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In vitro Evaluation of Antiviral Efficacy of Himachali Pahari Cattle Urine against Canine Parvovirus

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ABSTRACT

Background: Canine Parvovirus is a common virus found amongst canines and felines species causing severe gastroenteritis. Many inactivated and live attenuated vaccines have been introduced till now, the emergence of new antigenic variants have raised concerns regarding the efficacy of commercial vaccines. As no specific antiviral therapy has been introduced till date and only symptomatic treatment is done. Therefore, there is need to find alternatives to cure for CPV related issues. According to Ayurveda, cow urine has already been proven effective against various diseases having antibacterial, antifungal, anthelminthic, anticancer property etc, but it has not been tested for its antiviral property till now thus in this study we, have focussed on using Himachali Pahari cattle urine to test its antiviral effects against CPV.

Methods: CPV was confirmed through PCR from faecal samples of diarrhoeic dogs presented to the clinics and was adapted to MDCK cells and different preparations (Raw urine, Cow urine distillates (CUD) and Extracts) of Pahari pregnant and Pahari non pregnant cattle urine were tested at different concentration for cytotoxicity on MDCK cells and final safest concentration was used further against $TCID_{50}$ value of CPV.

Result: The results showed that raw urine was found to be cytotoxic at minimum concentration used and CUD of Pahari pregnant urine were found to be effective. Extracts of both the Pahari pregnant and Pahari non pregnant urine were found to be ineffective against CPV. Pahari pregnant urine distillates (CUD) was found to have mild antiviral effects against CPV as it reduces some virus titre and further studies need to be done for its substantial efficacy.

Key words: Canine parvovirus, Cow urine distillates, Cytopathic effect (Cpe), Pcr, $Tcid_{50}$.

INTRODUCTION

Viral diseases in animals pose significant challenges to both in terms of animal health and their treatment. The impact of viral diseases on animals can be devastating, leading to huge economic losses, reduced productivity with substantial morbidity and mortality. Unlike bacterial infections, viral infections are challenging to treat because viruses rely on the host cell's machinery for replication. Prominent among viral diseases are Foot and mouth disease (FMD), Canine Parvovirus (CPV), Canine distemper (CD, *Peste Des Petits* Ruminants, Marek's disease, Avian influenza, New castle disease, *etc.* that cause huge economic loss in different species of animals. In small animal practices, diseases affecting gastrointestinal tracts are very commonly seen among them is Canine Parvovirus, it is highly contagious virus that cause high mortality and morbidity in dogs especially young pups up to 6 months of age. Canine parvovirus is DNA virus belonging to the Parvoviridae family and currently have four antigenic variants *i.e.* CPV-2, CPV-2a, CPV-2b and CPV-2c. (Kaur *et al.,* 2015). At present, there is no specific antiviral drugs have been used and only symptomatic treatment is the only cure given to the patients (Nandi *et al.,* 2010). Thus, it is really necessary to find out an alternative which is cost effective and easily available. Cow urine has been considered as elixir of life in ayurveda (Jarald *et al.,* 2008). It has various therapeutic properties and known to be used against various diseases. Cow urine has already got a patent for having antimicrobial activity (Khanuja *et al.,* 2005). Pahari cattle of Himachal Pradesh is a small sized indigenous animal that thrives on grazing or foraging on herbal or medicinal vegetations. These animals therefore suffer less from diseases because of their effective immune system. No study has yet been done to check the antiviral activity of cow urine against any viral diseases among animals. Therefore, in this study we have focussed on evaluating the antiviral effects of Himachali Pahari cattle urine against Canine Parvovirus.

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MATERIALS AND METHODS

Five urine samples each from Pahari pregnant and Pahari non-pregnant cattle were collected from the hilly region of Himachal Pradesh on the basis of history of no disease and no artificial insemination. 1-2 litres of freshly voided urine samples were collected in morning in a sterile container. Thereafter, chemical examination of all the samples of urine was done by UriScan (Optima). Filtered raw urine was subjected to distillation to prepare cow urine distillates (CUD) and hexane and butane extracts of urine (CUE) were made using Rotary evaporator (Bucchi Rotavapor R-210, Bucchi Labor Technik AG, Switzerland) at 40°C temperature and 337 mbar pressure. These extracts were further lyophilized to obtain powdered form of extracts and stored in -20°C for future use. The powdered extracts were dissolved in distilled water to make stock solution of 10 mg/ml. Canine parvovirus was detected using CPV-2ab specific primers pair employing PCR that shows band at 681 bp (Senda *et al.,* 1995). Initially DNA of the CPV suspected samples and the virus harvested from the cell culture were extracted using conventional PCI method (Sambrook *et al.,* 2001). The quantification of DNA was done by using Bio drop spectrophotometer by measuring absorbance at 260/280nm to determine the optimal concentration of viral DNA. The PCR product was analysed by gel electrophoresis using 1.5% agarose gel made with 1X TAE buffer at 80V for 30 min. the gel was documented through gel documentation system. Madin Darby Canine Kidney (MDCK) cell line was used in the study because these cells are permissive to canine parvovirus which means virus can easily infect and replicate in the cells showing observable cytopathic effects (CPE). In this study, MDCK cells were procured from NCCS, Pune. The cells were propagated in growth medium *i.e.* Dulbecco Modified Essential Medium (DMEM) (HI Media, India) containing L-glutamine, glucose, 25mM HEPES and sodium bicarbonate supplemented with 10% Fetal bovine serum (FBS) (HI Media, India) and Maintenance media with 5% FBS. Subculturing of the cells was done by splitting in 1:3 ratio using growth media (DMEM+10% FBS) as per the standard protocol.

MDCK cells at 60-70% confluency was infected with 1 ml of the PCR positive faecal sample and after 2 hours of incubation, virus inoculum was removed and the maintenance media with 2% FBS was added in the flask and the flask was kept for incubation for 4-5 days at 37°C with 5% CO_2 for observing the cytopathic effects (CPE). Infected monolayers were collected on the fifth day post-infection at each passage level and the presence of Canine Parvovirus (CPV) was assessed using polymerase chain reaction (PCR). This molecular testing method allowed for the detection of viral genetic material, providing insights into the infection status at different passage levels. The virus was harvested after 5 days post-inoculation (DPI) by freeze-thawing thrice and the inoculum thus prepared was used for subsequent passages. The viruses were passaged 4 times and Canine parvovirus was titrated in MDCK cells to calculate TCID50 by Reed and Muench method (Parker and Parrish (1997). All the urine formulations *i.e.* Raw urine, CUD and CUE were tested for cytotoxicity to find the safest working dilutions and concentration to be used against CPV. For Raw urine and CUD, 2-fold and 5-fold dilutions of urine were tested on MDCK cells in 24 wells tissue culture plates. After 60-70% confluency reached, MDCK cells were treated with 200 µl of each dilution and urine was removed after 30 minutes of incubation and cells were again incubated at 37°C in CO_2 incubator. The cells were observed at 24, 48 and 72 hours of incubation. For CUE, Lyophilized products of each hexane and butane extracts of Pahari pregnant and Pahari non-pregnant urine were dissolved in distilled water to make stock solution of two different concentrations 10 mg/ml and 2 mg/ml. Two-fold dilutions was done of each extract and they were tested for cytotoxicity on MDCK cells in triplicates in 24-well tissue culture plates. MDCK cells were treated and tested similarly as done with CUD.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was also done to check the cell viability after treatment of different formulations of urine at different dilution. The ability of mitochondrial dehydrogenases to catalyse thiazolyl blue tetrazolium bromide (MTT) reduction to formazan crystals was used to measure cell viability. The amount of Formazan is directly proportional to the number of live cells. Final safest dilution of CUD and CUE were tested against $TCID_{50}$/ml of virus and virus titer reduction was also done to check for reduction of $TCID_{50}$/ml value of initial virus used for the assessment of the antiviral effect of urine.

RESULTS AND DISCUSSION

Cytotoxicity assay

Evaluation of cell viability was done using MTT solution. For this, MDCK cells were seeded in 96 well tissue culture plate with 10^4 cells/ 100 µl of DMEM. CUD in 2-fold dilution and CUE at concentration of (0.2 mg/ml, 0.1 mg/ml…) and (1 mg/ml, 0.5 mg/ml) were tested in triplicates on cells. The cells were kept at 37°C in CO_2 incubator. After 48 hours of incubation, 50 µl of MTT solution/well were added to the cells and plates were incubated for 4 hours and 100 µl of DMSO was added and incubated for 30 minutes. Purple colour produce and absorbance is measure at 650 nm and absorbance is taken at 650 nm. The graphs were made using Graph Pad Prism software 9.37. shown in Fig 1 showing the percentage viability of cells as per the dilution of cow urine distillates of both Pahari pregnant (P.P) and Pahari non-pregnant (PNP) CUD showing safe dilution is 1:8. Fig 2 shows the percent viability of cells using different concentration of hexane extracts of P.P and P.N.P urine showing safe concentration of 0.2mg/ml and Fig 3 shows safe concentration of butane extracts of P.P and P.N.P urine was 1 mg/ml.

Antiviral efficacy of urine

Raw urine was found to be toxic for MDCK cells thus not used further. 1:8 dilution of both the Pahari pregnant and Pahari non pregnant urine was found to be safe and was tested against virus titre of 106.31‘ $TCID_{50}$/ml. Cells were pretreated with the urine for 1 hour and then they were infected with the virus and incubated for next 1 hour. virus was removed after 1 hour of incubation (Zhou *et al.,*2019). It was observed that Pahari pregnant CUD was able to inhibit the virus till 72 hours of incubation whereas MDCK treated with Pahari non-pregnant CUD showed CPE within 24 hours of incubation. In case of CUE, it was observed

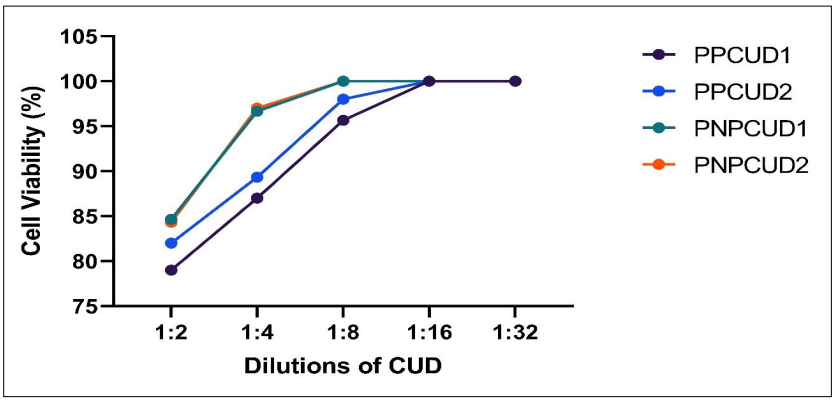

Fig 1: No. of viable cells after the treatment of 2-fold dilution of CUD of Pahari cattle on MDCK cells.

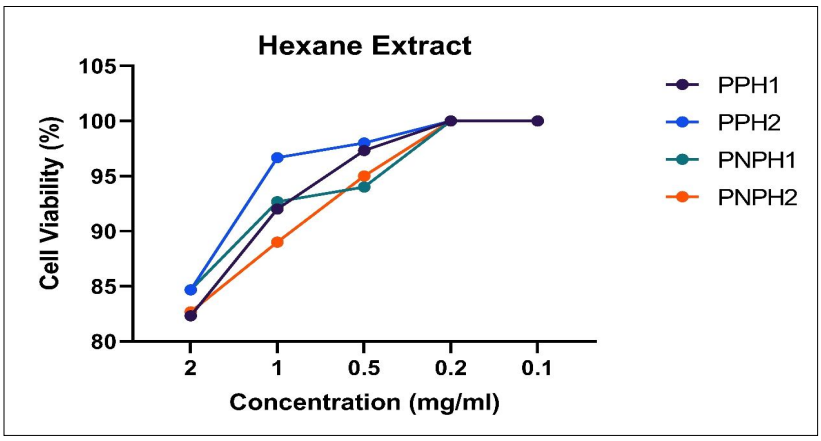

Fig 2: No. of viable cells after the treatment of different concentrations of Hexane extracts of Pahari cattle on MDCK cells.

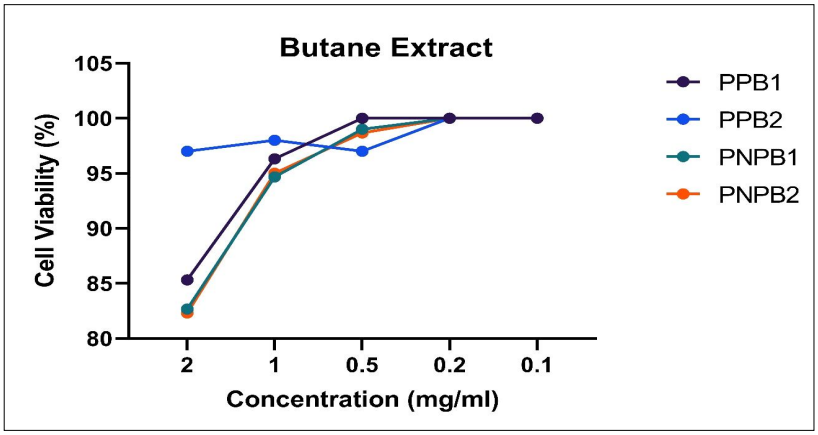

Fig 3: No. of viable cells after the treatment of different concentrations of Butane extracts of Jersey cattle on MDCK cells.

that all the Hexane and butane extracts of both the Pahari pregnant and Pahari non-pregnant urine was unable to inhibit the virus and showed CPE within 24 hours of incubation. There was marked rounding of cells with granulation of cytoplasm was observed. The results were compared with the virus control and normal MDCK cell control. In Virus titre reduction assay, virus was harvested using freeze and thaw method from the experiment control after 96 hours of incubation and titre was calculated using Reed and Muench method. It was found that Pahari pregnant CUD was able to reduce virus titre from 106.31$TCID_{50}$/ml to 105.81$TCID_{50}$ ml^{-1}. The results showed that Pahari pregnant urine had mild antiviral effect.

CONCLUSION

Canine parvovirus has already been known to cause high mortality in canines. Symptomatic treatment is the only possible way to treat the animals. As such no antiviral has been commercialised to be used against CPV, thus there is need to find out and a better cure which is cheap and easily available. In this study we have used Himachali Pahari cattle urine that is supposed to possess various medicinal properties and till now no research has been done to evaluate the antiviral activity of Pahari cattle urine. In this study, we prepared three urine formulations; raw urine, cow urine distillates (CUD) and cow urine extracts (hexane and butane). Initially they were tested for cytotoxicity on MDCK cells and it was observed that raw urine was found to be toxic as all the cells got damaged and sloughed off at the highest dilutions. CUD of both the Pahari pregnant and Pahari non-pregnant urine was found to be safe at 1:8 dilution. Hexane extracts of both the Pahari pregnant and Pahari non- pregnant urine was found to be safe at 0.2 mg/ml and butane fractions were safe at 1 mg/ml. These dilutions and concentrations were tested against 106.31 TCID50/ml of the virus and only Pahari pregnant CUD was found to be effective against CPV. All the CUE were not able to inhibit the virus by showing CPE after 24 hours of incubation. Only Pahari pregnant urine was able to reduce the titre of virus. This could be because pregnant cow urine may contain some stressors and hormones that might have some antiviral effects. This was a broad study done to evaluate the antiviral efficacy of Pahari cattle urine. Further studies need to be done to check for the exact constituent of urine through HPLC so that precise results could be obtained. So, through this study we can conclude that Pahari pregnant cow urine distillates was transiently effective against canine parvovirus.

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Conflict of interest

The authors declare that they have no conflict of interest. They are willing to submit this manuscript and they also declare that this manuscript has not been submitted or published in any other journal.

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