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Non-Invasive Fecal Based PCR Assays for Detection of Mouse Parvo Virus and Minute Virus of Mice in Laboratory Mice

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ABSTRACT

Background: Murine parvoviruses are a few of the most common pathogens of laboratory mice posing potential threats to mice colonies and experiments conducted in mice. We initiated this study to develop the fecal based PCR assays as an alternative to serology and to check its feasibility to detect the infections in mice caused by Mouse Parvo Virus (MPV) and Minute Virus of mice (MVM).

Methods: Primers targeting the VP2 gene of MPV and MVM were selected and their sensitivity was analysed in tenfold serially diluted gene template in the presence of negative mouse fecal DNA. Selected thirty-seven mice at the age of 6 to 18 weeks randomly and collected blood samples for serology and fecal samples for PCR assays.

Result: PCR assays of MPV and MVM detected as low as 0.4 fg of the target plasmid DNA. PCR assays in fecal samples of mice detected the presence of natural infections of MPV and MVM and their respective prevalence was 24% and 30%. Diagnostic sensitivity of MPV and MVM were 74% and 76% respectively. Our findings indicate that the fecal-PCR assay may be a useful, non-invasive and sensitive diagnostic tool in the ante-mortem detection of MPV and MVM in the health monitoring program.

Key words: Fecal PCR, Health monitoring, Minute virus of mice, Mouse parvo virus.

INTRODUCTION

Murine parvoviruses, Mouse Parvo virus (MPV) and Minute virus of mice (MVM), are among the common viral pathogens affecting laboratory mice despite housing in the hygienic barrier system. The asymptomatic and persistent natures of the infection with fecal shedding (Brownstein *et al*. 1991; Janus *et al*. 2008) pose threats to the mice colonies. Murine parvoviruses have a higher affinity for the dividing cells like lymphoid cells, enteroepithelial cells and neoplastic cells (Jacoby *et al*. 1995; Mckisic *et al*. 1996) and hinder researches related to immunology, transplantation, hematopoiesis and oncology (Mckisic *et al*. 1998; Smith *et al*. 1993).

A high degree of environmental stability of the parvovirus accelerates infection between research facilities (Clifford and Watson, 2008). Quarantine and health monitoring are important in limiting the transmission of infections (Boschetti *et al*. 2003).

In health monitoring, antibody testing has been routinely used in the indirect screening of parvoviral infection (Jacoby *et al*., 1996). The constraints faced in the importation and the high cost of commercial ELISA kits, lack of seroconversion (Janus and Bleich, 2012) in certain strains of mice and the persistent nature of the parvoviral infection stress the importance of developing a diagnostic tool alternative to serology.

Direct screening with PCR detects viruses even in early infections, in immunocompromised mice (Besselsen *et al*. 1995) and cell lines (Wang *et al*. 2013) but employing PCR as a post-mortem testing doesn't justify it as an alternative to serology in health monitoring.

MPV and MVM are relatively stable in feces at room temperature. Fecal based PCR assays offer the advantages of easy sample collection and antemortem testing of valuable animals (Bauer and Riley, 2006). On considering 3R's principle, non-invasive fecal based diagnosis of viral excretion of MPV and MVM is a very promising tool after considering the time frame of sample collection (Janus *et al*. 2008; Macy *et al*. 2009; Wang *et al*. 2013).

The most appropriate age for fecal testing of MVM is 5 to 7 weeks and can be detected till 11 weeks of age and in the case of MPV, the appropriate age was 5 to 11 weeks and can detect till 19 weeks of age (Bauer and Riley, 2006).

Hence this study was intended to develop non-invasive and sensitive fecal based PCR assays to detect active infections of MPV and MVM in mice of age 6 to 18 weeks, as an alternative to the serology based health monitoring in a lab animal breeding unit.

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MATERIALS AND METHODS

Animal maintenance

Present study was conducted using thirty-seven mice of Swiss Albino strain at the Laboratory Animal Medicine Unit

-a breeding unit of Tamil Nadu Veterinary and Animal Sciences University (TANUVAS). The study was carried out after the prior approval of the institutional animal ethical committee (IAEC), MVC, Chennai-07 (IAEC No. 172/DFBS/B/2013 dated 17.10.2013) and as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimentation in Animals (CPCSEA) Ministry of Environment, Forest and Climate Change, Government of India (GOI).

Animals were maintained in polypropylene cages with corn cobb bedding material and supplied with *ad libdum* feed and water. Cages, bedding materials and water bottles were autoclaved and purified water by Reverse Osmosis method and autoclaved before it was kept in each cage. Animals were maintained at room temperature 22±3°C and relative humidity 50±10% and ventilated with centralized air conditioning by the HVAC system.

Sample collection

Blood samples were collected from randomly selected weaned mice, from the lateral tail vein, after anaesthetizing the animal using 3% isoflurane in isoflurane anaesthetic apparatus. Clotted blood samples were centrifuged and the separated serum samples were stored as aliquots at -20°C till further use for ELISA. Fresh fecal samples were collected from the same animal, during the process of blood collection and were subjected to nucleic acid extraction immediately.

Isolation of DNA from fecal samples

A single mouse fecal pellet was suspended in 2mL of sterile Phosphate-buffered saline (PBS), pH 7.4. The suspension was centrifuged at 700 x g for 5 min at 4°C. A 100µL of the supernatant of the centrifuged was diluted with PBS at the ratio of 1:2 (Beckwith *et al*. 1997) and this fecal mixture was used for DNA extraction using High Pure Viral Nucleic Acid Kit (Catalogue number. 11858874001) as per the manufacturer's instruction. Purified viral nucleic acids (DNA) were used as a template for PCR assays. The concentration of DNA and their purity in the fecal DNA extracts were determined by measuring the *A*260/*A*280 optical density ratio with a UV-visible spectrum spectrophotometer.

Oligonucleotide primers

Primers specific for MPV (F5′-ACCACCAGCTCTTAACAACA-3′ and R5′- AAACTGGGTTCCAAGTAGCA-3′ targeting 377bp amplicon of VP2 gene) and MVM (F5′ - ACCATGAG CCAGCTTAACTT-3′ and R5′- CTGAAAGATCTCTGTCAA CGC-3′ targeting 268bp amplicon of VP2 gene), were designed using the NCBI primer designing tool based on the sequence from Genebank with accession no KC249524.1 and accession no DQ196317.1 respectively after multiple alignments with other parvoviruses using Lasergene analysis programme (DNASTAR, USA). Primers were synthesized and obtained from sigma.

To improve specificity, the regions of VP2 regions with maximum homolgy (based on consensus sequence) between the strains and the region exhibiting maximum heterology between the viruses were chosen. Among different primer sets, the primers on alignment with the related virus, exhibiting maximum mismatches and the mismatches in 3' end were selected. The forward primer and reverse primer of MPV, exhibits 7 and 4 mismatches (including 3' base of the reverse primer) respectively, in the VP2 gene of MVM. Similarly on aligning forward and reverse primers of MVM, with the VP2 gene sequence of MPV, exhibits 5 and 7 mismatches respectively (includes 3' base of both primers).

Gene template

Gene template containing the gene sequence (VP2 gene of MPV and MVM obtained from Genbank) synthesized in the PUC57 vector and obtained from genscript. Gene template was used as a positive control in all PCR assays and in the standardization of primers.

PCR assay

PCR assays were performed with a final volume of 20 µL containing 1 µL of each primer (10 pmol/µL), 20-30 ng of DNA, 10 µL of ready to use Taq DNA polymerase 2X master mix red (AMPLIQON, Denmark) and nuclease free water to make up the volume to 20 µL. All PCR assays included positive (Gene template) and negative controls (nuclease free water). An initial denaturation step of 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 45 sec, annealing 62°C for 30 sec (for both MPV and MVM) and extension at 72°C for 1 min and a final extension at 72°C for 7 min. PCR amplified products were analysed by agarose gel electrophoresis in the 1.5% agarose gel (Containing Ethidium bromide (10 mg/mL) added at 5 µL/100 mL just before casting gel). The results were visualized and recorded in the Bio-Rad gel documentation system. Amplicon size was compared with molecular weight markers.

Sensitivity and specificity

The sensitivity of the primers was determined by using a tenfold serial dilution of purified plasmid vector containing gene sequence (Concentrations of plasmid DNA ranged from 40 pg to 0.4 fg/PCR assay). To mimic diagnostic samples an equal volume containing 5 ng of mouse fecal DNA (Negative for MVM and MPV) was added to reactions containing serially diluted plasmid DNA. DNA samples positive for MVM and MPV were used in the PCR assay to analyse the specificity of MPV and MVM respectively.

Serology

Serum samples were analyzed by Sandwich ELISA using the available commercial kit (XpressBio Life Science Products, Thurmont, MD 21788, USA), for the presence or absence of antibody for MPV and MVM as per the protocol described by the manufacturer.

RESULTS AND DISCUSSION

The sensitivity of PCR assay

PCR assays of MPV and MVM consistently amplified as little

as 0.4 fg of the plasmid DNA template in the presence of negative mouse fecal DNA (Fig 1). Bauer and Riley (2006) and Wang *et al*. (2013) stated that the PCR assays detected as low as 100 copies of MVM and 10 copies of MPV and 10 copies of MVM and 50 copies of MPV respectively. In the current study, Plasmid DNA was used for the detection of the sensitivity of each set of primers; hence sensitivity assay was limited to the concentration of Plasmid DNA to 0.4fg, to avoid over-estimating the efficiency of primers.

The specificity of PCR assay

PCR assays of MPV and MVM amplified only in the presence of target viral DNA. Neither it amplified nonspecific mouse fecal DNA nor the target gene of the other parvovirus (*i.e* MVM PCR assay didn't detect reaction mix containing the VP2 gene of MPV as a template and vice versa) showing the high specificity of the primers to the targeted sequence in the PCR assay.

PCR and serology

Table 1 and Fig 2 shows the results of PCR and serology performed in this study. PCR in fecal samples showed that 9 out of 37 (24%) were positive for MPV and 11 out of 37 (30%) were positive for MVM. On examining serological results, 12 out of 37 samples were positive (32%) for MPV and 14 out of 37 samples (38%) were PCR positive for MVM.

With no reports across India for the prevalence of MPV by serology, 1-3% was reported in North America and Europe (Pritchett-Corning *et al*. 2009). Prevalence of MVM by serology is as low as 0.5% in North America and Europe (Gibellini *et al*. 2006), However in India, the prevalence in the nearby states namely Karnataka and Andhra Pradesh were 26.32% and 13% respectively and maximum of 50% prevalence was recorded in West Bengal across the country (Manjunath *et al.* 2015), which concurs with high prevalence in the present study by serology. Hence serological results of the present study were considered for analysing the diagnostic sensitivity and specificity of PCR assays.

Diagnostic sensitivity and specificity

Diagnostic sensitivity and specificity of PCR assay were determined by considering serology results as true positive and true negative results (Bauer and Riley, 2006; Kundave *et al*., 2017) and is shown in Table 1. Diagnostic sensitivity of the MPV and MVM PCR assays in the fecal samples are 75% and 79%, respectively. Seronegative animals in the colony were negative for PCR assay also, resulting in 100% diagnostic specificity. This result partially corroborates with

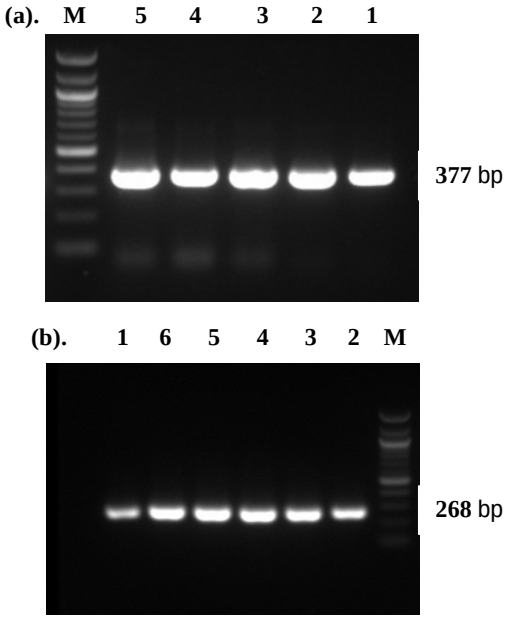

Fig 1: Sensitivity of PCR assay of (a) MPV and (b) MVM. (Lane-M- 100 bp marker, Negative fecal DNA + different Concentrations of Template DNA *viz*., 6- 40 pg, 5- 4 pg, 4- 400 fg, 3- 40 fg, 2- 4 fg, 1-0.4 fg).

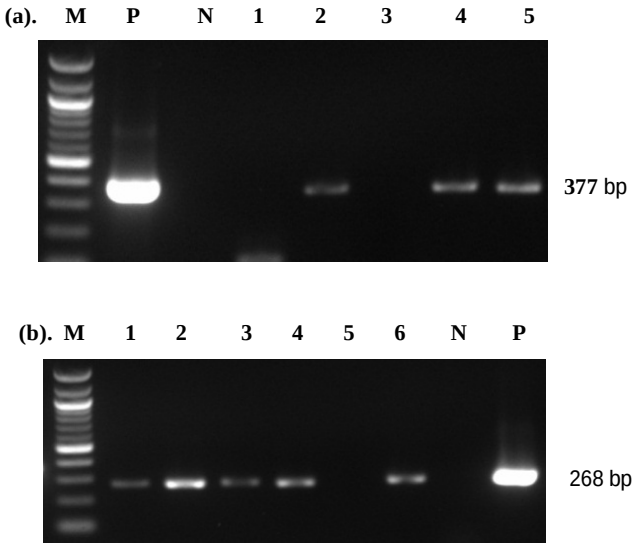

Fig 2: Agarose gel electrophoresis of PCR amplified DNA of (a) MPV and (b) MVM in fecal samples. (Lane - N- negative control, P- Positive control, M-marker, 1-6- Fecal samples of mice).

Table 1: Showing diagnostic sensitivity and specificity of serology and RT-PCR.

Virus	Test	Prevalence	Diagnostic sensitivity[#]	Diagnostic specificity[*]
MPV	ELISA	12/37 (32%)	100%	100%
	PCR in feces	9/37 (24%)	75%	100%
MVM	ELISA	14/37 (38%)	100%	100%
	PCR in feces	11/37 (30%)	79%	100%

[#]is [No. of true positive / (No of true positive + No. of false negative)] × 100.

[*]is [No. of true negative / (No of true negative + No. of false positive)] × 100.

the findings of Bauer and Riley (2006), who had reported that PCR in fecal samples showed diagnostic sensitivity of 74% and 54% in detecting MPV and MVM respectively. Enhanced diagnostic sensitivity of PCR for MVM in the present study may be attributable to the genetic background of mice and the age of exposure, which play critical roles in persistent infection and excretion of the virus (Janus *et al*. 2008; Macy *et al*. 2009).

In conclusion, the results of the present study indicate that the fecal-PCR assays may be useful as a non-invasive diagnostic tool for the rapid detection of natural viral infections caused by MPV and MVM after careful consideration of the age of the mice. Further evaluations of PCR sensitivity in comparisons of differences in the mouse strains are essential.

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