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Enzyme linked immunosorbent assay and polymerase chain reaction based surveillance of rabbit haemorrhagic disease in Indian and imported rabbits

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Rabbit haemorrhagic disease (RHD), an acute, highly contagious and often fatal viral infection of rabbits, is caused by RHD virus (RHDV). RHDV has been assigned to the family Caliciviridae (Meyers *et al.* 1991). Since its first report in China in 1984 (Liu *et al.* 1984), epidemics of RHD have caused severe economic losses to the rabbit industry in various parts of the world. The disease was known by various names like rabbit plague, rabbit viral septicaemia, rabbit viral hemorrhagic pneumonia and rabbit viral haemorrhagic disease. RHD is characterized by acute liver necrosis with high morbidity and mortality (60–90%) in adult rabbits (Xu and Chen 1989). RHD is widely distributed in different parts of Asia, Europe and Australia. So far, there is no report of RHDV infection in India and hence considered an exotic disease. In view of the deadly nature of the disease and as per the mandates of the institute to screen for exotic animal diseases, this study was carried out to screen for the presence of RHDV infection in Indian and imported rabbits.

Rabbit serum samples (514) collected from Andhra Pradesh (119), Madhya Pradesh (80), Meghalaya (55), Tamil Nadu (78) and Uttar Pradesh (182) were screened for the presence of RHDV antibodies by ELISA.

In 2001, German Angora rabbits (150), 3–to 12–month-old, were imported from Germany to India for breeding purpose. These rabbits during the quarantine period were examined for RHD as the disease is exotic to India. The rabbits were not vaccinated against RHDV. Serum samples (130) were collected from these imported rabbits for screening against RHD.

Screening by ELISA: A baculovirus expressed recombinant antigen based indirect ELISA was performed using a commercial kit as per manufacturer's instructions to detect antibodies to RHDV. Briefly, 100µl each of kit positive and negative controls, and 1:200 diluted test serum samples were added to the corresponding wells of recombinant antigen coated ELISA plate in duplicates and incubated for 1 h at 37°C. After 3 washes in washing buffer, 100µl of peroxidase conjugated protein-A was dispensed in all wells and incubated at room temperature for 1 h. After washing 5–times, 100µl of substrate solution (2,2'-Azino-bis(3–ethylbenzthiazoline–6–sulphonate, ABTS) was added and the plates were kept at 25°C for 10 min for color development. Finally the reaction was stopped by the addition of 100µl of 2M H_2SO_4. The optical density was read at 405nm in an ELISA reader. Samples with an optical density higher than 0.3 were considered as positive.

PCR optimization: PCR conditions to amplify the capsid region of RHDV genome were optimized using a 5.1kb referral clone obtained from Gregor Meyers, Federal Research Center for Virus Diseases of Animals, Germany. The clone was a pBluescript SK vector (Stratagene) containing a cDNA insert covering the RHDV genome from about nucleotide 1700 down to the poly-A tail. The sequences of the forward and reverse primers used are as follows: HSRHDV1, 5'-ACCCAGGCCGTCCAAAAGCACAC–3' (nt 6975–6997) and HSRHDV2, 5'-AATCAAACACTGGACTCGCCTGT–3' (nt 7380–7358). For optimizing the conditions, PCR was performed using PCR core system II (Promega) at different concentrations of dNTPs (0.1 to 0.2 mM each), primers (10 to 25 picomoles), Mg++ (0.5 to 3.0 mM) and Taq DNA polymerase (0.5 to 2.5 U), and annealing temperatures between 50 and 55°C. Best amplification was obtained when the reaction mixture consisted of 0.25µg template DNA, 0.2 mM of each of the 4 dNTPs, 14 picomoles of each primer, 1.5 mM of $MgCl_2$ and 1 U Taq DNA polymerase. Amplification was carried out in a thermal cycler with the following cycling conditions: an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 40 sec with a final extension at 72°C for 7 min.

Screening by RT-PCR: Blood samples in EDTA from 222 Indian and 31 imported rabbits were screened by RT-PCR. Total RNA was extracted from peripheral blood leukocytes

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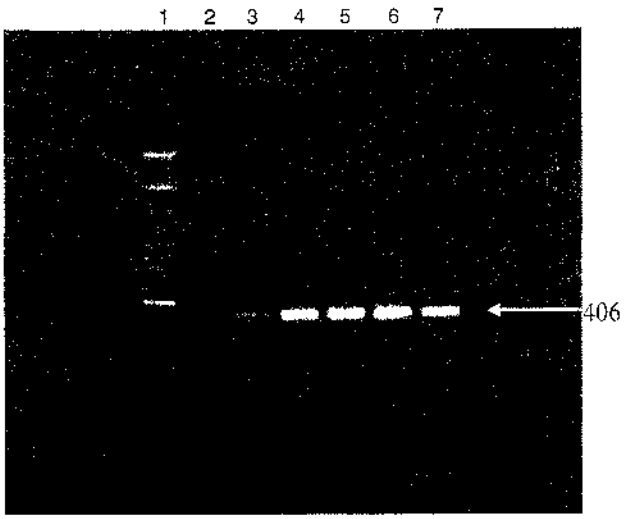

Fig 1. Amplification of 406bp DNA fragment of RHDV genome by PCR. Lane 1: 100 bp DNA ladder (MBI Fermentas, USA); Lane 2: Negative control; Lane 3–6: Positive samples from imported rabbits; Lane 7: Positive control

(PBL) using SV total RNA isolation kit. Reverse transcription was carried out at 42°C for 1 h with MMLV reverse transcriptase and random primers using RevertAid H-minus cDNA synthesis kit. PCR was carried out using PCR core system II using the optimized protocol. The PCR products were analysed by 1% agarose gel electrophoresis.

In Indian rabbits, none of the serum samples was positive for RHDV antibodies in ELISA. No specific amplification was obtained in RT-PCR from RNA extracted from the PBL samples. Results indicate the absence of RHDV infection in Indian rabbits.

In imported rabbits, 62 out of the 130 (47.69%) sera were found positive by ELISA. Ten out of the 31 (32.26%) rabbits tested showed specific PCR amplicon of 406bp size indicating the presence of RHDV genome. The results were communicated to the Department of Animal Husbandry and Dairying, Ministry of Agriculture, India, and all the rabbits were euthanised at the quarantine station as per quarantine norms. Thus the possible entry of RHDV into India was prevented.

Serological studies performed in Italy and the Czech Republic highlighted the existence of several rabbit populations with a high frequency of RHDV-seropositive individuals with no history of clinical symptoms or mortality (Capucci *et al.* 1996, Capucci *et al.* 1997). This correlates with the findings of the present study wherein presence of RHDV antibodies were detected in apparently healthy rabbits with no previous history of vaccination.

Cooke (2002) described the existence of persistently infected carrier rabbits, although the mechanisms of such infections are unknown. Existence of persistent infections with a potentially virulent virus was reported in rabbits from field sites endemic for virulent infections. Moss *et al.* (2002) reported that high proportion of serum samples that tested positive by RT-PCR were also positive for RHDV antibodies by ELISA, implying persistent RHDV infections in healthy immune adult rabbits inhabiting the endemic areas. Gall and Schirrmeier (2006) reported the persistence of RHDV genome for at least 15 weeks in vaccinated rabbits challenged with virulent RHDV. In our study, 7 out of the 10 RT-PCR positive rabbits were also positive in ELISA. Since the rabbits were imported from Germany, where RHDV infection is highly prevalent, these rabbits may be considered to be persistently infected. Results of screening of rabbit populations in India indicated the absence of RHDV infection in India. Introduction of such persistently infected imported rabbits would be a potential threat to the Indian rabbit populations.

SUMMARY

Rabbit serum samples (514) collected from different states of India and 130 rabbits imported from Germany were screened by indirect ELISA for the presence of RHDV antibodies. None of the Indian rabbits were positive while about 48% of the imported rabbits were positive for RHDV antibodies. PCR was optimized for the detection of RHDV capsid gene using a referral clone. RT-PCR was carried out on 222 Indian and 31 imported rabbit PBL. No specific amplification was obtained in Indian rabbits while 10 imported rabbits were positive for RHDV genome. Results indicated the absence of RHDV in Indian rabbits. Results of the tests on the imported rabbits were communicated to the Department of Animal Husbandry and Dairying, Ministry of Agriculture, India, and all the rabbits were euthanized at the quarantine station as quarantine norms. Thus the possible entry of RHDV into India was prevented.

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REFERENCES

Capucci L, Fusi P, Lavazza A, Pacciarini M L and Rossi C. 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit haemorrhagic disease virus but nonpathogenic. *Journal of virology* **70**: 8614–23.

Capucci L, Nardin A and Lavazza A. 1997. Seroconversion in an industrial unit of rabbits infected with a non-pathogenic rabbit haemorrhagic disease-like virus. *Veterinary Record* **140**: 647–50.

Cooke B D. 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. *Review in Science and Technology. Office Internationale Epizootes* **21**: 347–58.

Gall A and Schirrmeier H. 2006. Persistence of rabbit haemorrhagic disease virus genome in vaccinated rabbits after experimental infection. *Journal of Veterinary Medicine Series B Infectious Diseases and Veterinary Public Health* **53**: 358–62.

Liu S J, Xue H P, Pu B Q and Qian N H. 1984. A new viral disease in rabbits. *Animal Husbandry and Veterinary Medicine (in Chinese)* **16**: 253–55.

Meyers G, Wirblich C and Thiel H J. 1991. Genomic and subgenomic RNAs of rabbit haemorrhagic disease virus are both protein-linked and packaged into particles. *Virology* **184**: 677–86.

Moss S R, Turner S L, Trout R C, White P J, Hudson P J, Desai A, Armesto M, Forrester N L and Gould E A. 2002. Molecular epidemiology of rabbit haemorrhagic disease virus. *Journal of General Virology* **83**: 2461–67.

Xu Z J and Chen W X. 1989. Viral haemorrhagic disease in rabbits: a review. *Veterinary Research Communications* **13**: 205–12.