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

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Abstract: Using intraperitoneal (I.P.) inoculation of a commercial canine parvovirus vaccine we were able to prepare an antiparvovirus serum. By performing indirect immunofluorescence (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 (Ettlinger 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 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’ 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. Each fecal 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 then aliquoted and stored at −20 °C until further use.
2) Virus isolation assay
Crandell Feline kidney (CRFK) cell line was kindly provided by Professor Tomáš Žuľa 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 hours 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 flasks 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>
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<tr>
<th>Sample number</th>
<th>Origin</th>
<th>Breed</th>
<th>Sex</th>
<th>Age (month)</th>
<th>Vaccination against Parvovirus</th>
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<td>MB</td>
<td>F</td>
<td>12</td>
<td>NV</td>
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<td>F</td>
<td>2½</td>
<td>V</td>
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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

<table>
<thead>
<tr>
<th>Injection (days)</th>
<th>Mouse number</th>
<th>Inject. Vol. (ml)</th>
<th>Route of admin.</th>
<th>Harvest (day)</th>
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<td>11</td>
<td>0.5</td>
<td>IP</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>0.5</td>
<td>IP</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>11</td>
<td>0.5</td>
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<td>-</td>
</tr>
<tr>
<td>21</td>
<td>9</td>
<td>0.5</td>
<td>IP</td>
<td>28</td>
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</tbody>
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4) Immunofluorescence assay
The infection of the CRFK cell line with the different samples led to the appearance of a cytopathic effect CPEs 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 CO2 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 CO2 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 bent so 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 atmospheric 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             | T.C             |                     |                     |                     |                 |
| ECP 0 %         | ECP 0 %         |                     |                     |                     |                 |

b) Serum neutralization and identification
Beforehand, the reference serum is decompiled 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 shaked 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 atmospheric. 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 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 in 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 3 and 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.
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; Esfandiar 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 by maternally-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 means the 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.

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 canine fecal samples are parvoviruses and coronavirus. Rotavirus is seldomly found (Rimeezwaan et al., 1991). Clinical diagnosis not definitive and may lead to incorrect diagnosis and medication. Hence, laboratory tests are always needed to confirm the clinical
whether evaluate prevalence CPV al. variants for spindles CPV fast chara due inclusion of samples CPE CPE 2010 1993; amplification low Desario Mochizuki recent few multiply suggested 2008 Hirasawa et al. of Algeria. CPV of the infected CRFK cells was detected. This might be due to the use of 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 infections 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.

5. Acknowledgments

We would like to thank Prof. Tomáš Žuffa laboratory of MEVAK a.s Bratislava, for kindly provided us with CRFK cells. We also would like to thank Prof. Anne Morailon from Alfort National Veterinary School, France who kindly provided us with the anti-canine parvovirus serum. Besides our Thanks to our colleagues Yacine Mimouni, Moumen Bounaza and Nadir Benseghni for their collaboration in providing us the samples from suspected puppies with CPV.

References


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