated
Run-off from land spread with pig slurry	26	2 porcine enteroviruses
Cattle feedlot run-off	7	2 bovine enteroviruses
Surface water	33	1 porcine enterovirus
Ground water	36	0

enteroviruses were isolated (Table 2), one in June and the other in November, from different feedlots. Each was confirmed by reisolation of the virus from the same concentrate.

Isolation of viruses from surface water and ground water

Thirty-three samples of surface water and 36 samples of ground water were tested between November and January (Table 2). The only virus isolated from this material was a porcine enterovirus from a surface water sample collected in January.

DISCUSSION

Our findings on the slurry samples indicated that porcine enteroviruses were frequently present in slurry from a swine fattening house. Not only were enteroviruses regularly demonstrated in this material, but they were often isolated without concentration of the sample. This suggested a relatively high viral content, which was confirmed for the 3 samples which were titrated. The large number of isolations of enteroviruses is probably related to the high rate of excretion of these viruses by piglets (Derbyshire, Clarke & Jessett, 1966), to their rapid multiplication in cell culture and to the efficiency of the PE-60 adsorption procedure for the concentration of porcine enteroviruses (Hazlett, 1977). The lower prevalence of viruses in sow slurry corresponds with the lower excretion rate of enteric viruses from adult pigs compared with recently weaned piglets (Derbyshire *et al.* 1966). Our failure to isolate viruses from the cattle slurry may indicate that milking cows and older calves excrete viruses less regularly than recently weaned piglets, but an additional factor may be that the cattle slurry tanks were emptied less frequently than the pig slurry tank, so that while the pig slurry was always relatively fresh, much of the cattle slurry had been stored for a longer period. Since we had established a high prevalence of porcine enteroviruses in pig slurry, our subsequent investigations on soil and water related to the disposal of this material, although we did subsequently demonstrate bovine enteroviruses in cattle feedlot run-off.

Our findings indicated that, even under the relatively unfavourable conditions for virus survival, in terms of temperature and sunlight, which prevail during the summer, a porcine enterovirus can remain viable in surface soil for a period of at least 8 days after deposition in contaminated slurry. The survival of human

viruses in soil has been fairly well documented, and Gerba, Wallis & Melnick (1975) concluded that viruses survive in soil for at least as long as pathogenic enteric bacteria. Furthermore it was apparent from the results of our examination of run-off samples for porcine enteroviruses that these viruses can be eluted from the soil by rain water, and may be present in a viable form in concentrations of at least 1 TCID50 per ml of surface run-off. Our isolation of a porcine enterovirus from a sample of surface water suggests that, on occasion, run-off from agricultural land on which slurry has been spread may result in significant viral pollution of surface water supplies. Hanson & Schipper (1975) demonstrated the ability of a bovine herpesvirus to survive in river water for at least 12 h, and human viruses are found not uncommonly in surface water, usually as a result of contamination with inadequately treated sewage (Lamb, Chin & Scarce, 1964). The recovery of human viruses from ground water after effluent discharge onto soil has also been reported (Wellings *et al.* 1975), but in our study we failed to isolate viruses from ground water samples.

In conclusion, we have shown that pig slurry may contain significant amounts of porcine enteroviruses, and that these may retain their infectivity for several days when applied to agricultural land, and find their way into surface water supplies, presumably by elution from the soil by rain water. This could provide opportunities for the spread of viral infection among livestock, and might play a significant role in the epidemiology of certain viral diseases of animals.

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