e-planet **21** (1): 85-92 (June 2023)

Sero-prevalence of hepatitis-E virus in rhesus macaques (*Macaca mulatta*) in and around Kolkata, W.B., India

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Date of receipt: 23.02.2023 Date of acceptance: 02.06.2023

ABSTRACT

Hepatitis E virus (HEV) is endemic in many developing countries and becoming a major public health concern that is transmitted through the faeco-oral route. HEV can cross species barriers and infect rhesus macaques and chimpanzees, the most relevant surrogates for human infections. The objective of the study is to assess the prevalence of anti-HEV IgG in rhesus monkey both in free range and in captivity by indirect ELISA and confirmation by western blot. In the present study, serum samples were collected from 100 different individuals of rhesus monkeys both from free range and captive population. The sero-prevalence of hepatitis E virus in rhesus monkeys in and around Kolkata was found to be 47% in captive individuals whereas 23% in free range species. Liver function test of the anti-HEV IgG positive rhesus monkey samples show normal liver condition. Increasing trend of hepatitis E sero-positivity with the increase in age in both captive and free range signifies improper hygiene and sanitation.

Key words: Hepatitis E virus, Kolkata, rhesus monkey, sero-prevalence

INTRODUCTION

Viral hepatitis is caused by infection with one of the five known hepatotropic viruses, which are named hepatitis A virus (HAV), hepatitis B virus, hepatitis C virus, hepatitis D virus, and hepatitis E virus (HEV). The most common clinical consequence of infection with HAV or HEV, is an illness characterized by sudden onset of fever and systemic symptoms, which is followed a few days later by jaundice (Anonymous, 2016). HEV is an enterically transmitted virus that occurs primarily in Asia, Africa, and Central America, where it is the most common cause of acute hepatitis. Hepatitis E infection caused by HEV is an acute and self-limiting hepatitis of a wide range of susceptible domesticated and wild animals like bovines, caprines, swine, rodents, non-human primates, and chickens (Biswas et al., 2020). HEV infection occurs through the faeco-oral route. The virus enters the blood through the gastrointestinal tract, the primary site of viral replication, and reaches the liver where it replicates in the cytoplasm of hepatocytes (Krawczynski and Bradley, 1989).

In Indian subcontinent, HEV is found to be endemic in nature and the prevalence of IgG antibodies to HEV has been studied among pigs (54.6-74.4%), dogs (22.7%), rodents (2.1-21.5%) and cattle (4.4-6.9%) (Arankalle et al., 2001). However, anti-HEV IgG was also detected in Japanese monkeys, cynomolgus monkeys, rhesus monkeys and Taiwan monkeys (Hirano et al., 2003). HEV-specific antibodies and/or the genome of HEV or HEV-related viruses have also been detected in many other animal species, including primates, other mammals, and birds. Genotypes 3 and 4 infections are documented in many domestic, wildlife and zoo animal species (Spahr et al., 2017). Hepatitis E virus antibodies or genes have been

reported to exist in many species of mammals, including monkeys (Huang et al., 2011). HEV-like sequences were detected in seven (29.2%) of 24 Chimpanzees at the studied zoo, suggesting that if the sequences are from real viral particles, the virus may be a new type of HEV using non-human primate as its natural host (Zhou et al., 2014). The prevalence of IgG antibodies to HEV was observed among different Indian non-human primates like wild rhesus monkey 36.7%, bonnet monkey (19.1%) and langur (2%) (Arankalle et al., 1994a). The study was mostly restricted to southwestern part of India. Moreover, the data was fragmentary and did not reflect the distribution of HEV infection of the country.

Though the state of West Bengal is endemic to HEV (Bansal et al., 1998), very few studies have been done so far on the sero-prevalence of HEV in the rhesus monkey population that is the most abundant species in nature and in captivity in Eastern India. The objective of the present study is to assess the prevalence of anti HEV antibody (IgG) in rhesus monkey both in free range and in captivity by an indirect ELISA and confirmation by Western blot.

MATERIALS AND METHODS

The study was conducted in different places in Kolkata, West Bengal and the serum samples of rhesus monkey were collected from species of Animal Rescue Centre (ARC), Forest Department, Govt. of West Bengal, Marble Palace Zoo, Kolkata, and species from different areas of Kolkata under the custody of Madaris. The serum samples for prevalence study of anti-HEV IgG were stored at -20°C prior to analysis. For indirect ELISA, the purified recombinant open reading frame (ORF-2) protein of HEV in aliquots was obtained from National Institute of Cholera and Enteric Diseases, Kolkata, and Rabbit anti-HEV IgG (whole molecule) peroxidase conjugate was obtained from Sigma, USA. SDS-7 marker was used for Western blot analysis. To detect anti-HEV IgG in the serum samples of rhesus macaques, an indirect ELISA was performed using a purified recombinant ORF2 protein expressed in a baculovirus vector according to the method described by Li et al. (2000).

Screening of serum samples

Ninety-six wells flat bottomed micro-titer ELISA plates were coated with 50 µl of diluted ORD2 antigen @1 µl ml^{-1} in PBS-0.1 M. The plates were incubated at 4°C overnight and then washed three times at an interval of five minutes by washing buffer. The antigen sensitized plates were blocked by blocking buffer. The blocking was initiated by incubating at 37°C for 1 hour. The serum samples were diluted at 1:200 in 3% LAH diluents were dispensed in duplicate wells @ 50 µl per well. Then the plates were incubated at 37°C for 1 hour. Ag-Ab reaction was detected by addition of rabbit anti-monkey IgG HRPO conjugate (whole molecule) diluted at 1:3000 in EIA diluent keeping the volume per well was 50 µl and the EIA plates were incubated at 37°C for 1 hour. Then 50 µl of OPD substrate solution was added to all wells and were incubated at 37°C for 10 minutes. To stop the reaction 50 µl of 1 M H_2SO_4 solution was added to each well. The plates were read in an ELISA reader at 492 nm. (Multiskan, Labsyste, Model- 355) to give the O.D. values and the results were calculated. Control wells in duplicate for positive sera, negative sera and conjugate were kept separately in each plate. In each plate, blank wells were kept for estimating the test reagents every time. The samples were analyzed by applying standard protocol of electrophoresis and interpreted by different statistical analysis methods to derive the P value of the samples.

Estimation of anti-HEV IgG reactivity by Western blot analysis

The sera samples were also screened by Western blotting employing the recombinant antigen produced from putative capsid protein ORF2 region (Lee et al., 1994). For immuno-electrophoresis, the protocol described by Laemmli (1970) was followed with vertical mini slab gel electrophoresis system. 12.5% of separating gel solution was poured into the gel casting space between the glass plates until about 70% of the space was filled and layered with n-butanol. After polymerization of the separating gel, n-butanol was drained off by filtering the gel cast. 5% gel solution

was layered over the separating gel-well forming comb into the top of the gel casting area and inserted until both end of the comb stopped at tops of the slide spacer and over layering of n-butanol. The comb was removed after polymerization. The antigen sample was prepared by diluting the ORF2 antigen @ 1 µl/ml in 2X sample buffer. The sample containing equal amount of protein (40 µg μl^{-1}) was applied to each slot and electrophoresis was performed at a constant voltage of 50V at room temperature till the tracking dye reached the lower end of the gel. The data were recorded, and analysis was made by applying statistical methods to derive Fischer's Test value.

Estimation of molecular weight by SDS PAGE

A gel with the protein band was cut and stained with Coomassie blue staining solution followed by destaining with the destaining solution after 6 hours. Molecular weight was determined using standard protein markers. The mobilities of all the proteins were recorded. The Rm values were obtained from electrophoretic mobilities by calculating the ratio of mobility of protein to the mobility of the tracking dye.

Western blot

Transfer of protein from gel to nitrocellulose paper electroblotting

The proteins separated by SDS-PAGE (Laemmli, 1970) were transferred to the NCP according to the method of Svoboda et al. (1985). Rest of the procedure was followed according to Towbin et al. (1979). Electroblotting was performed in Transblot apparatus. The portion of the gel containing protein lanes to be blotted was cut and incubated in western blot transfer buffer for 10 minutes. An NCP membrane (Immobilon-NC) of required size along with 12 pieces of Whatman No. 3 MM chromatography paper slightly larger than the gel were cut and we re equilibrated with transfer buffer. The cathode was located at the highest position and the cover was opened. Transfer buffer was poured on the anode, enough to prevent anode from drying. Six absorbance papers (Whatman No. 3 MM chromatography papers) were layered, one by one, on the anode. The nitrocellulose membrane (NCP) was over layered, and a small amount of buffer was poured on the membrane. The gel was then over layered, and a small amount of buffer was poured on the gel. Care was taken not to trap air bubbles in the gel and the NCP. The cathode was lowered gently to press down the layered material and the cathode was locked. The cathodic and anodic wires were connected to the apparatus and to power supply (cathode to gel, anode to membrane). The transfer of protein was performed at 50 mA. per square centimeters at 4°C for 14 hours.

Post transfer processing

After turning off the power supply the NCP was kept in blocking solution at room temperature for 1 hour. The NCP was then washed (4 × 5 min) with washing solution (PBS-Tween20). The NCP membranes, duplicate in numbers for each sera sample, were probed with selected sera (positive and negative) @ 1:200 in PBS-Tween 20 con taining 1% BSA (diluent). The NCP was incubated at 37°C for 1 hour. Washing of NCP was carried out with washing solution (PBS-Tween20). HRPO conjugated rabbit anti monkey IgG was used @ 1:1000 dilution in diluents to counter stain the corresponding membranes. The NCP membranes were incubated at 37°C for 1 hour. Then, washing of NCP was done as above. Subsequently, the enzyme complexes were detected by addition of DAB (SIGMA, USA) substrate. Reactive protein bands appearing after few minutes were observed. Finally, the enzyme-substrate reaction was stopped by addition of distilled water. Then, the NCP was dried up and preserved.

Estimation of serum biochemical constituents

Estimation of serum total protein, albumin, globulin, and A:G

By using photoelectric colorimeter applying the green filter Serum total protein and Albumin were determined by Biuret method described by Reinhold (1953). Then, Globulin fraction was determined by subtracting Albumin from Total protein. All the values were expressed in g dl^{-1}. The Albumin and Globulin (A:G) ratio was finally calculated.

Estimation of serum alanine aminotransferase and serum aspartate aminotransferase

By applying the method described by Bergmeyer and Bernt (1974) serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) activities were estimated using 2,4 dinitrophenyl hydrazine. By using dilution of pyruvate solution, a standard curve was prepared with the help of the O.D. taken in a photoelectric colorimeter (Systronics) using green filter (520 nm). The enzyme activity was expressed in terms of µg pyruvic acid liberated per mg of protein in serum samples incubated for 30 minutes at 37°C temperature in case of ALT and for one hour at 37°C temperature in case of AST.

Estimation of total bilirubin, conjugated bilirubin, and unconjugated bilirubin

Conjugated (direct) bilirubin, Unconjugated bilirubin, and Total bilirubin were estimated colorimetrically by Jendrassik and Grof's method (Jendrassik and Grof, 1938) and was expressed in mg dl^{-1}.

RESULTS AND DISCUSSION

A total of 100 serum samples of 1 to 9 years old rhesus macaques were collected of which 46 were males and 54 were females. The macaques were restrained from different localities of Kolkata out of which 70 individuals from free ranging areas of Animal Rescue Centre (ARC), Kolkata, 20 individuals from Marble Palace Zoo, Kolkata and 10 individuals from Madaris. All the macaques were found in captivity in different habitats.

The ORF2 ELISA has been performed to optimize baseline reactivity for rhesus monkey sera with the foremost aim of determining 'negative' population. Rhesus monkey sera demonstrated wide variation in level of reactivity, whereas some of them demonstrated low and relatively uniform reactivity. As shown in Fig. 1a, 1b and 1c, the rhesus monkey sera demonstrated widely varying levels of reactivity either in free range or captive sera samples. Analysis of reactivity for low reactive samples showed an approximately normal distribution, as observed with bovine sera samples (FCS) whereas other sera had significant numbers of 'outlying ' considered as highly reactive samples. Therefore, low reactive samples were considered to represent an 'HEV negative' rhesus monkey population in all the cases and the mean + 3 S.D.' of those negative 'cut- off" value for subsequent studies as described by Hirano et al. (2003). In practice, the optical density (O.D.) for a single reference serum on each plate (serving as negative control) was multiplied by a fraction of 1.5, calculated to be equivalent to the 'cut off". The serum samples showing an absorbance value greater than the 'cut off" values was determined to be positive (Choi et al., 2003).

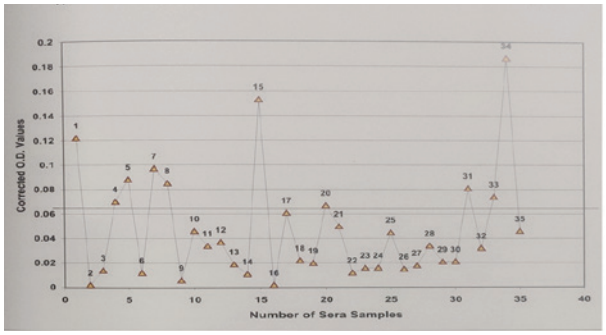

Fig. 1a. Sero-reactivity of Monkey Sera against HEV ORF-2 Antigen

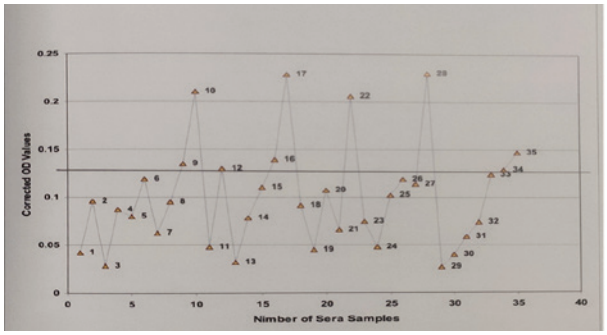

Fig. 1b. Sero-reactivity of Monkey Sera against HEV ORF-2 Antigen

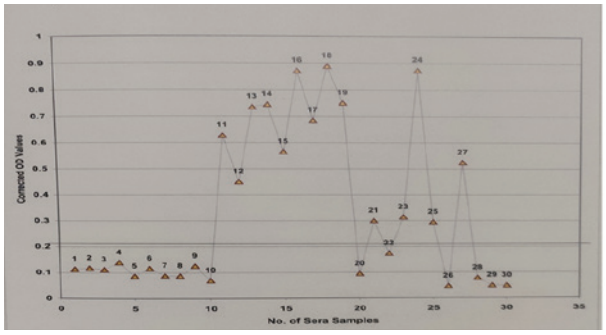

Fig. 1c. Sero-reactivity of Monkey Sera against HEV ORF-2 Antigen

Based on the above calculation, the 'cut off' values for ELISA plates (Fig. 2a and 2b) were found to be 0.065, O.131 and 0.201 respectively. The O.D. values of sample numbers 1-70 (free range) vary from 0.002 to 0.230 and that of sample nos. 71-100 (captive) varies from 0.069 to 0.888. Thus, in al1, 16 nos. of sera from 70 free ranging rhesus monkeys (23%) and 14 nos. of sera from 30 captive rhesus monkeys (47%) were found to be positive.

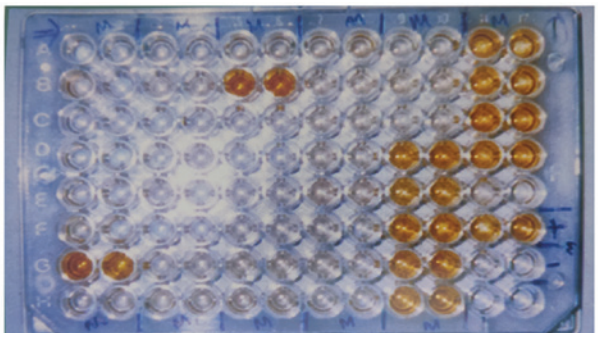

Fig. 2a. ELISA Plate showing positive and negative sera samples

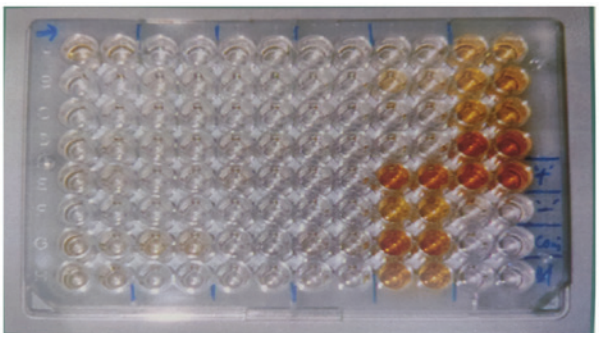

Fig. 2b. ELISA Plate showing positive and negative sera samples

The seropositive rate of anti-HEV IgG increase with age: 33% in 0-3 year old monkeys, 43% in 3-6 year old monkeys and 54% in 6-9 year old monkeys in captive rhesus monkeys whereas in the free range 18% in 0-3 year old monkeys, 23% in 3-6 year old monkeys and 37% in 6-9 year old monkeys (Table 1). No significant differences were observed between males and females.

Table 1. Prevalence of anti-HEV IgG in rhesus monkey of both free ranging and captive

Age	Free ranging (Percentage of positive)	Captive (Percentage of positive)	P Values
0 - 3	5/27 (18%)	1/3 (33%)	P = 0.50 (Fisher's Exact Test)
3 - 6	8/35 (35%)	6/14 (43%)	P = 0.16 (X2 = 1.96)
6 - 9	3/8 (37%)	7/13 (54%)	P = 0.39 (Fisher's Exact Test)
	16/70 (23%)	14/30 (47%)	P < 0.02 X2 = 5.57

This study demonstrates the presence of an antibody reactive to a recombinant HEV protein in rhesus monkeys in and around Kolkata. Although antibody prevalence varied across species and habitats, rhesus monkeys with anti-HEV antibody were found in virtually all the locations sampled. This agrees with the findings of Hirano et al. (2003) in Japanese macaques captured in various geographic regions of Japan. Rhesus monkeys are susceptible to infection with HEV like cynomolgus macaques found experimentally by Erker et al. (1999) and in wild by Hirano et al. (2003). Persistence of IgG anti-HEV antibodies for a long time and protection offered by low titered antibody against re-infection (Arankalle et al., 1999) is also proved by the fact that seroprevalence of hepatitis E virus in the zoo and with the *Madaris* is more than free range (Table 1). The seropositivity of free-range rhesus monkeys (23%) is more than the seroprevalence rate of 3.6% in free range rhesus macaques in Japan (Hirano et al., 2003), where HEV is not endemic and less than that of 36.7% in wild rhesus monkeys in south India (Arankalle et al., 1994b) where HEV is endemic. But again, the seroprevalence of captive rhesus monkeys kept in the zoo and with the *Madaris* is much higher (47%).

Sixteen (16) out of 70 free range rhesus monkeys (23%) and 14 out of 30 captive rhesus monkeys (47%) were positive for anti-HEV IgG tested by ELISA. These results indicated that HEV is circulating among monkeys belonging to the genus *Macaca*. The difference between the free range and captive monkeys is statistically significant

($P < 0.02$; $x^2 = 5.57$). Hirano et al. (2003) concluded that there is an increasing trend in seropositive rate among the Japanese macaques where the prevalence of anti-HEV IgG is higher in sexually matured adults over 5 years of age. The same finding had been found both in free range and in captivity along with the increase in age. This study demonstrates that the prevalence of anti-HEV IgG is higher in sexually matured adults over 6 years of age than in sexually mature individuals of less than 3 years of age.

Analysis of western blot of the serum samples

Assessment of the molecular weight of the HEV ORF2 antigen by SDS page is shown in Fig. 3a and 3b. All the positive representative rhesus monkey samples, exhibiting anti-HEV IgG reactivity in ELISA showed predominant and specific reaction to recombinant antigen prepared from capsid protein ORF2 in western blot. These reactivities of the selected positive serum samples were compared with sera from negative population also, where no band for anti-HEV reactivity appeared against the ORF2 antigen. The result for the reaction of positive and negative sera samples in Western immunoblot is shown Fig. 3A and 3B. These results co-related with the findings of Chandler et al. (1999) and Tam et al. (1991).

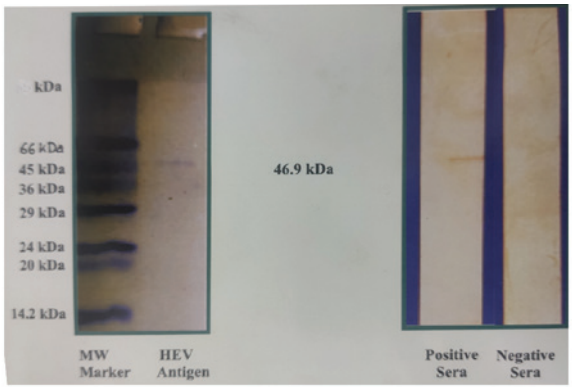

Fig. 3a. SDS-PAGE (12.5% Gel) showing peptide profile of ORF-2 Antigen of Hepatitis-E virus and Western Immunoblot of Positive and negative anti-sera.

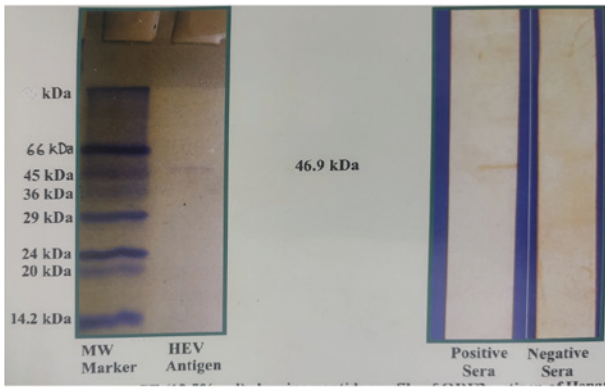

Fig. 3b. SDS-PAGE (12.5% Gel) showing peptide profile of ORF-2 Antigen of Hepatitis-E virus and Western Immunoblot of Positive and negative anti-sera.

SDS -PAGE of ORF2 antigen showed polypeptide profile of MW of 46.9 kDa against the marker antigen showing polypeptide profile of MW from 66 to 14.2 kDa. The representative positive sera sample showed one band of 46.9 kDa when the ORF2 antigen was resolved in 12.5% SDS PAGE and it was found to be immunoreactive when analyzed by Western blot.

The issue of persistence of anti-HEV antibodies is of great concern in countries like India where hepatitis E is highly endemic. HEV antibodies elicited by either natural or experimental infection do not distinguish between different strains of the virus and therefore a single serotype of HEV appears to be in circulation (Arankalle, 1994b; Bradley, 1988).

Results of level of serum protein and serum bilirubin

Depending on the type of test system used for detection of IgG anti-HEV antibodies, disappearance of antibodies was noted up to 6-12 months (Goldsmith et al., 1992), 1-4 years (Favorov et al., 1992) and up to l4 years (Khuroo et al., 1993). Moreover, experimental infection of rhesus with HEV and based on recombinant baculovirus derived frown ORF2 protein-based ELISA, a long-lasting IgG anti-HEV antibodies has been observed for 7 years by Arankelle et al. (1999). In this study there is no rise in ALT levels of all the sero-positive HEV rhesus monkeys. As such, it can be it can be inferred that the captive sero-positive population were already infected previously. The mean (±S.E.) of serum globulin of the rhesus monkeys in the study is found to be higher than the normal values

as reported by Wallach and Boever (1983). Kaneko et al. (1997) reported that with increasing age, the plasma proteins are seen to increase above the normal adult levels because of a small decrease in albumin and a progressive increase in the globulins. The level of serum bilirubin in the rhesus monkeys both in free range and in captivity is found to be within the normal limits which indicate the normal liver function.

CONCLUSION

Association of primates with human being dates to the evolution of man on earth. Among the primate population, rhesus monkey is the most abundant species in India. This study documents that HEV is infecting rhesus monkeys in natural environment both in free range and in captivity. It is noteworthy and alarming that a high prevalence of anti-HEV IgG is observed in rhesus monkeys in captivity (47%) compared to that in free range (23%). This may be because the monkeys in captivity are getting infection leading to a higher percentage of HEV infected population either from the persons handling the animals or due to the captive environment where there is a source of HEV infection. In captive environments, especially in the zoos, the animal houses are cleaned manually by the keepers which lead to animal human interaction. Moreover, lack of proper sanitation is a vital cause for spread of hepatitis E infection. In Kolkata, there is a good population of monkey keepers showing road shows with rhesus monkeys. These so called 'madaris' keep the monkeys confined in their houses and share the same food and drinking water. Proper hygiene is not at all maintained in these cases. These are the probable causes of HEV infection and subsequent development of anti-HEV IgG in the monkeys as found in the present study.

ACKNOWLEDGEMENT

The authors are thankful to Animal Resources Development Department, Government of West Bengal for providing permission to collect the serum samples of free ranging and captive rhesus monkeys. Authors express gratefulness for Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, West Bengal for providing a platform for undertaking the study. The help and guidance of Dr. Moloy Kumar Saha, Virology Division, NICED, Kolkata and Dr. Supriyo Bhattacharya, Animal Resources Development Department, Government of West Bengal are highly acknowledged.

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