



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Studies on canine parvovirus infection: preparation of challenge virus

L. MACARTNEY, I. A. P. McCANDLISH, H. THOMPSON, H. J. C. CORNWELL, *Department of Pathology, Glasgow Veterinary School, Glasgow G61 1QH*

Two techniques, adsorption on to hydroxylapatite and density gradient centrifugation, were investigated as prospective methods for the large scale purification of canine parvovirus from faecal suspensions. Adsorption with hydroxylapatite successfully removed virus from faecal material. However, the resultant virus was contaminated and some virus was left behind in the faecal suspension. Repeated adsorption with hydroxylapatite appeared to result in some damage to the virus particles. In contrast, density gradient centrifugation provided a simple, economical method of purification which yielded uncontaminated, infectious virus. The final method, using both isopyknic and rate zonal centrifugation is described.

CANINE parvovirus (CPV) emerged in 1978 and its rapid rise to the status of a major canine pathogen necessitated study of the pathogenesis of the infection and the development of effective vaccines. In both cases a potent challenge with virus of high infectivity and virulence is required if, in the first instance, one is to reproduce the natural disease in an experimental situation and, in the second, if one is to test adequately the efficacy of a vaccine.

The present report describes the preparation of challenge virus making use of the unique characteristics of CPV and the fact that large amounts of virus are excreted in the faeces of infected dogs (McCandlish et al 1981). The challenge virus was obtained from CPV contaminated faeces by two methods which were investigated and compared. In the first, separation of virus from faeces was attempted by adsorption on to a solid phase medium hydroxylapatite (HAP) (Smith and Lee 1978). In the second, virus was purified from faeces by density gradient centrifugation using both isopyknic and rate zonal techniques.

Materials and methods

Faeces from clinical cases of CPV enteritis were obtained from the samples submitted to the diagnostic service of the Canine Infectious Disease

Research Unit of Glasgow University Veterinary School. The faecal specimens were diluted 1:10 with phosphate buffered saline (PBS) pH 7.2 and the suspensions screened for CPV haemagglutinins as previously described (Macartney et al 1984b). Samples with titres in excess of 1:2048 by the haemagglutination test were then used in the purification experiments.

Purification by hydroxylapatite

HAP was prepared according to the method of Tiselius and co-workers (1956). Two grams of HAP powder were washed with 80 ml of 0.15 M phosphate buffer (PB) pH 7.4, the solid phase allowed to settle, the supernatant discarded, and the washing step repeated.

Positive faecal samples were suspended in 0.15 M PB clarified by centrifugation at 1500 g for 15 minutes and then by ultracentrifugation at 70,000 g for 30 minutes. The resultant supernatant was added to the HAP slurry and stirred for 18 hours at 4°C. After this adsorption period, the HAP was sedimented, washed with 0.15 M PB, resedimented and finally suspended in high ionic strength (0.5 M) PB to elute the attached virus from the HAP. This suspension was stirred at 20°C for 30 minutes and the HAP removed by centrifugation. The supernatant fluid was retained. This elution procedure was repeated three more times and on each occasion the supernatant was collected.

Purification by density gradient centrifugation

Positive faecal samples were suspended in PBS pH 7.2 and clarified by centrifugation at 1500 g for 15 minutes and then at 70,000 g for 30 minutes.

For isopyknic centrifugation, gradients were formed by allowing solutions of caesium chloride density 1.2 g ml⁻¹ (D) to 1.5 D to diffuse at 4°C for 24 hours in Beckman ultraclear tubes. A protective layer of 1 ml of 1.2 D sucrose solution was gently added to the top of the gradient just before the addition of 5 ml of the clarified faecal suspension. For rate zonal centrifugation a pad of 1 ml of 1.5 D caesium chloride was added at the bottom of the tube

and then 6 ml of a 66 per cent w/v sucrose solution was added gently. The clarified faecal suspension (5 ml) was then layered on to the sucrose solution in each tube and centrifugation carried out using a Beckman SW41 rotor. A time of 18 hours at 270,000 g was allowed for centrifugation following a pilot experiment. After centrifugation, fractions of the gradient were collected by piercing the bottom of the centrifuge tube with a heated 20 gauge hypodermic needle. Ten drops of fluid were collected for each fraction.

The gradient fractions were checked by the haemagglutination test to assay the presence of viral antigen and then examined by negative stain electron microscopy. The quantity of infectious parvovirus present was measured by a modification of the method described by Macartney and co-workers (1984b).

Infectivity titration

Feline embryo cells were seeded into roller tubes and allowed to settle at 37°C for two hours. Serial 10-fold dilutions of the test fraction were inoculated on to the cultures. Four tubes were used per dilution, 0.2 ml per tube. The tubes were incubated for a further two hours at 37°C after which a total volume of 1 ml growth medium was added to each tube. The cultures were incubated at 37°C for four days and then each tube was frozen and thawed three times. The cell debris was removed and the supernatant checked for haemagglutinating activity. The infectivity titre was taken as the reciprocal of the highest dilution which permitted detectable viral multiplication in 50 per cent of the inoculated cultures.

Results

Purification by adsorption on to HAP

A faecal suspension with a very high haemagglutination titre was subjected to the procedures described above and the results are presented in Table 1. Adsorption on to HAP was successful and the adsorbed virus could be eluted by the 0.5 M PB washes. Most of the virus was released following the first two washes. On electron microscopic examination of the supernatants from the first and second washes numerous parvovirus particles were seen. Some of these particles permitted the entry of the stain and the capsid was apparently empty but the majority did not allow the entry of stain and were full complete virions. In addition long filamentous structures approximately 20 nm in diameter and ranging in length from 100 to 200 nm were also present.

As a high haemagglutination titre was still detect-

TABLE 1: Purification of canine parvovirus from faeces: adsorption by hydroxylapatite (HAP)

	Haemagglutination*	Electron microscopy
First adsorption		
0.5 M wash 1	524,288	Contaminated
0.5 M wash 2	16,384	Contaminated
0.5 M wash 3	512	ND
0.5 M wash 4	128	ND
Post HAP supernatant	16,384	ND
Second adsorption		
0.5 M wash 1	8192	Contaminated, damaged
0.5 M wash 2	2048	Contaminated, damaged
0.5 M wash 3	128	ND
Post HAP supernatant	2048	ND

ND Not done

* Results expressed as reciprocal of highest dilution giving complete agglutination

able in the supernatant which remained after adsorption with HAP, the procedure was repeated using fresh HAP. Following this second adsorption more virus was removed and could again be eluted (Table 1). Electron microscopic examination revealed that contamination with the filamentous structures was still a problem and although numerous parvovirus particles were present, many were apparently damaged with the normal symmetrical outline of the virions being disrupted.

Purification by density gradient centrifugation

Initial studies demonstrated that the optimum conditions for complete sedimentation of CPV in a non-viscous (saline) solution were a centrifugation time of 18 hours at 270,000 g.

Two faecal samples containing viral antigen were then clarified, ultraclarified and concentrated by pelleting. The pellets were resuspended in 1 ml PBS and layered on to the density gradients to be centrifuged at 270,000 g for 18 hours. The final preparation layered on to gradient 1 had an haemagglutination titre of 65,536 while the second preparation placed on gradient 2 had a titre of 525,288. Following centrifugation the gradients were examined by incident light and the fractions collected. The results are presented in Table 2. Two distinct light-refractive bands could be seen in gradient 1.

Significant haemagglutination titres were found between fractions 3 to 8 with the highest levels in fractions 4, 5 and 6. Electron microscopy revealed that fraction 4, which corresponded to the lower band seen with incident light, contained mainly full virus particles whereas fractions 5 and 6 corresponding to the upper band contained a significant proportion (30 per cent and 70 per cent, respectively) of empty

TABLE 2: Purification of canine parvovirus from faeces: isopyknic centrifugation

Fractions	Gradient 1		Gradient 2	
	Haemagglutination*	Electron microscopy	Haemagglutination	Electron microscopy
1	<4	ND	<4	ND
2	<4	ND	<4	ND
3	64	ND	4096	Contaminated 90% complete virus
4	4096	Clean, complete virus	4096	Contaminated 70% complete virus
5	4096	Clean, 70% complete virus	4096	Contaminated 50% complete virus
6	4096	Contaminated, 70% empty virus	4096	Contaminated 50% complete virus
7	32	ND	4096	Contaminated 50% complete virus
8	16	ND	4096	Contaminated 70% empty virus
9	8	ND	512	ND
10	8	ND	512	ND
11	8	ND	256	ND
12	8	ND	128	ND

ND No data available

* Results expressed as reciprocal of highest dilution giving complete agglutination

particles. In addition fraction 6 was contaminated with filamentous structures identical to those observed in the HAP study.

Gradient 2 was cloudy and no distinct banding was observed on incident light examination. An irregular filament was observed at the level of 1.4 D. Haemagglutination titres were present from fractions 3 to 12, with the highest level between 3 and 6. Fraction 3 contained the highest proportion of full particles but all the fractions examined were contaminated with filamentous structures.

The contaminated fractions of gradient 2 were pooled, diluted 1:4 in PBS and subjected to rate zonal centrifugation through 66 per cent sucrose at 270,000 g for 90 minutes with a pad of 1 ml 1.5 D caesium chloride solution at the bottom of the tube. Following centrifugation the gradient (2A) was

examined by incident light and fractions were collected and examined as before. The results are shown in Table 3. A mass of filamentous material was present at the interface between the sucrose solution and the basal caesium chloride pad. Significant haemagglutinin titres were detected in fractions 2 to 7. Both empty and full particles were observed in these fractions and they were now clean of filamentous structures.

Fractions taken from gradient 1 and 2A were assayed for infectivity (Table 4). In gradient 1, fraction 4, containing full viral particles, had the highest level of infectivity. Fractions 3, 5 and 6 had a similar infectivity despite the much lower haemagglutination titre in fraction 3. This suggests that the virus present in fraction 3 may also have been composed of full complete virus particles. In gradient 2A the infectivity titres correlated with the haemag-

TABLE 3: Purification of canine parvovirus from faeces — rate zonal centrifugation

Fractions	Gradient 2A	
	Haemagglutination*	Electron microscopy
1	<4	Contaminated
2	4096	Clean
3	4096	Clean
4	4096	Clean
5	4096	Clean
6	2048	Clean
7	64	ND
8	8	ND
9	8	ND
10	8	ND
11	8	ND
12	8	ND

ND No data available

* Results expressed as reciprocal of highest dilution giving complete agglutination

TABLE 4: Infectivity titration of fractions following density gradient centrifugation

Fraction	Infectivity titre	
	Gradient 1	Gradient 2A
1	ND	ND
2	ND	2.5 log ₁₀ TCID ₅₀ ml ⁻¹
3	2.5 log ₁₀ TCID ₅₀ ml ⁻¹	2.5 log ₁₀ TCID ₅₀ ml ⁻¹
4	5.25 log ₁₀ TCID ₅₀ ml ⁻¹	4.5 log ₁₀ TCID ₅₀ ml ⁻¹
5	2.0 log ₁₀ TCID ₅₀ ml ⁻¹	ND
6	2.0 log ₁₀ TCID ₅₀ ml ⁻¹	ND
7	1.25 log ₁₀ TCID ₅₀ ml ⁻¹	ND
8	Positive	ND
9	ND	ND
10	ND	ND
11	ND	ND
12	ND	ND

ND No data available

glutination titres reflecting the non-separation of full and empty particles.

Discussion

There are a number of experimental studies on the pathogenesis of CPV enteritis but in only a few have the severe clinical signs and pathological lesions of the natural disease been recorded. Three sources of virus have been used for experimental challenge; tissue culture adapted virus (Pollock and Carmichael 1982), filtered tissue homogenates (Carman and Povey 1982, Pollock 1982) and purified faecal virus prepared by the method described in this paper (Macartney et al 1984a,b). Tissue culture virus given by the oral and parenteral route has been used most frequently and although infection, as measured by seroconversion and viral excretion, is recorded there has been a lack of severe clinical signs in most infected dogs in the published reports.

Carman and Povey (1982) observed severe illness in 22 out of 24 dogs infected with filtered mucosal scrapings prepared from clinically infected dogs. As a potent method of challenge, the use of filtered scrapings would appear to be satisfactory but the technique has certain important disadvantages. First, it is difficult to ensure that this type of inoculum is uncontaminated by adventitious agents and, secondly, only small amounts of virus may remain in tissues at the time of clinical disease (Macartney et al 1984a,b, Meunier et al 1985).

Like the challenge devised by Carman and Povey (1982), purified faecal virus has been shown to produce clinical disease (Macartney et al 1984a,b). As an infected dog excretes vast amounts of virus and it is possible to collect faeces over several days, large quantities of infectious material is readily available (McCandlish et al 1981). A technique to purify such material should ensure that adventitious agents are removed, the infectivity of the parvovirus is unimpaired and that the methodology is simple and economic.

Hydroxylapatite has been used to partially purify viruses from large volumes of fluid in the past (Smith and Lee 1978) and when successful has proved an alternative to high speed centrifugation. In the present situation HAP adsorption was disappointing. Large amounts of virus were left in the original faecal supernatant after adsorption and attempts to increase the yield of virus by repeated adsorption resulted in morphological damage to the virions.

More important, however, was the contamination of the eluted parvovirus by filamentous structures. The appearance and dimensions of the latter was consistent with bacteriophage fragments.

In contrast, purification of parvovirus from faeces by ultracentrifugation fulfilled all three criteria: the method was simple, large amounts of uncontaminated virus were obtained and the virus had high infectivity. The isopyknic technique had the additional advantage of separating full and empty particles provided the gradient was not overloaded. The high density of CPV makes it very suitable for separation by this means. The larger enteric viruses, for example, coronavirus with a density of 1·14 D, paramyxovirus 1·2 D, adenovirus 1·34 D and rotavirus 1·3 D would all be left behind at their position of isodensity as CPV sedimented down to the region of 1·4 D. When contamination with bacteriophage fragments was present they could be removed by further zonal centrifugation.

The main limitation to density gradient centrifugation is the high capital cost of an ultracentrifuge. Given that such equipment is available to most laboratories, isopyknic centrifugation followed by rate zonal centrifugation where necessary is therefore a practical method of obtaining pure virulent parvovirus. This final method has now been used many times to prepare purified CPV for studies on the pathogenesis of the infection and for vaccine challenge studies.

References

- CARMAN, S. & POVEY, C. (1982) *Canadian Journal of Comparative Medicine* **46**, 47-50
- MACARTNEY, L., McCANDLISH, I. A. P., THOMPSON, H. & CORNWELL, H. J. C. (1984a) *Veterinary Record* **115**, 201-210
- MACARTNEY, L., McCANDLISH, I. A. P., THOMPSON, H. & CORNWELL, H. J. C. (1984b) *Veterinary Record* **115**, 453-460
- McCANDLISH, I. A. P., THOMPSON, H., FISHER, E. W., CORNWELL, H. J. C., MACARTNEY, L. & WALTON, I. A. (1981) *Veterinary Record Supplement In Practice* **3**, 5-14
- MEUNIER, P. C., COOPER, B. J., APPEL, M. J. G. & SLAUSON, D. O. (1985) *Veterinary Pathology* **22**, 60-71
- POLLOCK, R. V. H. (1982) *Cornell Veterinarian* **72**, 103-119
- POLLOCK, R. V. H. & CARMICHAEL, L. E. (1982) *Cornell Veterinarian* **72**, 16-35
- SMITH, R. G. & LEE, S. A. (1978) *Analytical Biochemistry* **86**, 252-263
- TISELIUS, A., HJERTEN, S. & LEVIN, O. (1956) *Archives of Biochemistry and Physiology* **65**, 132-135

Received March 2, 1987
Accepted October 5, 1987