Viruses and virus-like particles in the faeces of dogs with and without diarrhoea

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SUMMARY: Negative staining electron microscopy was used to identify viruses in 157 normal and 29 diarrhoeal faecal samples collected from 156 dogs admitted to an animal shelter during an 8 month period (March to October) in 1982. Seven distinct viral types were detected: 21-26 nm parvovirus-like particles, 28-31 nm astrovirus-like particles, a previously undescribed 34-35 nm "round" virus particle, coronavirus, coronavirus-like particles (CVLP), rotavirus and papova-like virus. Parvovirus-like particles alone were detected in 14 diarrhoeal and 50 normal faeces, astrovirus-like particles in 3 normal faeces, "round" viruses in 4 normal faeces, coronavirus in 2 diarhoeal and 5 normal faeces, CVLP in one diarrhoeal and one normal faeces, rotavirus in 2 normal faeces, papova-like virus in one normal faeces, both parvovirus-like particles and coronavirus in 2 diarrhoeal and 2 normal faeces, parvovirus-like particles and rotavirus in one normal faeces and parvovirus-like and papova-like virus in one normal faeces. The significance of these findings in canine and human disease is discussed.
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Introduction

Viral gastroenteritis has emerged in the last decade as a major cause of morbidity and mortality in many animal species including man. Four main types of virus, namely parvovirus, rotavirus, astrovirus and coronavirus have been demonstrated in the faeces of dogs and are known to cause, or to be associated with, canine gastroenteritis (Appel *et al* 1979; Pastoret *et al* 1980; Williams 1980; Carmichael and Binn 1981; Hammond and Timoney 1983). There is also evidence that at least parvovirus, coronavirus and rotavirus can be shed by apparently healthy dogs (Roseto *et al* 1979; Roseto *et al* 1980a; Roseto *et al* 1980b; Hoshino *et al* 1982).

In this study the nature and frequency of virus excretion by diarrhoeal and non-diarrhoeal dogs were examined.

Materials and Methods

Collection of Specimens

Faeces were collected, usually on a weekly basis, from March to October 1982, from dogs admitted to the Lort Smith Animal Shelter and Hospital, Melbourne. This shelter admits both healthy and sick, stray and owned dogs. Dogs are generally housed in separate enclosures but are usually released into a communal exercise yard during the day. Enclosures are cleaned twice daily. A total of 186 faecal samples (29 diarrhoeal and 157 normal) were collected from 156 dogs.

Details such as status (owned, stray), ill, injured or healthy, age, sex, date of admission and medication or surgery required were recorded in most cases. The study included dogs ranging in age from pups to mature adults; very young or neonatal pups were not encountered.

Preparation of Faecal Samples

Faecal samples were processed immediately after collection as follows: a 20% (w/v) faecal suspension in Hank's complete balanced salt solution was vigorously shaken and the mixture centrifuged at 3,500 g for 15 min at 4°C. The supernatant fluid was collected and centrifuged through a one ml 45% (w/v) sucrose/0.002 M Tris buffer (pH 7) cushion for 2 h at 150,000 g using a Beckman SW41 rotor at 4°C. The supernatant fluid was then discarded and the pellet resuspended in 6 drops of the Tris buffer.

Negative Staining Electron Microscopy (EM)

The purified concentrated faecal specimens were examined after negative staining with 3% phosphotungstic acid (pH 7) on 400 mesh Formvar-carbon coated grids. At least 5 grid squares were examined for each specimen using a Philips 301 electron microscope.

Virus and virus-like particles were photographed and measured from the photographic negatives. Catalase crystals§ with half the principal lattice spacing taken to be 8.6 nm (Wrigley 1968), were used as calibration standard. The mean virus diameter was calculated from measurements of 3 to 18 virus particles in each case. Approximate numbers of particles per grid square were recorded.

Immune Electron Microscopy (IEM)

Immune electron microscopy (IEM) was carried out to identify human hepatitis A virus and canine and feline parvovirus. Human hepatitis A antiserum was provided by N. I. Lehmann of Fairfield Hospital. Feline parvovirus antiserum, raised in rabbits, was derived from the study of Studdert and Peterson (1973). Canine parvovirus antiserum was obtained from greyhound pups bled 4 weeks after infection. Negative (control) serums were also used for the tests. Appropriate dilutions of the antiserums (which resulted in the formation of distinct immune complexes) were established in preliminary experiments using hepatitis A virus (from N. I. Lehmann) and purified canine parvovirus. After mixing the concentrated faecal extract with antiserum, the mixture was incubated at 34°C for one h, left overnight at 4°C and then examined after negative staining.

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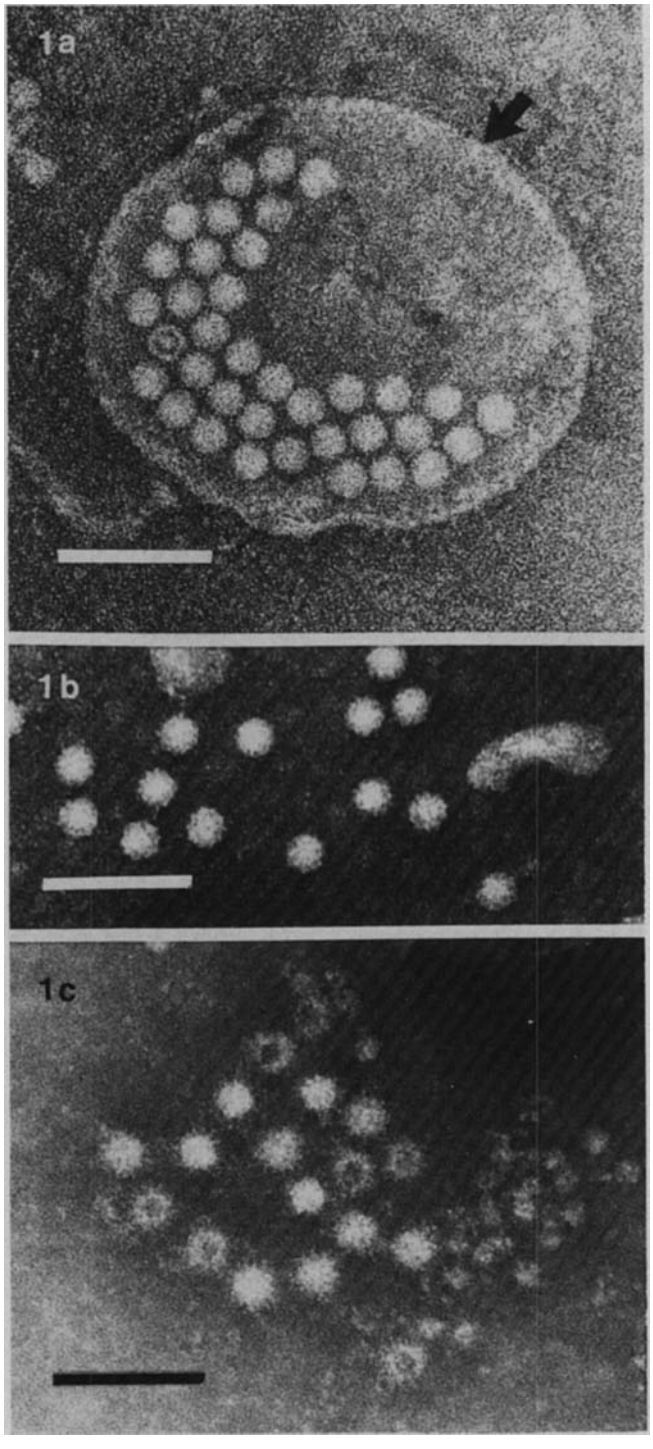

Figure 1. Parvovirus-like particles.
(a) Clump of particles associated with membrane (arrow);
(b) free virus;
(c) immune complex of virus. The bar represents 100 nm.

Virus Isolation

Virus isolation was attempted by inoculating aliquots of one drop of the purified, concentrated faecal sample into a variety of cell lines including cynomolgus monkey kidney epithelial cells (MK), heteroploid cynomolgus monkey embryonic kidney cells (MEK), rhinovirus sensitive HeLa cells (HeLa), Borrie cells (Bo) (Kennett *et al* 1974), human embryonic lung fibroblasts (HEL) and human heteroploid epithelial cells (HEp-2 cells). Details of the procedure are given in Kennett *et al* (1972).

Results

Virology

On the basis of morphology 7 distinct viruses or virus-like particles were identified in the dog faeces. IEM studies

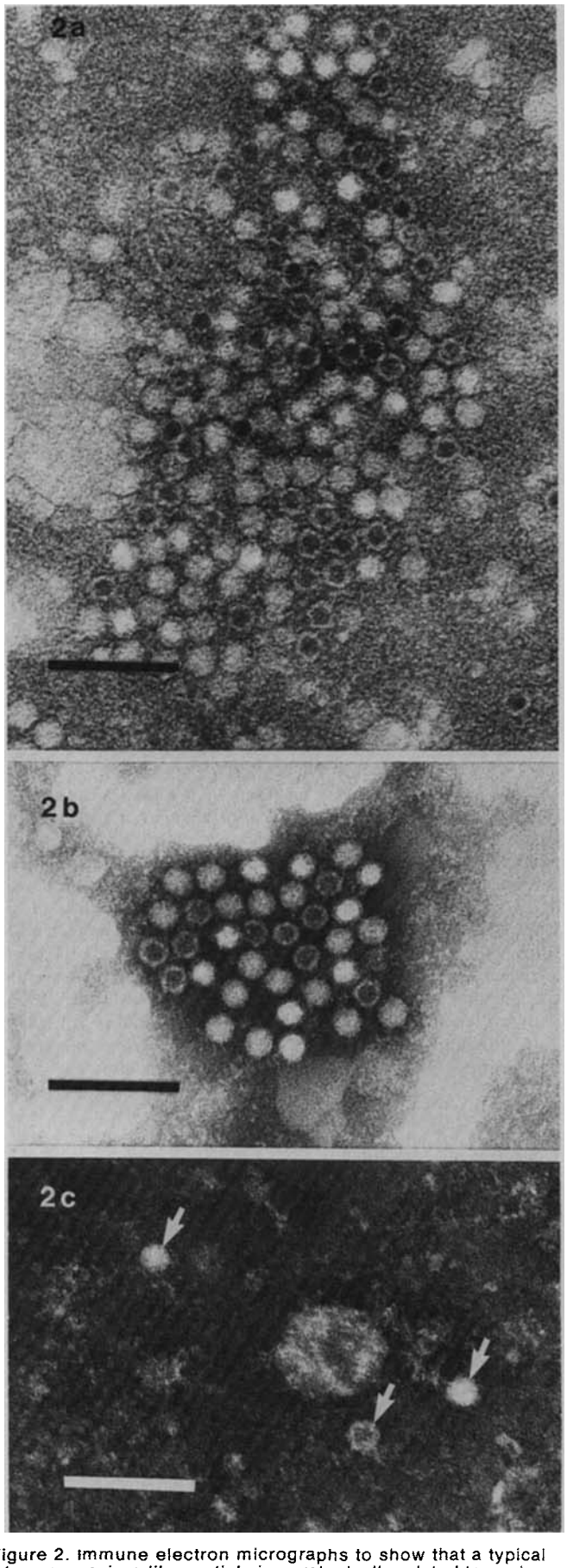

Figure 2. Immune electron micrographs to show that a typical 24 nm parvovirus-like particle is serologically related to canine and feline parvovirus.
(a) Immune complex formed by interaction of the virus and canine parvovirus antiserum;
(b) immune complex formed by interaction of the virus and feline parvovirus antiserum;
(c) uncomplexed virus (arrows) after interaction with the negative control serum. The bar represents 100 nm.

confirmed the identity of parvovirus. All attempts at virus isolation were unsuccessful.

Parvovirus-like particles — Particles, 21-26 nm in diameter (mean ± S.D. 23.3 ± 1.3 nm; n=70) were the most common detected and will be referred to as parvovirus-like particles. These small, round, typically featureless virions occurred in clumps (Figure 1a), as discrete particles (Figure 1b), and/or as immune complexes (Figure 1c). The particles were sometimes associated with membrane (Figure 1a). IEM studies using a morphologically typical (24 nm) strain showed it to be serologically related to canine and feline parvovirus (Figure 2).

Astrovirus-like particles — These relatively rare virus particles could be distinguished from parvovirus-like particles by their larger size, (28-31 nm) and the observation that they sometimes displayed a star-shaped staining pattern reminiscent of astrovirus (Figure 3). IEM tests for hepatitis A with 2 of these 3 strains, as well as virus isolation studies using MK, MEK, HeLa, Bo and HEL cells indicated they were not hepatitis A or cultivable human enteroviruses despite morphological similarity to these viruses.

"Round" virus particles — Roughly round particles, measuring 34-35 nm in diameter, were detected in 4 faecal samples, and are unlike any virus previously reported in canine faeces. It is possible they are tailless phage. In 2 cases the viruses were frequently clumped, probably due to agglutination by antibody (Figure 4). Although the particles were roughly spherical in outline, they occasionally displayed an irregular surface. Virus isolation studies were attempted with one strain using MK, MEK, HeLa, Bo, HEL and HEp-2 cells.

Coronavirus — Coronavirus could be recognised with confidence as roughly round, ovoid or pear-shaped pleomorphic particles measuring about 70 to 100 nm along the greatest dimension with petal-shaped spikes measuring about 17 nm in length (Figure 5). Virus isolation studies were attempted with two strains using MK, MEK, HeLa, Bo, HEL and HEp-2 cells.

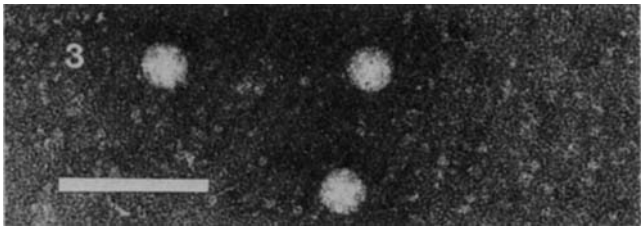

Figure 3. 28-31 nm astrovirus-like particles. Note the star-shaped staining pattern. The bar represents 100 nm.

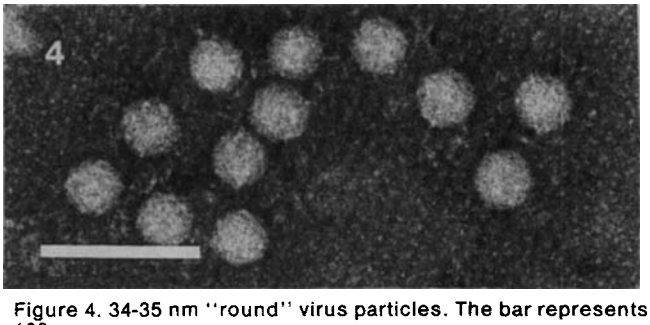

Figure 4. 34-35 nm "round" virus particles. The bar represents 100 nm.

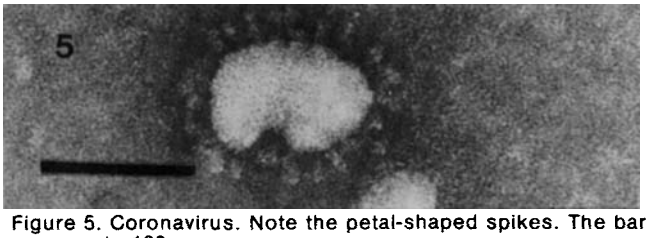

Figure 5. Coronavirus. Note the petal-shaped spikes. The bar represents 100 nm.

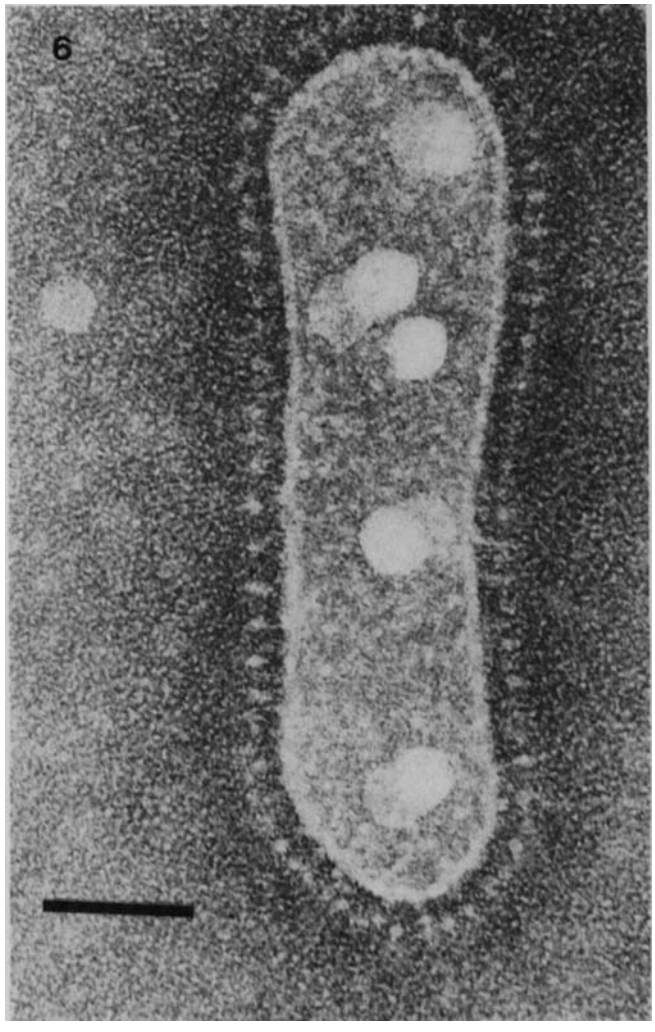

Figure 6. Coronavirus-like particle (CVLP). Note the fringe of club-shaped spikes. Bar represents 100 nm.

*Coronavirus-like particles (CVLP)** — CVLP were recognised as pleomorphic particles with a fringe of club-shaped spikes about 23 nm in length (Figure 6). The particles were highly variable in size and up to about 1000 nm along their greatest dimension. Virus isolation studies were attempted with one strain using MK, MEK, HeLa, Bo, HEL and HEp-2 cells.

Rotavirus — Rotavirus particles were identified with ease on the basis of their characteristic size and morphology (see Martin *et al* 1975) (Figure 7).

Papova-like virus — Papova-like viruses about 50 nm in diameter (Figure 8) were identified by their size and similar morphology to human and simian papovaviruses (see Madeley 1972).

Other viruses — Tailed phage was noted in most specimens; 27 nm particles, intermediate in size between the parvovirus-like particles and astrovirus-like particles, were noted in 2 cases. Their correct classification could not be made on morphological grounds and they are not considered further.

Clinical and Epidemiological Observations

Since more than one faecal sample was collected from some dogs, the clinical and epidemiological analysis below is based on both total faecal samples and total dogs. Table 1 summarises the chief clinical findings for total faecal samples.

Parvovirus-like particles — Although the proportion of diarrhoeal faeces with only parvovirus-like particles was greater than the proportion of normal faeces with only these

termed "alternate coronavirus-like particles" (CVLP) by Williams (1980).

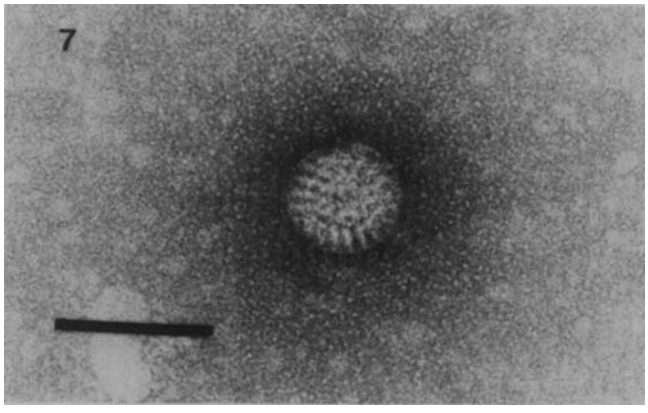

Figure 7. Rotavirus. Bar represents 100 nm.

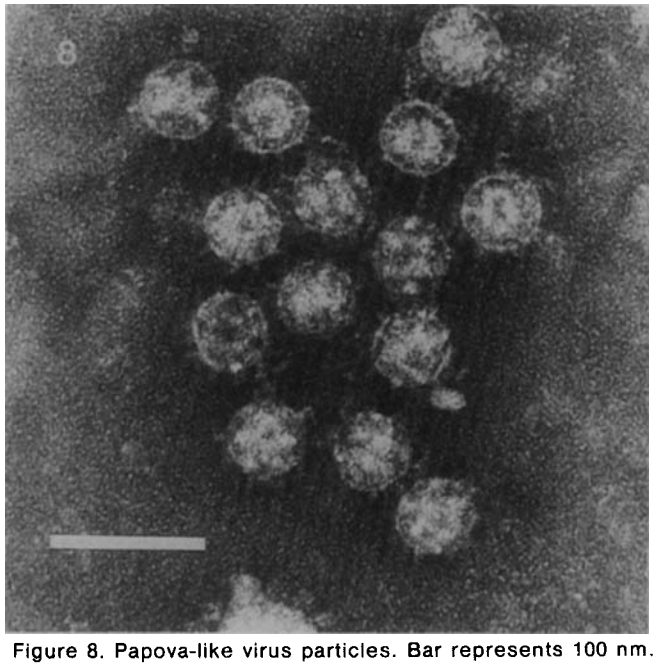

Figure 8. Papova-like virus particles. Bar represents 100 nm.

particles (Table 1), this difference was not statistically significant (chi-square test; $P > 0.05$). However, of 14 diarrhoeal faeces with only parvovirus-like particles, 6 (43%) had > 50 virus particles per grid square compared to 6 of 50 (12%) normal faeces containing only these particles; the chi-square test showed the difference between these percentages was significant ($P < 0.01$). Thus, when dogs are excreting only parvovirus-like particles, it is very high levels of virus excretion rather than infection *per se* which is associated with diarrhoea.

TABLE 1

Association between virus type and diarrhoea

Virus type(s) present	No. of diarrhoeal faecal specimens*	No. of normal faecal specimens†
Parvovirus-like	14 (48.3)	50 (31.9)
Astrovirus-like	0 (0)	3 (1.9)
"Round"	0 (0)	4 (2.6)
Coronavirus	2 (6.9)	5 (3.2)
CVLP‡	1 (3.5)	1 (0.6)
Rotavirus	0 (0)	2 (1.3)
Papova-like virus	0 (0)	1 (0.6)
Parvovirus-like and coronavirus	2 (6.9)	2 (1.3)
Parvovirus-like and rotaviris	0 (0)	1 (0.6)
Parvovirus-like and papova-like virus	0 (0)	1 (0.6)

* Figure in brackets gives percentage of 29 diarrhoeal specimens.
† Figure in brackets gives percentage of 157 normal specimens.
‡ Coronavirus-like particles.

Of the 156 separate dogs studied, parvovirus-like particles were detected at least once in 63 cases (40%). It is probable, however, that most, if not all dogs remaining in the shelter longer than 5 or 6 days became infected with the virus (Figure 9), since excretion of virus rose progressively from the first to the sixth day post admission then levelled off to about 30 to 40% of all dogs thereafter.

Serial collection of faeces from 2 dogs (Figure 10), furthermore, suggested that while the period of excretion might be shortlived (dog 2), dogs could show a cyclic pattern of virus excretion over very long periods (dog 1).

No relationship between age and excretion of the virus was found. Dogs of all ages excreted the virus.

The parvovirus-like particles were detected in each of the 8 months of the survey although the percentage of faecal samples positive for the virus in any given month varied from 19% to 64%.

Six of the 70 faecal samples positive for the parvovirus-like particles were also positive for coronavirus, rotavirus or papova-like virus (see Table 1 and below).

Astrovirus-like particles — The astrovirus-like particles were detected in 3 dogs (1.9%), aged about 3 to 6 months; none had diarrhoea.

"Round" virus particles — These particles were detected in 4 dogs (2.6%), 2 of which were described as "mature" and the others aged about 8 and 20 months respectively. None had diarrhoea.

Coronavirus — Coronavirus was detected in 11 dogs (7.1%) of which 4 had diarrhoea; (2 diarrhoeal and 2 non-diarrhoeal dogs had both coronavirus and parvovirus-like particles). Ages varied from about 3 months to "old".

CVLP — CVLP was detected in 2 dogs (1.3%), one aged about 5 months and the other about 5 years. The younger dog, whose faecal sample had counts of hundreds of particles per grid square, had diarrhoea; the older dog, whose faecal sample had counts of about 10 particles per grid square, did not.

Rotavirus — Rotavirus was detected in 3 dogs (1.9%). The dogs were recorded as "young", about 18 months old and "at least 2 years old". None had diarrhoea. One of these dogs had both rotavirus and parvovirus-like particles.

Papova-like virus — Papova-like virus was detected in faeces from 2 dogs (1.3%) collected about 2 months apart. Data was inadequate to determine if the same dog had been re-admitted; both dogs were listed as female, about 5 years old. Both faecal samples were normal. One of these dogs was also excreting parvovirus-like particles.

Discussion

The chief finding of this study is that viruses of 7 distinct types, some of known pathogenicity, can be excreted by non-diarrhoeal, mature, apparently healthy dogs; of 157 normal faeces studied 34% contained parvovirus-like particles, 5% contained coronavirus, 3% contained a previously undescribed 34-35 nm "round" virus particle, 2% contained rotavirus, 2% contained astrovirus-like particles, 1% contained papova-like virus and 1% contained CVLP.

Since the viruses were classified chiefly on morphological grounds, each category could include more than one virus type. However, since the characteristics of the viruses described in this study are either imperfectly understood, or in some cases totally unknown, and since methods of detection other than EM are in most cases difficult or unavailable, the clinical and epidemiological analysis below provides valuable information in the first instance.

The data indicates that excretion of parvovirus-like particles is widespread in the dog community and occurred throughout the 8-month period of the study. No relationship between age and excretion of these particles was found. The data suggests that infection is readily spread when dogs are in close contact. A periodicity in the shedding of parvovirus-

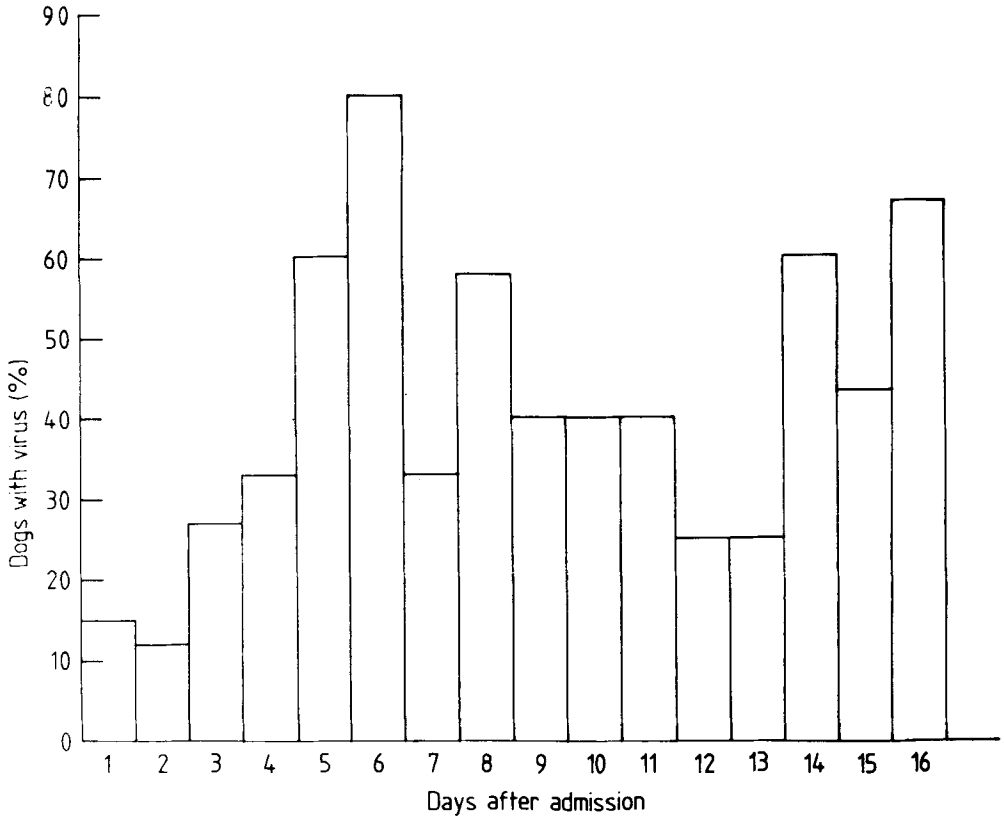

Figure 9. Detection rate of parvovirus-like particles relative to time in the shelter.

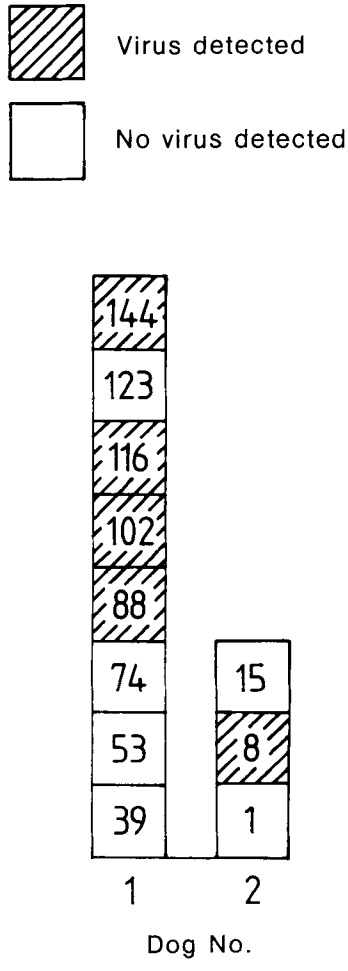

Figure 10. Periodic detection of parvovirus-like particles in 2 dogs. The numbers in the boxes refer to the days post admission on which the faeces were collected.

like particles noted for one dog (Figure 10) indicated that either reinfection or periodic shedding could occur. A correlation between diarrhoea and the excretion of the virus was not found except when the virus was excreted in high concentration.

The emergence of canine parvovirus in 1978, presumably as a host range mutant of the closely related feline or mink parvoviruses, and the dramatic pandemic which rapidly followed, also focuses on the possibility of interspecies transfer of enteric viruses. Early reports of association of human diarrhoea with canine parvovirus were refuted (Lenghaus and Studdert 1980), but the emergence of further host range mutants of parvovirus must be considered a distinct possibility.

Although human picornaviruses have been isolated from canine faeces (Carmichael and Binn 1981), no such viruses were isolated in the 2 experiments carried out in this study. The 2% detection rate of astrovirus-like particles probably corresponded to the previously described canine astrovirus (Williams 1980).

A round virus particle 34 to 35 nm in diameter was found in about 3% of dogs sampled, and does not appear to have been described previously. While the identity of this virus is uncertain — it could be tailless phage — it is worth noting its similarity to the recently described Otofuke and Sapporo agents, which have been associated with outbreaks of human gastroenteritis in Japan (Taniguchi *et al* 1979, 1981; Kogasaka *et al* 1981).

Coronavirus was detected in about 7% of all dogs; a third of these had diarrhoea. Some limited success has been achieved in the cultivation of the human coronavirus strain 229E in this laboratory (M. L. Kennett personal communication), but attempts to cultivate the virus in this study were unsuccessful.

CVLP was clearly distinguishable from coronavirus and has previously been described in both dogs (Schnagl and Holmes 1978) and people (Caul *et al* 1975; Schnagl *et al* 1979; Marshall *et al* 1982). The nature and association of CVLP with disease in dogs and man, however, is problematical. CVLP has been detected in apparently healthy dogs and people (Schnagl and Holmes 1978; Schnagl *et al* 1979;

Marshall *et al* 1982) and dogs and people with diarrhoea (Caul *et al* 1975; Clarke *et al* 1979; Williams 1980; Marshall *et al* 1982). In this study, a dog excreting very high concentrations of the particles had diarrhoea, while another excreting low concentrations did not. If virus, then pathogenicity may be dose related.

It is now known that rotavirus from one species may infect another and sometimes cause diarrhoea (Bridger *et al* 1975; Middleton *et al* 1975). Furthermore Engleberg *et al* (1982) recently reported a correlation between dog ownership and rotavirus gastroenteritis in a population of Indians in the San Carlos Apache Reservation. The finding of rotavirus in mature non-diarrhoeal dogs in this study may therefore have epidemiological significance for both canine and human gastroenteritis.

The detection of papova-like virus particles in canine faeces does not appear to have been reported previously. The origin of this virus was probably oral or gastrointestinal papilloma.

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Death in sheep following dosing with copper diethylamine oxyquinoline sulphonate as a commercial injectable copper preparation

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SUMMARY: Death occurred in sheep following diethylamine oxyquinoline sulphonate (DOS) copper injections given at recommended dose rates. The copper content in unused portions of DOS copper packs was normal and free of bacterial contamination. Liver and blood copper levels in dead and sick sheep were not high. Sick sheep showed signs of hepatic encephalopathy and dead sheep were generally piled against fences and scrub. Deaths were associated with acute, severe, generalised, centrilobular, hepatocellular necrosis and live sheep had elevated circulating levels of liver enzymes consistent with liver damage. In recovered sheep there were no residual complications. It would appear that even at 0.5 mg/kg of DOS copper the safety threshold may sometimes be exceeded in some sheep.

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Introduction

In the treatment of copper deficiency, injectable copper preparations have an advantage over orally administered copper, because they by-pass the inhibitory effects on copper availability of sulphur and molybdenum in the gut (Underwood 1981). However, in sheep some injectable copper compounds such as copper-methionate, and to a lesser extent copper calcium EDTA, produce tissue reactions at the injection site and result in variable and short-lived protection when compared with copper as diethylamine oxyquinoline sulphonate (copper DOS) which produces uniform long-lived protection and negligible tissue reaction (Suttle 1981). Because of the rapid availability of copper from copper DOS it is potentially more toxic than other injectable copper preparations (Cunningham 1959; Mahmoud and Ford 1981). Nevertheless, few cases of copper DOS poisoning have been reported. In Australia deaths have occurred in sheep and goats in Western Australia after normal doses of DOS copper

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