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Molecular typing of canine parvovirus strains circulating from 2008 to 2012 in an organized kennel in India reveals the possibility of vaccination failure

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ABSTRACT

Canine parvovirus-2 (CPV-2), which emerged in 1978, is considered as the major viral enteric pathogen of the canine population. With the emergence of new antigenic variants and incidences of vaccine failure, CPV has become one of the dreaded diseases of the canines worldwide. The present study was undertaken in an organized kennel from North India to ascertain the molecular basis of the CPV outbreaks in the vaccinated dogs. 415 samples were collected over a 5 year period (2008–2012). The outbreak of the disease was more severe in 2012 with high incidence of mortality in pups with pronounced clinical symptoms. Molecular typing based on the VP2 gene was carried out with the 11 isolates from different years and compared with the CPV prototype and the vaccine strains. All the isolates in the study were either new CPV-2a (2012 isolates) or new CPV-2b (2008 and 2011 isolates). There were amino acid mutations at the Tyr324Ile and at the Thr440Ala position in five isolates from 2012 indicating new CPV mutants spreading in India. The CPV vaccines used in the present study failed to generate protective antibody titer against heterogeneous CPV antigenic types. The findings were confirmed when the affected pups were treated with hyper-immune heterogeneous purified immunoglobulin's against CPV in dogs of different antigenic types.

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1. Introduction

Canine parvovirus 2 (CPV-2), emerged in 1978 and is considered as the major etiological agent causing severe hemorrhagic gastroenteritis in canine population characterized by depression, loss of appetite, vomiting and leucopenia (Kelly, 1978; Appel et al., 1979; Decaro and Buonavoglia, 2012). CPV, probably derived from a very closely related virus in cats, feline panleukopaenia virus (FPLV) or a closely related carnivore parvovirus (FPLV-like virus), has become endemic in the global canine population. CPV-2 is highly contagious and cause high neonatal mortality.

The genome of CPV-2 is a single stranded DNA of about 5.2 kb in length, which comprises of two open reading frames, one coding in a nested fashion for capsid proteins VP1 and VP2 while the other frame codes for non-structural proteins NS1 and NS2 which are involved in replication of the virus as well as some gene products resulting from alternate splicing (Cotmore and Tattersall, 1995). VP3 capsid is formed by proteolytic cleavage of VP2 capsid protein in the full capsids (Weichert et al., 1998). Parvovirus capsid proteins are highly antigenic which play a major role in determining viral host range and tissue tropism.

Amino acid substitutions in the capsid protein VP2 is known to have significant biological consequences including the antigenic properties and the host range of the virus (Strassheim et al., 1994). A few years after the emergence of CPV-2, two new antigenic variants, named CPV-2a and CPV-2b were characterized (Parrish et al., 1985, 1991; Decaro et al., 2005). The antigenic types CPV-2a and CPV-2b differ from the ancestor (CPV-2) in at least five or six amino acid positions in the VP2 protein affecting the affinity to react with monoclonal antibody, binding to feline transferrin receptor (TfR) and replication in cats (Truyen and Parrish, 1992). Another antigenic variant of CPV-2 emerged in Italy around 2000 which has an amino acid substitution at 426 position and was designated as CPV-2c (Buonavoglia et al., 2001). An amino acid substitution in recent times has been observed in the 297 position

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(Ser297Ala) in the CPV-2a/2b/2c population which has been designated new CPV-2a/2b (Ohshima et al., 2008; Decaro et al., 2009a). All the three CPV-2 antigenic types, namely new CPV-2a, new CPV-2b and CPV-2c have been reported throughout the world (Decaro et al., 2005, 2007, 2011).

The original CPV-2 strain though no longer circulates in the field is still being employed in many commercial vaccine formulations. There is a concern that these current vaccines may fail to protect pups against the CPV-2 variants (Decaro et al., 2008, 2009b). A plan to use current strains in vaccine formulations has led to a CPV-2b based vaccine (Martella et al., 2005).

The present study was undertaken in an organized kennel located at Meerut, Northern India. The ibid kennel has been following stringent routine/recommended vaccination regime in its adult dogs and the pups. Surprisingly, significant outbreaks of CPV infection with high incidence of mortality in the pups were observed in the recent times. Attempts were made to isolate the virus in MDCK cell line and to characterize the antigenic region of the VP2 gene by sequencing so as to determine the antigenic types of the circulating strains. Amino acid substitutions in the VP2 protein of the present isolates were compared with the prototype strains and commercial vaccine strains (being commonly used in India) so as to infer the molecular basis of the outbreaks.

2. Materials and methods

2.1. Clinical samples

A total of 415 rectal swabs were collected from either morbid dogs or from the intestinal lumen of the post-mortem samples from dogs of different age groups and sex suspected of CPV infection and in-contact dogs from the ibid kennel, Meerut, India from 2008 to 2012. The samples were emulsified in Hanks Buffered Salt Solution (Sigma–Aldrich, St Louis, USA) containing Streptomycin (1 mg/ml) and Penicillin (1000 IU/ml), and incubated for 1 h at room temperature. The emulsified solutions were centrifuged at 10,000g for 15 min at 4 °C and the supernatant was passed through 0.45 μm filter (Millipore, CA, USA). The filtrate was collected and stored at −20 °C until further use.

2.2. Extraction of DNA, polymerase chain reaction (PCR) and sequencing

Total nucleic acid from all the clinical specimens was extracted using either QIAamp DNA Stool Mini Kit or QIAamp DNA Tissue Mini Kit (Qiagen, Hilden, Germany) as per recommendation of the supplier. The extracted DNA was stored in −20 °C until further use.

Primers were designed within the VP2 gene segment using Gene Tool Lite 1.0 software (BioTools Inc., Edmonton, Canada).

The PCR-amplification of the antigenic region of the VP2 gene was performed by using 100 ng DNA, 5 μL 10× PCR buffer, 2 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 10 μM of forward and reverse primers (CPV-FP: ^{3590}TGATTGTAAACCATGTAGACTAAC^{3613} and CPV-RP: ^{4135}TAATGCAGTTAAAGGACCATAAG^{4157} (the primer position is based on CPV-2 GenBank Accession No. M38245), 2.5U of Pfu DNA Polymerase (Fermentas, Lithuania) and the volume was made up to 50 μl with Nuclease Free water (NFW). The PCR amplification consisted of initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 70 °C for 1 min, followed by a final extension at 70 °C for 10 min. The PCR amplified products were resolved on 1% agarose gel in Tris acetate EDTA (TAE) buffer (1×) and visualized under UV transilluminator (AlphaImager®EP, Alpha Inootech, San Leandro, CA, USA).

2.3. Virus isolation

0.5 ml of inoculums from all the suspected clinical samples which were found positive by PCR was used for virus isolation in MDCK cell line. The infected monolayer was harvested after 3 times of freeze thawing 5 days post infection. After each passage level, the virus supernatants were used for nucleic acid isolation and subsequent PCR or stored at −20 °C until further use.

The PCR-amplified products (567 bp) were excised from the gel and extracted using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The purified products were assessed for quality and quantity. The sequencing was carried out using the Big dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA, USA) following the manufacturer's instructions on an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzer, Applied Biosystems, CA, USA) at BioServe Biotechnologies (India) Pvt Ltd, Hyderabad, India.

2.4. Bioinformatics analysis

The sequence chromatogram was visualized in BioEdit v 7.0.5 analysis software (Isis Therapeutics, Carlsbad, CA, USA). Mega Blast was performed with the deduced sequence within the non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov/Blast) to confirm the presence of the gene specific to CPV. VP2 gene sequences of CPV-2, CPV-2a, CPV-2b and CPV-2c from different geographical locations within India and rest of the world were retrieved from the NCBI nucleotide database. Multiple sequence alignment was performed using the in-built ClustalW algorithm in MEGA5 software (Tamura et al., 2011). Selection of the best-fit nucleotide substitution model for the dataset confined to the antigenic region of the VP2 gene (598 bp) was conducted in MEGA5. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura 3-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2055)). The branch lengths were measured in terms of the number of substitutions per site. Bootstrap test (1000 replicates) was performed to evaluate the clustering authenticity of the taxa.

3. Results

Of the total 415 samples analyzed in the present study, 28 samples were found positive for CPV. A single band of expected size of around 567 bp was observed on amplification of the partial VP2 gene in all the positive samples including the vaccines which were used as positive controls. There were only seven incidences of CPV infection in 2008 out of the 208 samples screened when vaccination was practiced with CPV-2 type vaccine strain. From 2009 onwards, vaccination was practiced with CPV-2b type and no incidences of CPV infection was observed in 2009 and 2010. In 2011, two incidences of CPV infection was observed from the 25 samples screened. These sporadic incidences of CPV infection pattern changed drastically in March, 2012 causing high incidences of mortality in the pups of 1–3 months of age. In 2012 outbreak, there were 19 incidences of CPV infection out of the 36 samples screened (52.78% positive cases). A subset of PCR positive samples from different years of isolation was sequenced in the present study.

On BLAST search, the CPV isolates matched with either with new CPV-2a or new CPV-2b and showed a similarity of 98–99% at the nucleotide level with most of the other isolates. The details of the isolates, CPV type, breed of the dog, age, isolation source, year of isolation, vaccination status, prognosis and accession numbers of each sequence is enumerated in Table 1. The interesting finding of the present study revealed that all the 2012 isolates

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