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Isolation and Identification of Canine Parvovirus Type 2 in Dogs from Algeria

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Running title: Detection of canine parvovirus

Abstract: ***Using intraperitoneal (I.P.) inoculation of a commercial canine parvovirus vaccine we were able to prepare an anti-parvovirus serum. By performing indirect immuno- fluorescence (IFd) we characterized three virus isolates from the fecal samples of 12 dogs presenting with clinical signs of canine parvovirus (CPV) disease. Serum neutralization tests on CRFK cells with the reference serum confirmed the identity of these isolates as CPV thereby representing the first isolation of this canine virus in Algeria.***

Keywords: Canine parvovirus (CPV), CRFK, Algeria

1. Introduction

Canine parvovirus designated CPV-2 was identified for the first time in 1978 when it was recognized as being responsible for a new pathology in dogs (Parrish. 1990). CPV is responsible for highly contagious and fatal acute hemorrhagic gastro-enteritis in dogs of all ages and fatal myocarditis in young pups (Kelly. 1978; Appel et al., 1979). CPV is a very common virus infecting domestic and wild dogs (Parrish et al., 1988). CPV can cause high morbidity and has a mortality rate up to 50% (Appel et al., 1979; Johnson and Spradhow. 1979). It causes periodic outbreaks with CPV variants varying in their pathogenicity. CPV-2a is more virulent than CPV-2b (Moon et al., 2008). It is stable in the environment and can survive for months or even years (Ettinger et al., 1995). It is highly contagious and is spread via the fecal-oral route and is shed in very high titer in the feces of infected dogs 8-12 days after infection (Parrish. 1990; Carmichael. 1994; Decaro et al., 2005).CPV infection is routinely diagnosed using serologic tests such as hemagglutination inhibition assay, ELISA, immunofluorescence and virus isolation. More recently, PCR-based assays have become a routine diagnostic test. Genomic sequencing and real time PCR are used in the detection and differentiation between various CPV antigenic variants (Decaro et al., 2005; Ohshima et al., 2008). CPV is a member of the autonomous parvovirus and belong to the Parvoviridae family (Parrish. 1999; Berns et al., 2000). It is a small, non-enveloped, icosahedral virus with linear, single-stranded DNA genome of 5 kb in length (Paradiso et al., 1982). The CPV genome has two major ORFs. One encodes for two non-structural proteins NS1 and NS2 and the other encodes for two structural proteins, VP1 and VP2 (Parrish et al., 1999).

In 1979 the CPV-2 strain was replaced by CPV-2a and become distributed worldwide (Parrish et al., 1988). A few years later, a new CPV-2b strain emerged and together with CPV-2a become distributed worldwide (Decaro et al., 2007). These two variants were later slowly replaced by a new antigenic variant, CPV-2c, which was first detected in Italy and then in many other countries (Buonavoglia et al., 2001; Decaro et al., 2006; Calderon et al., 2009).

In Algeria, CPV disease was identified and recognized after 1987 and is thought to be widespread according to veterinarians working in private clinics and in veterinary schools (unpublished data). Algeria has a large population of dogs mostly in rural areas with many being malnourished and suffering from various infections. CPV has caused high mortality rates within young pups and several interventions have failed to control and stop the disease. The only study on Parvoviridae family members in Algeria was carried out by Vincent (1971) at Algiers's Pasteur institute, which culminated in the isolation of four bovine parvovirus stains.

The aim of this study was to identify the causative agent of hemorrhagic gastro-enteritis in young pups in Algeria. We sought to isolate at least one strain of CPV for further investigations including immunological characterization of the prevalent CPV variant types so that they could be used as the basis for the development of local Algerian vaccine to control local CPV infection.

2. Materials and Methods

1) Fecal specimens

The collection of fecal samples from dogs suspected of being infected with CPV was carried out with the help of fellow veterinarians. A total of 12 fecal samples were collected from vaccinated and non-vaccinated dogs with clinical signs of CPV infection. The samples were collected from the national veterinary school of Algiers and from private veterinary clinics in the suburban area of the capital. The samples were stored at –20 °C. Eachfecal sample submitted to our laboratory was accompanied by its respective data shown in Table1. The samples were vigorously homogenized (10% w/v) for 1 min in PBS buffer (pH 7.2) containing penicillin, streptomycin and fungizone and subsequently clarified by centrifugation at 3100 rpm for 15 min. The supernatant was filtered first through 0.45 µm then through 0.22 µm membrane filter (Millipore, France) and thenaliquoted and stored at – 20 °C until further use.

2) Virus isolation assay

Crandell Feline kidney (CRFK) cell line was kindly provided by Professor TomášŽuffa Laboratoire MEVAK a.s Bratislava. The cells were maintained in DMEM media (Gibco BRL, France) supplemented with 5% of fetal bovine serum and 100U/ml penicillin G and 100 µg/ml streptomycin and 0.25 µg/ml fungizone. Twenty four hour after trypsinization, the cells were inoculated with aliquots from different samples. After adsorption for 2 hr at 37°C, complete DMEM medium was added and the cells were incubated for 48 hr at 37°C then transferred to 33°C incubator. The cells were monitored daily to detect any cytopathic effect (CPE). When the CPE become more prominent, the tissue culture flakes were freeze-thawed and the infected cells were harvested by centrifugation at 2000 rpm for 10 min. The supernatant was aliquoted into 2 ml vials and stored at – 20°C. Several passages were carried out for each aliquot.

Table I: Technical records of the clinical specimens from dogs suspected of CPV infection. GS: German shepherd. MB: mixed breed. D: Doberman. PC: private clinic. ENV: national veterinary school. NV: non-vaccinated, V: vaccinated

Sample number	Origin	Breed	Sex	Age (month)	Vaccination against Parvovirus
1	PC	MB	F	12	NV
2	PC	GS	M	5	NV
3	PC	D	F	7	NV
4	PC	GS	M	3	NV
5	ENV	GS	F	9	NV
6	ENV	GS	M	2 ½	NV
7	ENV	GS	F	3	NV
8	PC	GS	M	2 ½	V
9	ENV	GS	M	3	NV
10	ENV	GS	F	4	NV
11	ENV	MB	F	6	NV
12	PC	GS	F	2 ½	V

3) Preparation of anti-parvovirus serum in mice

The anti-parvovirus serum was prepared as follows: 5 doses of Enduracell vaccine (live attenuated homologous strain, Lot 3597, SmithKline Beecham) were diluted in 25 ml of DMEM tissue culture medium. The virus suspension was then inactivated by beta-propiolactone (BPL) at 1: 4000. The mixture was slowly stirred for 3 hr at room temperature and for 24 hr at 4°C. The inactivated vaccine was stored at 4°C until needed. The production of anti-parvovirus serum was carried out in a group of NMRI strain mice which received 0.5 ml of the inactivated virus by the intra-peritoneal route (Table.2). The blood of 9 mice that had received 4 inoculations of the inactivated vaccine was harvested. After clarification, the serum was diluted in 1:4 in PBS buffer and filtered with 0.22 µm filter, aliquoted and stored at – 20°C.

Table II: Immunization protocol in mice inoculated by inactivated commercial CPV vaccine. IP: intra peritoneal

Injection (days)	Mouse number	Inject. Vol. (ml)	Route of admin.	Harvest (day)
0	11	0.5	IP	-
7	11	0.5	IP	-
14	11	0.5	IP	-
21	9	0.5	IP	28

4) Immunofluorescence assay

The infection of the CRFK cell line with the different samples led to the appearance of a cytopathic effect CPEas detected by indirect immunofluorescence assay after a few passages. The assay was carried out for every passage and for the 3 samples (P5, P9, P12) that showed a CPE. Following trypsinization, the cells were grown in Leighton tubes and incubated for 3 hr at 37°C in complete DMEM tissue culture media. The media was removed and the cells were infected with 0.5 ml of viral suspension. Following virus adsorption for 1 hr, 0.5 ml of complete media was added to each Leighton tube and incubated for 48 and 72 hr at 37°C. At the end of the incubation time, the cells were fixed. Then the cells were rinsed in PBS buffer and hyperimmune serum raised in mice was added at dilution 1:32 and incubated for 30 min at 37°C in CO_2 incubator. The cells were rinsed in PBS buffer and then treated with anti-rabbit IgG conjugated with FITC for 30 min at 37°C in CO_2 incubator. Finally, the cells were thoroughly rinsed in PBS buffer and the slides were examined under fluorescence microscopy. In this work, the inoculum added on CRFK cells attached to the support flask, Labtek slides. In this case, the infection occurs on young cells fixed on the support which have begun the process of mitosis, prophase stage estimated. In comparison with freshly trypsinized cells floating in the culture medium in rounded form and that is estimated at the interphase stage. In the nucleus, the CPV begins its propagation and is trained in the process related to cell mitosis. It will go through the different phases namely prophase, metaphase and finally telophase. It will be conveyed along the mitotic spindle on one side or the other depending on the progress of the carrier chromosomes.

Fig.4 shows a cell in the final telophase stage. There is an unequal distribution of newly formed virus in the future daughter cells. It is indicated by the intensity of the fluorochrome. The simultaneous analysis of the figure (3 and 4) shows that the end of phase telophase viral particles is predominantly aggregated in a form of fluorescent mass, homogeneous at the apex (c, d). On the Figure 3, lateral fluorescents extensions (b), are connected with a form of release of neo formed virus.

5) Serum neutralization Assay

In our laboratory, control of the protective power of inactivated rabies vaccines is through the NIH test. During this test, the intraperitoneal route is used on a large number of mice (up to 60). By this route, the immune response against the rabies virus is good. We readily selected and tested this way for introducing inactivated parvovirus. The development of the indirect immunofluorescence technique (IFd) , its application to the search for traces (inclusions) of canine parvovirus on CRFK cells shows that this route of introduction of antigens in mice is convenient, fast and effective . Including serum antiparvovirus product in the IFd, highlighting of intranuclear inclusion shows some specificity. The use of Serum neutralization secondly can validate its quality for the IFd. CRFK cells, DMEM with 8 % fetal calf serum (FCS) decomplemented, antibiotics. For this test we use somematerial like: Plate 24 holes, Lab tek 8 chamber, Nunc Inc. USA, Multipettes. Petri dishes glasses (sterile). Serum antiparvovirus provided by Anne Moraillon (ENVA). Antiparvovirus serum: In a first step, we determine

the framing ECP of percentage from viral dilutions allowing the use of a antiparvovirus serum with unknown title. Freshly trypsinized CRFK cells are distributed in the wells of a plate 24. From a suspension with 10^5 cells / ml, 1.2 ml per well was distributed into twice aliquots. The plate is incubated at 37 ° C under CO2 atmospheric. The confluence of cells was reached after 48 hours.

a) Infection with sample P5

The viral suspension is diluted to 1 /2, 1/4⋯ in the medium. The tubes containing viral dilutions are placed in crushed ice. The wells are emptied of their maintenance medium using a plastic syringe of 10 ml. The needle is bentso that the suction of the medium is carried out. From each dilution, 200 ml per well is poured starting from the wells emptied first. The plate was manually stirred to cover the cells with the inoculums. During incubation, manual agitation is performed every 15 minutes. The plate is incubated at 37 ° C under CO2 atmosphic for 1 hr. After this period, completed with 1 ml of complete medium and the plate is placed under the same conditions. Reading: A daily observation of wells allows noting the type of cellular changes (ECP).

Table III CPE is obtained with the virus dilutions from 1/2 to 1/32. The effect of the virus not appears in Beyond the 1/64 dilution

1/2 ECP 100%	1/2 ECP 100%	1/4 ECP 80 %	1/4 ECP 80 %	1/8 ECP 40 %	1/8 ECP 50%
1/16 ECP 30 %	1/16 ECP 25 %	1/32 ECP 8%	1/32 ECP 10 %	1/64 ECP 0 %	1/64 ECP 0%
1/128 ECP 0 %	1/128 ECP 0%				
				T.C ECP 0 %	TC ECP 0 %

b) Serum neutralization and identification

Beforehand, the reference serum is decomplemented by incubation at 56 ° C for 30 min. On the basis of the results of ECP obtained previously with the P5 sample, we selected the wells so as to frame the ECP between 80% and 25% of infection. Viral dilutions ranging from 1/4 to 1/16 are retained. The test was redone 3 times under the same conditions. The results are very close and confirm the choice of framing dilutions retained for further work.

c) Preparation of virus serum mixture

An aliquot of P5 viral supernatant is thawed. From deductions of viral dilutions between 1/4 and 1/16), 35 µl are removed and mixed with equal volume to the reference serum. For the control virus, 2 holes receive 70 µl mixtures of equal volumes of virus with medium. These tubes are incubated for 1 hr at 37 °C in water bath. Every 10 min., the tubes are shaqued then returned to incubation. Just before the end of this step, a cell suspension is prepared, 350 µl of the suspension of CRFK cells containing about 100, 000 cells are distributed in Labtek chambers. Serum-virus contacting time being up, the distribution of 60 ml of each virus mixture in duplicate on cells that have just been allocated, not fixed to the support. The lab tek slides are incubated at 37 ° C in CO2 atmosphic. Sterile glass Petri dishes were used as support for lab tek, which facilitates movement between the luminary flow and the CO2 incubator and then to the microscope. Monitoring cells changes is achieved by daily observation in an inverted microscope. Observations: It begins with the positive control and spread to other chambers of the lab tek blades. The same method was used for the other samples. The assessment of the percentage in ECP, with regard to different dilutions of the virus shows that after 8 days of incubation, all wells have an ECP. It will be estimated between 100% and 25%. Dilution 1/4 matches to about 80 %, whereas the 1/16 dilution is estimated about 25%. In terms of the neutralization test, the ECP presence in the two chambers with positif control and the lack of CPE in the chambers having received the serum / virus confirms virus neutralization by serum. For the other samples, we obtained results that follow the same logic. It shows that the three isolated viruses are identified as CPV.

3. Results

With the exception of the 3 samples P5, P9 and P12, the other samples did not show any CPE in CRFK cells. The samples P5, P9 and P12 showed distinctive CPE after just 3 passages in CRFK cells in the form of destruction of the cell monolayer. The daily observation of CRFK cell infected with other samples was continued but no CPE was detected and these samples were considered negative. The time of CPE appearance was longer in the first 5 passages but become shorter and shorter at the later passages. Earlier passages were incubated for 16 days. The CPE of the three samples was as follows: The cells infected with P5 showed both isolated and floating cells or a mass of rounded and dark cells (granulated) attached to the monolayer with some holes (Figure.1). The cells infected with P9 had generally similar features as in P5 but with more cells detached from the monolayer and more holes. These holes were surrounded by fusiform cells in the form of stars. The cells infected with sample P12 showed a very prominent CPE from the beginning of the culture. A lot of floating cells, individually and in dark masses were observed. Small holes of similar size were observed all over the cell monolayer. In some passages, a strip of cells formed the dominant feature. These large masses of dark cells were seen floating in the supernatant media. At an advanced passage stage and after 5 to 7 days of incubation, we observed the same features in all samples. Dark, round cells were scattered along the monolayer 48 to 72 hr p.i. later on these cells detached from the monolayer leaving holes (Figure.2). Intra-nuclear inclusions were observed by indirect immunofluorescence assay carried out after various passages. The same inclusion type was observed for the samples P5, P9 and P12. The most constant feature was a large inclusion occupying the whole nucleus with the exception of the nucleolus which appeared black. These inclusions were seen 24 hr p. i and beyond 4 days p.i. Two other types of inclusions were also observed

on the mitotic spindle or microtubule fibers and produced very fine extensions in the spindle and in lateral direction (Figure 3, b). Overall, these extensions ended with a tiny bulge resembling a budding particle (figure 4 c, d). The contrast between the bright and dark parts contrast the mitotic spindle of dividing cells harbouring structures which might represent CPV virus (Figure 3and 4). The second type of inclusion looked like a racket as if the virus was moving to one direction of the spindle leaving the other part without virus (figure. 4). These two types of inclusions that occupied partially or completely the dividing nucleus were observed most of the time during the early 24 hr to 72 hr p.i. The supplied anti-parvovirus serum was lightly cytotoxic to CRFK cells in its pure form or at dilution of 1:2. CPE caused by the samples P5, P9 and P12 was completely neutralized by the supplied anti-parvovirus serum at dilutions of 1:4 and 1:8.

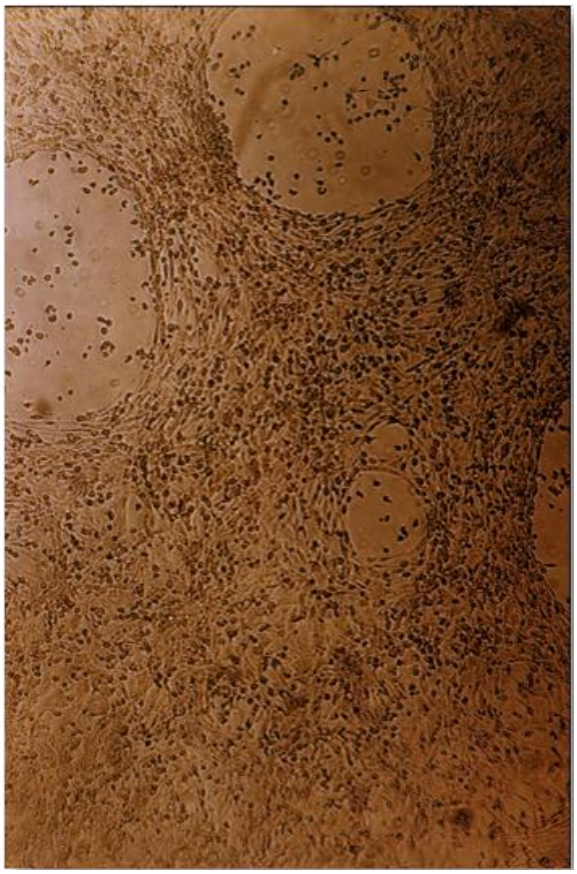

Figure 1

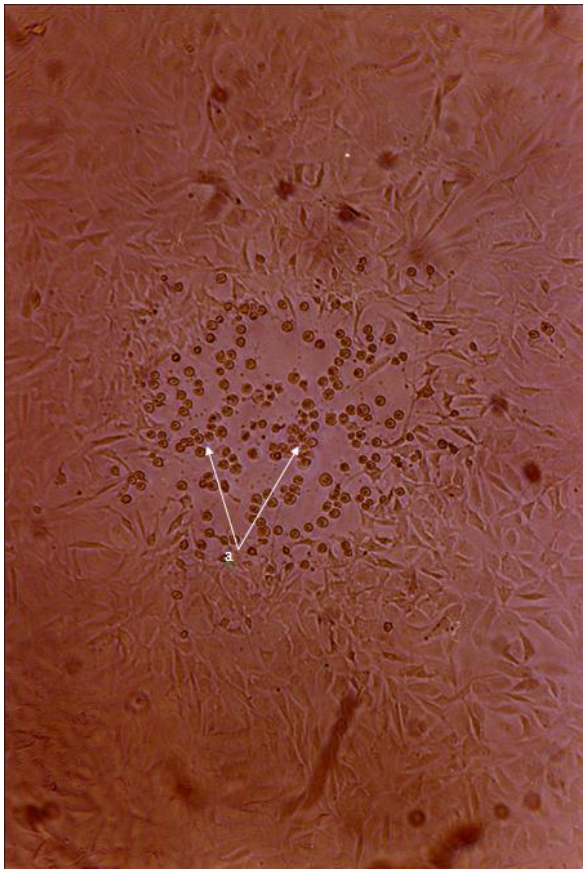

Figure 2

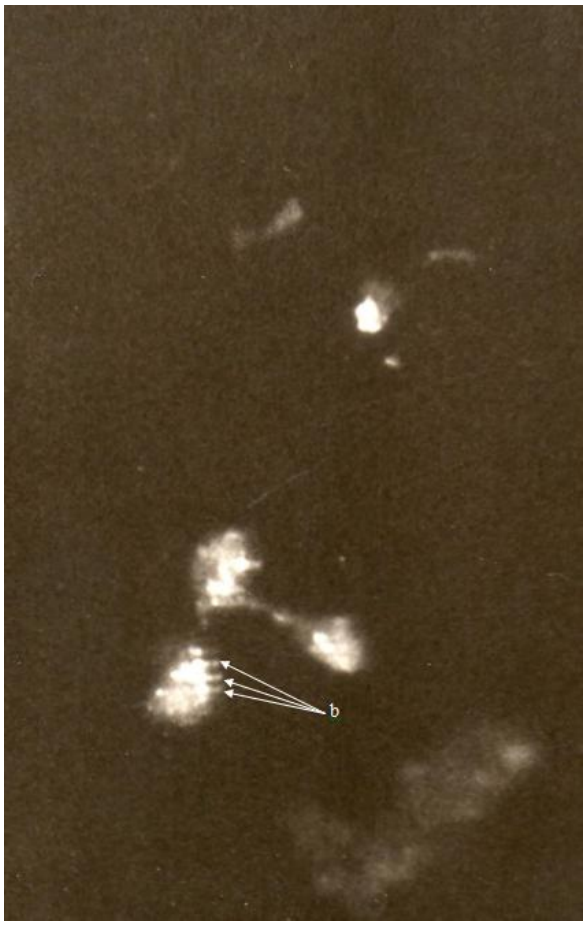

Figure 3

Figure 4

Fig. 1CPV isolation in CRFK cells. CRFK cells were infected with the suspected P5 sample and incubated for 16 days at 33°C. The CPE caused by CPV showed a mass of round and dark (granulated) cells with few islets of different sizes (magnification X40).

Fig. 2 CPV isolation in CRFK cells. CRFK cells infected with the suspected P5 and incubated for 16 days at 33°C showing a cytopathic effect. The CPE showing an islet of small, round and dark cells floating in a clear supernatant media (phase contrast magnification X40).

Fig. 3 Detection of CPV by indirect immunofluorescence assay. All the three suspected samples P5, P9 and P12 had similar intranuclear inclusion observed after 4 days of incubation in 33°C on cells telophase stage.

Fig. 4 Detection of CPV by indirect immunofluorescence assay. Intranuclear inclusions in the form of a racket were observed at 24 hr p.i to 72 hr pi in all the three suspected samples during CRFK cell division (end of telophase stage)

4. Discussion

Gastro-enteritis in canines can be caused by viruses, bacteria or parasites. Faeces of dogs suspected of canine parvovirus disease are the most commonly used substrate in the diagnosis of gastro-enteritis (Carmichael and Binn. 1981). The most common viruses found in caninefecal samples are parvoviruses and coronaviruses. Rotavirus is seldomly found (Rimelzwaan et al., 1991). Clinical diagnosisis not definitive and may lead to incorrect diagnosis and medication. Hence, laboratory tests are always needed to confirm the clinical diagnosis and the presence of CPV in fecal specimens. Commercial kits for the detection of CPV and other viruses are available. Conventional serological assays are commonly used but are time consuming, less specific and less sensitive compared to molecular assays (Mochizuki et al., 1993; Esfandiari and Klingeborn. 2000).

Among 12 samples collected and submitted to our Institute, only 3 were found positive after passages in CRFK cells. The number of positive samples might have been much more if more sensitive molecular techniques such as PCR or real-time PCR had been used (Parthiban et al., 2011). Another study showed more than 95% of suspected fecal samples were positive when using molecular assays (Filipov et al., 2011).

The first incubations of the infected CRFK cells were long ranging from 9 to 15 days after which CPE could be seen. The positive samples were from dogs of German shepherd breed with the age of 2 and half month (P12), 3 months (P9) and 9 months (P5). The vulnerability of young pups to CPV infections might be due to infection with new and heterologous CPV variants which maternal antibodies either cannot protect against or for which maternal antibodies were insufficient (Truyen. 2006). The P12 sample was from a vaccinated puppy. In this case the failure of vaccine to protect pups against CPV infection might be due to interference bymaternally-derived antibodies as previously reported (Buonavoglia et al., 1992; Waner et al., 1996). It is not yet known whether the CPV we detected is a field or vaccine strain. The differentiation between vaccine and field strains can be performed by real-time PCR assay with minor groove binder probes (Decaro et al., 2006). Generally CPV vaccine strains can be found together with field strains that infect dogs shortly before or after vaccination (Decaro et al., 2007). Since vaccinated puppies developed clinical signs of CPV disease this meansthe commercial vaccine used in Algeria may not protect against local CPV disease indicating a need to develop new vaccines based on local CPV strains. Our finding that vaccinated puppies presented with clinical signs of disease is in accordance with previous reports (Decaro et al., 2009; Ntafis et al., 2010; Filipov et al., 2011). Ntafis et al (2010). In fact nearly half the CPV infections detected by conventional PCR were from dogs that have been vaccinated with commercial live virus vaccines (Ntafis et al., 2010). Vaccine failure might suggest that a new and emerging CPV variant has replaced the CPV-2a strain used as a basis for most commercial vaccines (Touihri et al., 2009).

The CPE observed in the 3 positive samples was similar to a previous report (Hirasawa et al., 1985). Our results differ from Parthiban et al (2011) in which only 3 out of 18 samples with high titer of CPV showed mild CPE. It is not known why their other samples confirmed as positive did not cause CPE in CRFK cells (Parthiban et al., 2011). In our study, CPE was used as a reference guide to pick samples harbouring CPV. Even though virus isolation is a routine test, its use is limited because of possible cell-toxic substances found in the feces, which are hard to eliminate (Mochizuki et al., 1993). In addition, feces may contain inactivated CPV which cannot be detected by virus isolation. Other viruses present in the feces can develop similar CPE

(Mochizuki et al., 1993). Virus isolation assay sensitivity is low and can gave false results because of the presence of antibodies in the fecal samples of infected dogs which bind to CPV particles and prevent them from binding and infecting cells (Desario et al., 2005). The method of choice in this case is to use either conventional PCR or real-time PCR which is able to detect CPV that is undetectable by HA (Desario et al., 2005). PCR is now widely used as the method of choice for routine diagnosis of CPV because of its sensitivity and efficiency compared to serological assays. However, care needs to be taken to eliminate carry over PCR contamination (cross-contamination) in addition to false negative resulting from inhibitors of DNA amplification present in fecal samples (Mochizuki et al., 1993; Hirasawa et al., 1994; Pereira et al., 2000; Buonavoglia et al., 2001; Decaro et al., 2005; Ntafis et al., 2010).

CPE is not a valid assay for CPV detection since negative CPE does not exclude the presence of CPV particles (Kang et al., 2008). Virus isolation is not practical for clinical practice since it is costly, requires specialized equipment, is labor intensive, is not suitable for analyzing large number of samples and is not definitive since it requires identification of the virus by other assays such as HA or immunofluorescence.

Using anti-parvovirus serum prepared in mice we were able to confirm the presence of CPV in the form of intra-nuclear inclusion bodies by immunofluorescence assay. However, very few infected CRFK cells were detected. This might be due to the use of an old vaccinated strain that generated antibodies that were unable to detect current CPV antigenic types. Another explanation might be due to the characteristics of CPV infection in which inclusions are rare because the infected cells detach from the monolayer very fast as suggested by a previous study (Aubert et al., 1980). The observed inclusions on the mitotic spindle show that CPV multiply preferentially in dividing cells. These mitotic spindles seen in CPV infected CRFK cells are due to the binding of CPV to transferrin receptors that are expressed at high density on actively dividing cells (Parker et al., 2001). This may explain the requirement of actively dividing cells for CPV infection (Truyen. 2006).

In recent years, real-time PCR has been increasingly used because of its high sensitivity, specificity and reproducibility. It can detect and quantify CPV DNA within a few hours (Decaro et al., 2005; Desario et al., 2005). Real-time PCR alone or coupled with Taq man probes or MGB (minor groove binding) probes is the best method for identifying, quantifying and differentiating between various CPV variants (Decaro et al., 2005; Decaro et al., 2006; Filipov et al., 2011). However, real-time PCR may be too costly for laboratories in developing countries.

We have confirmed in this investigation the presence of CPV in Algeria. It would be interesting to investigate the prevalence, pathogenesis and spread of various CPV variants in the Algeria's dog populations to determine the prevalent CPV variants. In addition there is a need to evaluate immunity conferred by current vaccines to check whether current vaccines are effective or whether there is a need to develop a new vaccine based on currently prevalent local CPV variants. Currently a new variant termed CPV-2c (Buonavoglia et al., 2001) with mutation Glu-426 has emerged in many countries and is slowly replacing the previous CPV variants, CPV-2a and CPV-2b. It will be necessary to identify and monitor the antigenic variants circulating in Algeria because of the rapid evolution of CPV strains. Otherwise CPV could develop into a major public health threat and get out of control. Because of its closeness and cross-border trade, the prevalence and spread of CPV variant types in Algeria might follow similar patterns found in neighbouring Tunisia in which all 3 CPV variant types were evenly distributed (Touihri et al., 2009). In conclusion, CPV was isolated for the first time in Algeria confirming that CPV is distributed worldwide in canine populations.

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References

[1] M.J.G Appel, W.F Scott, L.E.Carmichael (1979). Isolation and immunization studies of canine parvo-like virus from dogs with haemorrhagic enteritis. Vet. Rec. 105, 156-159.

[2] Aubert, C. Convert, F. Mottet (1980). Gastro-enterite à parvovirus du chien. Rec.Med.Vet 131, 767-774.

[3] K.I. Berns, M. Bergoin, M.Bloom, M. Lederman, N. Muzyczka, G. Siegel, J.Tal, P.Tattersall (2000). Family Parvoviridae. In. Virus Taxonomy, VIIIth Report of the ICTV (Edited by: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA), Chap.2 pp. 315-317. London: Elsevier/Academic Press 2005.

[4] C. Buonavoglia, M.Tollis, D. Buonavoglia, A. Puccini (1992). Response of pups with maternal derived antibody to modified-live canine parvovirus vaccine. Comp. Immunol. Microbiol. Infect. Dis 15, 281-283.

[5] C. Buonavoglia, V.Martella, A. Pratelli, M. Tempesta, A. Cavalli, D. Buonavoglia, G. Bozzo, G. Elia, N. Decaro, L, E. Carmichael (2001). Evidence for evolution of canine parvovirus type-2 in Italy. Journal general of virology 82, 1555-1560.

[6] M.G. Calderon, N. Mattion, D. Bucafusco, F. Fogel, P. Remorini, J. La Torre (2009). Molecular characterization of canine parvovirus strains in Argentina: detection of the pathogenic variant CPV-2c in vaccinated dogs. Journal of virological methods 159, 141-145.

[7] L.E. Carmichael, L.N. Binn (1981). New enteric viruses in the dog. Adv. Vet. Sci.25, 1-37.

[8] L.E. Carmichael (1994). Canine parvovirus type-2. An evolving pathogen of dogs. Ann. Vet. Med. 25, 1-27.

[9] N. Decaro, C. Desario, D.D. Addie et al.(2007). Molecular epidemiology of canine parvovirus. Europe. Emerg. Infect.Dis 13, 1222-1224.

[10] N. Decaro, F.Cirone, C. Desarioetal (2009). Severe parvovirus in a repeatedly vaccinated 12 year old dog. Veterinary Record 164, 593-595.

[11] N. Decaro, C. Desario, A. Parisi, V.Martella, A.Lorusso, A. Miccolupo, V.Mari, M.L. Calaianni, A. Cavalli, L.D. Trani, D.Buonavoglia (2009). Virology 385, 5-10.

[12] N.Decaro, C. Desario, G. Elias, M. Campolo, A. Lorusso, V. Mari, V. Martella C. Buonavoglia (2007). Occurrence of severe gastroenteritis in pups after canine parvovirus vaccine administration: a clinical and laboratory diagnostic dilemma. Vaccine 25, 1161-1166.

[13] N. Decaro, G. Elia, C. Desario, et al. (2006). A minor groove binder probe real-time PCR assay for discrimination between type 2-based vaccines and field strains of canine parvovirus. J.Virol. Methods. 136, 65-70.

[14] N.Decaro, G. Elia, V. Martella, C.Desario, M.Campolo, L. Di Trani, E. Tarsitano, M.Tempesta, C. Buonavoglia (2005). A real-time PCR assay for rapid detection and quantitation of canine parvovirus type2 DNA in the feces of dogs. Veterinary Microbiology 105, 19-28.

[15] C. Desario, N. Decaro, M. Campolo, A. Cavalli, F.Cirone, G. Elia, V. Martella, E. Lorusso, M. Camero, C. Buonavoglia (2005). Canine parvovirus infection: which diagnostic test for virus?. Journal of Virological Methods 126, 179-185.

[16] J. Esfandiari, B. Klingeborn (2000). A comparative study of a new rapid and one-step test for the detection of parvovirus in faeces from dogs, cats and mink. J. Vet. Med. B. 47, 145-153.

[17] S.J. Ettinger, E.C. Feldman (1995). Textbook of veterinary internal medicine, 4th edn. (W.B.Saunders company).

[18] C. Filipov, N. Decaro, C. Desario F. Amorisco, R. Sciarretta, C. Buonavoglia (2011). Canine parvovirus epidemiology in Bulgaria. Journal of veterinary Diagnostic investigation 23, 152-154.

[19] T. Hirasawa, N. Tsujimura, S-I. Konishi (1985). Multiplication of canine parvovirus in CRFK cells. Jpn. J.Vet.Sci 47, 89-99.

[20] T. Hirasawa, T. Kaneshige, K Mikazuki (1994). Sensitive detection of canine parvovirus DNA by the nested polymerase chain reaction. Vet. Microbiol 41, 135-145.

[21] R, H. Johnson, P.B. Spradbrow (1979). Aust. Vet. J. 55, 151

[22] B.K. Kang, D.S. Song, C.S. Lee, K.I. Jung, S.J. Park, E.M. Kim, B.K. Park (2008). Prevalence and genetic characterization of canine parvoviruses in Korea. Virus Genes 36, 127-133.

[23] W.R. Kelly (1978). An enteric disease of dogs resembling feline panleukopenia. Aust. Vet. J. 54, 593.

[24] M. Mochizuki, M.C. San Gabriel, H. Nakatani, M.Yoshida (1993). Comparison of polymerase chain reaction with virus isolation and haemagglutination assays for the detection of canine parvoviruses in faecal specimens. Research in Veterinary Science 55, 60-63

[25] H.S. Moon, S.A. Lee, S.G. Lee, R. Choi, S.Y. Jeoung, D Kim, C. Hyun (2008). Vet Microbiol 131, 47.

[26] V. Ntafis, E. Xylouri, I. Kalli, C. Desario, V. Mari, N. Decaro, C. Buonavoglia (2010). Characterization of canine parvovirus 2 variants circulating in Greece. Journal. Vet. Diagn. Invest 22, 737-740.

[27] T.Ohshima, M. Hisaka, K. Kawakami, M. Kishi, Y.Tohya, M. Mochizuki (2008). j.vet.med.sci 70, 769-775.

[28] S. Parthiban, H.K. Mukhopadhyay, D. Panneer, P.X. Antony, R.M. Pilla (2011). Isolation and typing of canine parvovirus in CRFK cell line in Puducherry, south India. Indian Journal of Microbiology 51, 456-460.

[29] P.R. Paradiso, S.L. Rhode, I.I. Singer (1982). Canine parvovirus: a Biochemical and ultrastructural characterization. Journal of General Virology 62, 113-125.

[30] J.S. Parker, W.J. Murphy, D.Wang, S.J. O'Brien, C.R. Parrish (2001). Canine and feline parvoviruses can use human or feline transferring receptors to bind, enter, and infect cells. J.Virol 75, 3896-3902.

[31] C.R.Parrish (1999). Host range relationships and the evolution of canine parvovirus. Vet. Microbiol 69, 29-49.

[32] C.R.Parrish, P. Hawe, W.J. Foreyt, J.F. Evermann, M. Senda, L.E. Carmichael (1988). The global spread and replacement of canine parvovirus strains. J. Gen. Virol 69, 1111-1116.

[33] C.R. Parrish (1990). Emergence, natural history, and variation of canine, mink, and feline parvoviruses. Advances virus research 38, 403-450.

[34] C.A.D. Pereira, T, A. Monezi, D.U. Mehnert, M. D'Angelo, E.L. Durigon (2000). Molecular characterization of canine parvovirus in Brazil by polymerase chain reaction assay. Veterinary microbiology 75, 127-133.

[35] G.F. Rimelzwaan, J. Groen, H. Egberink G.H.Borst, F.G. Uytdehaag, A.D. Osterhaus (1991). The use of enzyme-linked immunosorbent assay systems for serology and antigen detection in parvovirus, coronavirus and rotavirus. Vet. Microbiol. 26, 25-40.

[36] L. Touihri, I. Bouzid, R. Daoud, C. Desario, A.F. ELGoulli, N. Decaro, A.Ghorbel, C, Buonavoglia, C.Bahloul (2009). Molecular characterization of canine parvovirus-2 variants circulating in Tunisia. Virus Genes 38, 249-258.

[37] U. Truyen (2006). Evolution of canine parvovirus- a need for new vaccines?. Veterinary Microbiology 117: 9-13.

[38] J.Vincent (1971). Isolement en Algérie de quatre souches de parvovirus bovin. Archives de l'institut Pasteur 121, 811-814.

[39] T.Waner, A. Naveh, I.Wudovsky, L.E. Carmichael (1996). Assessment of maternal antibody decay and response to canine parvovirus vaccination using a clinic-based enzyme-linked immunosorbent assay. J. Vet. Diagn. Invest 8, 427-432.