International Journal of Basic and Applied Biology
Print ISSN: 2394-5820, Online ISSN: 2349-2539, Volume 2, Number 3; October-December, 2014 pp. 173-179

http://www.krishisanskriti.org/ijbab.html

Tick Borne Viral Diseases in India : Hen's Chick Embryonic Fibroblasts and Silkworm's Hemocytes as Proposed in Vitro Models for Tick Borne Virus Isolation

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Abstract: *Kyasanur Forest Disease (KFD) virus is vectored by the tick Haemaphysalisspinigera(Ghosh et.al. 2007) and chick embryo tissue culture vaccine is available. Crimean Congo Haemorrhagic fever (CCHF virus) is vectored by unknown Hyalomma species (Ghosh et.Al. 2007) and no vaccine is available.Ganjam virus (GANV)is a member of genus Nairovirusof family Bunyavirdae. Neutralizing and complement fixing antibodies to GANV have been detected in animal and human sera collected from different parts of the country. Thirty three strains of GANV have been isolated from India, mainly from Haemaphysalisticks (A.B. Sudeep, R.S. Jadi& A.C. Mishra 2009). Tick borne encephalitis is reported in humans, rodents, carnivores. Colorado Tick fever in humans, rodents and canine (http://animalrange.montana.edu/courses/ANSC410/Ticks%20Part%202%20Diseases.pdf).*
It is important to study the tick borne viral diseases of livestock and humans. The ixodid ticks as vectors of each of these viral pathogens are to be studied. The types of viruses, their structure, genome, virulence and disease caused is to be studied. The regions where the tick borne viral diseases are reported be studied to prevent the spread of the virus. The viral pathogens be detected with ELISA, PCR, sequencing, phylogeny and vaccines and drugs be developed. In addition, the control measures for tick vectors causing various tick borne viral diseases in livestock and humans be devised.

Keywords: *Ticks, Viral diseases, Vaccines*

1. INTRODUCTION

Ticks are ectoparasites and are feeding on blood of livestock, birds, reptiles and humans. They belong to Phylum arthropoda, class arachnida. There are 2 types of ticks, hard ticks (ixodidae) and soft ticks (argasidae). There are 7 genera of ixodid ticks in India namely Ixodes, Dermacentor, Haemaphysalis, Hyalomma, Rhipicephalus (Boophilus), Nosomma andAmblyomma. They are vectors of many pathogens causing pathogenesis and disease in livestock and in humans. They are vectors of bacteria, rickettsia, viruses and protozoa. Identification of these ectoparasites is therefore important to control the pathogens and the spread of them in order to prevent the disease caused by them in the livestock and humans. Thus, after correct identification of ticks species, vaccines may be developed for ticks and for tick borne pathogens.

*Hyalommaanatolicumanatolicum*and *Haemaphysalisspinigera*are the two important species of ticks present inIndia which are responsible for causing the fatal tick-borne viral diseases of Crimean–Congo hemorrhagic fever (CCHF)and Kyasanur forest disease (KFD) respectively. (Mourya et.al. 2014).

Classification for *Hyalommaanatolicumanatolicum*(http://www.gbif.org/species/7344309/classification)
Kingdom :animalia
Phylum :Arthropoda
Class :Arachnida
Order :Ixodida
Family :Ixodidae
Genus :hyalomma
Species :anatolicum
Classification for *Haemaphysalisspinigera*
(http://www.gbif.org/species/2183262)
Kingdom :animalia
Phylum :Arthropoda
Class :Arachnida
Order :Ixodida
Family :Ixodidae
Genus :Haemaphysalis
Species :spinigera
As per the literature, there are reports of tick borne viruses in India viz. Ganjam virus (Sudeep et al 2009), Crimean Congo Haemorrhagic fever virus (Yadav PD et al 2014, Appannanavar SB and Mishra B 2011) and the Kyasanur

forest Disease virus (http://www.icmr.nic.in/pinstitute/niv/KYASANUR%20FOREST%20DISEASE.pdf). These viruses were isolated from the hard ticks belonging to the family ixodidae. Different genera are involved for vectoring these viruses. Also, these viruses were isolated from different regions of the country. These viruses infect livestock and also humans. Each virus causes pathogenesis which may be fatal. Although there are high risk areas of these virus prevalence and reported mortalities, these viruses are not found in other regions of the country although preventive measures towards their spread is necessary.

2. GANJAM VIRUS

GANV was first isolated from *Haemaphysalisintermedia*ticks collected from goats,suffering from lumbar paralysis from Orissa, India, during 1954-55 and named after the place of isolation. Subsequent studies have yielded several isolationsmainly from *Haemaphysalis*ticks and a few frommosquitoes, sheep and man. Recently, for thefirst time, the virus was isolated from *Rhipicephalushemaphysaloids*ticks.Ganjam virus (GANV), a member of genus *Nairovirus*of family *Bunyavirdae.*GANV is antigenically related to Nairobi sheep diseasevirus (NSDV) of Africa, which is highly pathogenic for sheep and goats causing 70-90 per cent mortalityamong the susceptible population. Recent molecular studies have demonstrated that GANV is an Asianvariant of NSDV and both these viruses are related to the dreaded Crimean Congo haemorrhagic fever(CCHF) group viruses. The versatility of the virus to replicate in different arthropod species, its abilityto infect sheep, goat and man makes it an important zoonotic agent.CCHFV is one of the mostpathogenic human viruses among *Nairoviruses*, whichhas a wide geographic distribution in Africa, Europeand Asia. In India,disease associated with GANV in humans has neverbeen reported at an epidemic level. All the human cases recovered aftera brief illness. The virus, however, was found highlypathogenic to exotic and crossbred sheep and goatscausing high morbidity and mortality(Sudeep et al 2009).

Ganjam virus is classified as Biosafety level 3 agent (http://www.cfsph.iastate.edu/Factsheets/pdfs/nairobi_sheep_disease.pdf).

3. .KFD

Heavy mortality in two species ofmonkeys viz. the black faced langur (*Semnopithecus entellus*) and the redfaced bonnet monkey (*Macacaradiata*)in March 1955 in the forested areas ofShimoga district, Karnataka State led tothe discovery of the disease. Investigations resulted in the isolation ofthe virus from monkeys, man and ticks.The disease was named after the forestarea where it was first discovered asKyasanur Forest Disease (KFD) and thevirus was named as KFD virus. The initialfocus of about 100 sq km in Sagar taluk,Shimoga district, widened in subsequentyears and the disease has beenrecorded from Uttar Kannada, Udipi,Mangalore and Chikmagalore districtsof Karnataka state. KFD virus belongs to Russian SpringSummer Encephalitis group,amember of family Flaviviridae.KFD virus has been isolated from 16 species of ticks. However, *Haemaphysalisspinigera*is considered as themain vector. In enzootic state, KFD virus circulates through small mammals such as rodents, shrews, ground birds and an array of tick species including *H.spinigera*. When monkeys come in contact with the infected ticks, they get infected, amplify and disseminate the infection creating hot spots of infection. The people who pass through the forest are bitten by the infected nymphs of *H.spinigera*, which are highlyanthropophilic.The virus has been isolated fromnaturally infected *Semnopithecusentellus* (langur),*Macacaradiata*(bonnet monkey), *Rattusblanfordi,Rattusrattuswroughtoni*(rat),*Suncusmurinus (*shrew) and a bat*Rhinolophusrouxi*.NIV has developed an inactivated chick embryo tissue culture vaccine against KFD. Internationally, KFD virus is ranked as one of the highest risk categories of pathogens belonging to Bio Safety Level-4.http://www.icmr.nic.in/pinstitute/niv/KYASANUR%20FOREST%20DISEASE.pdf]

4. CCHF

During December 2010, National Institute of Virology, Pune detected Crimean-Congo hemorrhagic fever virus specific IgG antibodies in livestock serum samples from Gujarat and Rajasthan states. Subsequently, during January 2011 Crimean-Congo hemorrhagic fever virus was confirmed in a nosocomial outbreak, in Ahmadabad, Gujarat, India. Retrospective investigation of suspected human samples confirmed that the virus was present in Gujarat state, earlier to this outbreak. This disease has a case fatality rate ranging from 5 to 80 %. The evidences of virus activity and antibodies were observed during and after the outbreak in human beings, ticks and domestic animals (buffalo, cattle, goat and sheep) from Gujarat State of India. During the year 2012, this virus was again reported in human beings and animals. Being a high risk group pathogen, diagnosis is a major concern in India where only a few Biosafetylevel 3 laboratories exist and it needs to be addressed immediately before this disease becomes endemic in India(Yadav P.D. et. al. 2014)

CCHF viral infection had not been reported in humans from India before, though previous seroprevalence studies have shown viral antibodies both in animals and humans. In 1973, Shanmugam *et al.*, in their study, tested a total of 643 human sera from all over India; of these, nine samples from Kerala and Pondicherry were positive for anti-CCHF virus antibody. In the same study, 34 of 655 serum samples collected from sheep, horse, goat, and domestic animals from all over India showed evidence of CCHF virus. Subsequently, in 1977, Kaul *et al.*, conducted a survey of ixodid ticks to determine the Crimean hemorrhagic fever (CHF) virus activity in Jammu

and Kashmir state of India but CCHF virus was not isolated in any of the 138 pools comprising eight species under six genera of ticks. However, a related species of the genus *Nairovirus* – Ganjam virus – that belongs to the Nairobi Sheep group is transmitted locally by *Hemaphysalis* ticks. This virus has veterinary importance in India and has been demonstrated in mosquitoes, man, and sheep. The recent outbreak of CCHF viral infection in Gujarat is the first notable report from India. The striking feature of this outbreak was high fatality and rapid spread among treating medical team(Appannanavar S.B. and Mishra B. 2011)

In addition to the bunya-, nairo-, and phlebovirustick-bornemembers of the Bunyaviridae, there are at least 15 other tick-borne viruses that have yet to be assigned to a particular genus. The best known of these is Bhanja virus isolated from ticks of the genera Haemaphysalis, Boophilus, Amblyommaand Hyalommain India, Africa (Nigeria, Cameroon, Senegal) and Europe (Italy, Balkan states). Palma virus is an isolate from Portugal closely related to Bhanja virus. (Labuda and Nuttal 2004).

Thus, for tissue culture isolation of KFD, GANV,CCHFV hen's CEFs and silkworm hemocyte cultures are proposed as in vitro cell culture models.

NIV has developed an inactivated chick embryo tissue culture vaccine against KFD (http://www.icmr.nic.in/pinstitute/niv/KYASANUR%20FOREST%20DISEASE.pdf). Similarly, the hen's (Gallus domesticus) Chick Embryonic Fibroblasts (CEFs) and silkworm (Bombyxmori) hemocytescan be applied for isolation and tissue culture vaccine production of tick borne Ganjam virus, KFD virus, CCHF virus and Bhanja virus in biosafety vevel 4.

Hen's (*Gallus domesticus*) CEFs cultures: From 8 to 10 day old hen's chick embryo, primary culturescan be prepared from whole specimen aseptically with mechanical dissociation, cultured in 5 ml Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % Fetal Calf Serum and antibiotic and antimycotic solution in T-25cm^2 flasks and cultured at 37^{0}C with 5 % CO_2. After attaining confluency and monolayer formation in 2 days depending on seeding density, 10^4 cells/ml can be seeded for subcultures and CEFs can be maintained with trypsinization. The primary cultures can also be initiated with enzymatic dissociation and seeding of primary explants for cell cultures.

Addition of tick borne viruses to CEFs : These CEFs can be utilized for isolation of tick borne viruses. The infected serum from humans and livestock and tick extract can be added to monolayers of CEFs, observed under inverted microscope at 5X, 10X and 40X and photographed for CytoPathicEffects (CPEs).The viral antigens in CEFs can be localized with immunofluorescence and ELISA with PCR.

Thus, CEFs can be tissue culture model for propagation of tick borne viruses. The hen's eggs of 8-10 days can be obtained from the hatcheries for CEFs culture. These fertilized hen's eggs possess tissues to start about 20 primary cultures.

As mentioned in literature, the tick borne viruses propagate in other cells lines such as BHK-21, Vero, (Moureau et al 2008),they may also propagate in hen's CEFs. The receptors for these viruses on CEFs can be studied.

Silkworm (*Bombyxmori*) hemocytecultures :Silkworm larvae of 4th to 5th instar stage can be collected from farmers or rared from fertilized silkworm eggs in sericulture. From these 4th to 5th instar stage silkworm larvae, about 180 microlitres of hemolymph can be obtained. The hemolymph is colorless viscous liquid consisting of 5 types of circulating hemocytes classified as prohemocytes, granulocytes, plasmatocytes, spherulocytes and oenocytoids (Deshpande TM and Chaphalkar SR 2014). Plasmatocytes adhere to the substratum within 1 hour after seeding.The hemolymph can be collected from 4th to 5th instar stage silkworm larve aseptically from anal horn, seeded in T-25cm^{2}flasks, cultured in 5ml Grace insect medium with 10% Fetal Calf Serum and antibiotic and antimycotic solution at room temerature. Depending on seeding density, confluent cultures can be obtained in 2 days and 10^4 cells/ml can be subcultured.

Addition of tick borne viruses to silkworm hemocytescultures : These silkworm hemocytes cultures can be utilized for isolation of tick borne viruses. The infected serum from humans and livestock and tick extract can be added to monolayers of silkworm hemocytes cultures, observed under inverted microscope at 5X, 10X and 40X and photographed for CytoPathicEffects (CPEs).The viral antigens in CEFs can be localized with immunofluorescence and ELISA with PCR.

Thus, silkworm hemocytes cultures can be insect cell culture model for propagation of tick borne viruses. The silkworm's life cycle can be continued after collection of hemolymph without harm to silkworms.Many primary cultures from silkworm hemocytes can be started from many 4th to 5th instar silkworm larvae as seeding can be done from one 4th to 5th instar stage larva per one T-25cm^2 flask to get monolayers.

As mentioned in literature, the tick borne viruses are also vectored by mosquitoes (Ganjam virusSudeep et al 2009 and Issyk-Kul virus Labuda and Nuttal 2004) hence, they may also propagate in silkworm hemocytes in vitro. The receptors for these viruses on silkworm hemocytes can be studied.

Since the tick borne viruses are highly pathogenic, their propagation in hen's CEFs and silkworm hemocytes in vitro as tissue culture models may be done in BSL-4 facilities.

In addition to the above tissue culture models for tick borne virus isolation, the ixodid tick cultures may also be developed for tick borne virus isolation. The primary cultures and cell lines may be initiated from *Haemaphysalisspinigera*, *Hyalommaanatolicumanatolicum*speciesfrom tick hemocytes, salivary glandsinLeibovitz L-15 medium with additives such asTryptose Phosphate broth, 10% Fetal Calf Serum and antibiotic and antimycotic solution. Silkworm haemolymph as media supplement may also be added in the culture medium to study its growth promoting effects on tick cells in vitro.Earlier reports on tick cell lines are available from *Haemaphysalisspinigera*Newmann, *Haemaphysalisobesa*Larrousse and *Rhipicephalussanguineus*Latreille (Guru et al 1976) and applications of tick cell cultures from Haemaphysalisspinigera for growth of arboviruses (Banerjee et al 1977). Organs cultures of *Hyalommaanatolicumanatolicum*were reported for development of protozoa*Theileriaannulata*(Bell 1984).Hence, new primary cultures and continuous cell lines from*Haemaphysalisspinigera*, *Hyalommaanatolicumanatolicum*and from other ixodid tick genera may be initiated and developed for isolation of tick borne viruses.

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