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CONFERENCE ABSTRACTS

Abstracts of the Papers (Oral/Poster) Presented During the XXI National Conference of Indian Virological Society (IVS) on "Immunobiology and Management of Viral Diseases in 21st Century", Held from 8–10 November, 2012, at Indian Veterinary Research Institute (IVRI) Mukteswar Campus, Mukteswar, Nainital 263138, Uttarakhand

Transcriptional Targeting of Viral Oncogenes: A Novel Approach to Control Tumor Progression

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Specific types of high risk Human Papillomavirus (HR-HPV) types, particularly the HPV type 16 and HPV 18 are known to cause cervical cancer. The expression of two viral oncogenes E6 and E7 responsible for tumorigenic transformation, is mainly dependent on specific host cell transcription factor, Activator Protein 1 (AP-1) which acts as a signaling epicenter for cervical cancer. Although recently two prophylactic HPV vaccines have been developed, there is no therapeutic molecule available for the treatment of cervical lesions. We demonstrate that Curcumin (Diferuloylmethane), a yellow pigment, used in traditional medicines and present in the dietary spice turmeric (*Curcuma longa*), can selectively down regulate HPV 18 transcription as well as AP-1 binding activity. Curcumin can also reverse the expression dynamics of c-fos and fra-1 in tumorigenic HeLa cells by mimicking their expression pattern in normal cells. But the bioavailability of curcumin is extremely poor due to its hydrophobicity, rapid metabolism and lack of specificity in targeting cancer cells. Therefore, a folic acid-curcumin conjugate for mutation has been developed by attaching one molecule of curcumin with two molecules of folic acid (Cur-2FA) to make curcumin hydrophilic, enhance its bioavailability and to target only cancer cells which specifically express high level of folate receptors. The effect of both native curcumin and Cur-2FA on HPV and AP-1 has been analyzed in HPV positive cervical cancer and HPV negative breast cancer cell lines, by band shift assay, confocal microscopy, flow cytometry and western blotting including their cytotoxic potential and targeted cellular uptake of curcumin.

In comparison to native curcumin, curcumin-2FA has been found to be at least two times more effective in inducing down regulation of HPV transcription, AP-1 activity and expression of its components particularly c-fos and fra-1. Curcumin-2FA was found to be more potent than curcumin in inducing apoptosis inhibits tumor cell proliferation and Cur-2FA conjugate was specifically taken up by cancer cells. In mice, curcumin-2FA was more bioavailable and had a longer half-life than curcumin.

We demonstrate that this nontoxic low molecular weight curcumin-2Folic acid conjugate formulation specifically targets cancer cells which overexpress high affinity folate receptor and enhanced cellular uptake facilitating increased bioavailability and targeted delivery to cancer cells.

Emerging Viral Infections in 21st Century and Their Implications on Health System

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Concerned with emergence of many new diseases during the last decade the World health Assembly adopted International Health Regulations in 2005. Accordingly, each country is having obligation to monitor infectious diseases of international concern by developing minimum capacity for surveillance and diagnosis. Awareness has also increased in communities and vague diagnosis like viral fever or viral aetiology is no more acceptable to patients. Amongst infectious diseases viral diseases are the most important but capacity for surveillance and diagnosis is limited in most of the countries. Therefore, it is a major challenge to countries to develop virology to meet national and international expectations.

The major viral outbreaks in India during last decade include Nipah virus, SARS, Chandipura, Avian Influenza, Chikungunya and Crimean Congo haemorrhagic fever. Nipah virus outbreak in Siliguri in 2001 exposed ill preparedness of national laboratories to provide timely diagnosis and lack of infection control practices in hospitals. The disease created unparalleled scare to health care workers.

SARS posed extra ordinary pressure on health system due to peculiar nature of spread and resultant monitoring of airport and sea ports to detect cases, contact tracing and quarantine of suspected patients in infectious disease hospitals.

Discovery of Chandipura as a causative agent of encephalitis in central India was a masterpiece investigation. Involvement of virus has been proved year after year and is fully established. Being disease of low socioeconomic strata there is little interest to commercialize the diagnostics and vaccines developed by the institute. This brings important issue of governmental policy for neglected important diseases of specific populations in the country.

Avian influenza is caused by highly pathogenic Influenza H5N1 virus. This virus kills almost 100% infected chickens and causes 40–90% mortality in humans in different countries. The virus became greatest worry to public health and also to the food economy. Influenza viruses have notorious history of world wide spread in shortest possible time and with widespread occurrence of this virus in many countries it was considered as the prime candidate for the pandemic. Unprecedented pandemic preparedness plans were prepared by countries causing huge financial burden on health systems around the world.

Chikungunya outbreak after 34 year took everyone by surprise. The main vector *Aedes aegypti*, which was earlier designated as urban mosquito has developed strong foothold in rural areas also. As a result both urban and rural communities experienced massive outbreak. To add to problem the *Aedes albopictus* also played important role in some ecological settings in places like Kerala.

Finally in 2009, H1N1 pandemic appeared to test all the claims of our preparedness to contain infectious diseases. The virus appeared in Mexico/USA and within a month travelled to most part of the word once again to prove that spread of this virus remains unmatched. The virus caused millions of the cases and many deaths causing panic in public health system. It was real life time experience to be part of the team responsible for management and control of the pandemic.

Quick identification and fantastic public health response to Crimean Congo Haemorrhagic Fever outbreak in Gujarat was reassuring and satisfactory and greatly appreciated. The highly fatal and obnoxious disease is a real threat to health care workers particularly young doctors and nurses in Gujarat and Rajasthan states of the country.

Aetiology of many outbreaks like encephalitis in Gorakhpur and other parts of eastern UP remained elusive. Similarly, several outbreaks of haemorrhagic manifestations are also unidentified. The epidemics and pandemics provided tremendous pressure on public health system of the country and posed unique challenge to laboratories and scientists of the country. On one hand progressive evolution in public health preparedness and response has been reassuring and satisfactory but on the other hand lot more is expected from government and scientists to meet future s challenges. Newer diagnostics, standard and uniform protocols, quality control and assurance, vaccines and antivirals need to be developed. Hospital records and infection control practices need to be modernized. Infectious disease hospitals are in pathetic state and need review and fresh investment by governments.

A Journey of Citrus Virology in India

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Citrus originated in South-east Asia including N.E. India. Many of the important virus diseases now recognized in citrus appear to have originated in India and the orient, and to have moved with citrus as it was carried to other parts of the world through traders. Economic importance of citrus viruses has been realized world over. Therefore, for the first time delegates from 11 countries participated in a conference of citrus virus diseases in Riverside, California in 1957. At this meeting in California, an organization now known as 'International Organization of Citrus Virologists' (IOCV) was established. IOCV has organized 18 conferences since its inception in 1957. These conferences are held once in three years in different citrus growing countries. The 12th IOCV conference was held in India at Indian Agricultural Research Institute, New Delhi in 1992. To avoid introduction of viruses through the movement of citrus germplasm, the International Organization of Citrus Virologist, (IOCV), FAO, and IBPGR jointly published *FAO/IBPGR Technical Guidelines for the Safe Movement of Citrus Germplasm* and the I was a member of this team. Citrus psorosis was the 1st virus disease reported in 1933 in California and budwood certification was started against it in 1938. Many important discoveries were made between 1950 to 1975 on virus chemistry and structure, Identification of new pathogens like mycoplasma-like organisms and viroids and discovery of density gradient centrifugation which helped the purification of viruses.

In India, Citrus was grown as seedling trees. Horticultural techniques like budding and grafting started by 1920. Since then grafted plants were raised in various parts of India. Decline or dieback started in sweet orange on rough lemon and became a baffling problem by 1960. Dieback was attributed to various causes like Fungi, physiological disorders, deficiency, and soil etc. After the discovery of greening disease in India, dieback was suggested to be caused by Greening + fungi like *Diplodia*, *Fusarium* etc. In India, the researches on citrus viruses were restricted only to symptomatology, transmission, vector, and virus-vector relationship by 1980. The research work on virus and virus-like diseases was strengthen after the establishment of the Advanced Centre for Plant Virology at IARI, New Delhi in 1984 with the assistance of UNDP of FAO and establishment of a National Research Centre for citrus (NRCC) at Nagpur in 1986 by ICAR. In citrus, more than 40 virus and virus-like diseases have been described from most citrus growing countries but in India, 21 virus and virus like diseases are known to occur. Some of the economically important diseases were reported and worked out by us. Important among these are: *Indian citrus ringspotvirus* (*Mandarivirus*), *Citrus yellow mosaic virus* (*Badnavirus*), Citrus yellow vein clearing virus, Pummelo yellow mosaic rhabdovirus, Citrus yellow corky vein viroids. After 1990, reliable serological and molecular diagnostics were developed for detection of viruses in citrus and many viruses infecting citrus were sequenced. Details of these events will be discussed.

Veterinary Virology in India: In Retrospective and Perspective

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Both DNA and RNA viruses cause disease in livestock and poultry. RNA viruses are more prone to mutations and hence the diseases caused by RNA viruses, such as Foot and mouth disease, Bluetongue, African horse sickness, rabies are difficult to control. Carrier status and reservoirs of viruses pose further problem in this regard. Bats and wildlife are known as carriers/reservoirs of many viral diseases. There are instances where the virus can be maintained and excreted by the healthy individual for a long period as has been reported for rabies virus in dog saliva, herpes and lentiviruses in animals and man. For bluetongue virus cattle serve as reservoirs and for malignant catarrhal fever virus sheep act as reservoir. Pigs are known to amplify FMD and influenza viruses. Since in developing countries, sheep, goats, cattle, buffaloes and pigs often graze or live together in the company of humans, these close contacts increase the chances of spread and maintenance of existing viral diseases as well appearance of new viral diseases. Immunosuppression by herpes and retroviruses further complicate the epidemiology of animal viral diseases. As a result of this, non-existent forgotten diseases like Tuberculosis have re-emerged. Prions responsible for Mad cow disease in cattle and Scrapie in sheep have belied the notion that an infectious agent need to have nucleic acid. Origin of viruses and place of viruses in total micro biome still remains less understood, particularly when there are viruses of animals, birds, fishes, lower animals, bacteria, mycoplasma, fungi, etc. Changing life style, societal development, climate change has made visible impact on the occurrence of new viral diseases. In recent decades, occurrence of new viral diseases has gone up and now a new disease appears almost every year or alternate years, 75% of which are zoonotic in nature. Similarly, about 63% of the known 1465 existing infectious diseases are zoonotic. Besides causing diseases, there are some positive aspects of viruses. For example, the Newcastle disease virus has been shown beneficial application in resolving tumors in humans due to its oncolytic properties. Bacteriophages have been shown to have the potential of using for prevention/cure of mastitis caused by Staphylococci and Streptococci.

Rinderpest, also known as Cattle Plague, is a viral disease of cattle, buffalo, sheep, goats, pigs and other wild ruminant species. It is difficult to trace back to the time when the disease was recorded for the first time, it is believed that the disease originated from Asia and it ravaged the livestock in Asia and Europe from time immemorial. Though the disease in India was first recorded in Assam during 1752, it must have been prevalent through centuries. Rinderpest has been linked with the development of modern veterinary science globally. This devastating disease, in the absence of any known treatment or vaccine used to cripple the economy as it resulted in high morbidity and mortality in bovines, adversely affecting livestock and agricultural production leading to poverty of rural masses. The mortality could be as high as 80% or more. Efforts to counteract Rinderpest have inspired many developments in the history of veterinary science including the creation of first veterinary school at Lyon in France in 1762. It also triggered the establishment of public veterinary services in Europe and creation of Indian civil veterinary services in India in 1891. Considering the high morbidity/mortality and enormous economic losses due to Rinderpest, the Govt. of India in 1868 appointed "The Cattle Plague Commission" to study the problem and suggest remedial measures. Ever since the establishment of Imperial Bacteriological Laboratory (IBL) at Pune in 1889 on the recommendation of this Commission, priority was given for conducting research on Rinderpest. Sustained research work started after shifting of the IBL to Mukteshwar (Indian Veterinary Research Institute) in 1893. The research work laid emphasis on diagnosis and vaccine development to control/prevent the disease in domestic livestock. Though the efforts to effectively control Rinderpest were initiated very early in 20th century, major thrust came after 1950s.

The presence of Rinderpest in India was confirmed by Cattle Plague Commission in 1871. It was reported that hundreds of thousands cattle died every year. Agricultural activity like ploughing of fields and transportation of agricultural products were drastically affected. On the recommendation of Cattle Plague Commission the Imperial Bacteriological laboratory (now known as Indian Veterinary Research Institute (IVRI) was established at Pune on 8th December, 1889 for undertaking researches on Rinderpest and other infectious diseases of livestock and poultry.

Because of the huge losses and socio-economic implications of rinderpest, the Government of India invited Prof. Robert Koch and his team to Mukteshwar in 1897 to provide his scientific inputs to control Rinderpest. Prof. Robert Koch and his team along with Dr Alfred Lingard, the then Head of the Laboratory, conducted experiments on the use of bile from infected animals as vaccine with inconclusive results. However, Prof. Koch confirmed that the disease prevalent in India and the one he was working in South Africa were similar. In 1899- Anti-Rinderpest serum was developed at IVRI, Mukteshwar and used as a method of passive immunization. The protection conferred was for about 14 days only. Thereafter, serum simultaneous method of vaccination was standardized at Mukteshwar and shown to be superior to the Anti-Rinderpest serum. While the antiserum provided immunity for about a week only, in serum simultaneous method, involving the use of live virus along with the serum, there was a risk for the spread of the disease to the susceptible in contact animals. This method remained in vogue till the epoch making discovery of Dr. J.T. Edwards for successfully adopting and fixing the Rinderpest virus in heterologous host (goat) made a great breakthrough for the control of Rinderpest in India and abroad and also paved the way for the development of other attenuated viral vaccines which provided lifelong immunity. The serum simultaneous method of vaccination was replaced by goat tissue Rinderpest virus (GTRV) vaccine in 1931 for its use among plain cattle of India.

Further refinement in the development of goat tissue Rinderpest virus vaccine (GTV) was responsible for launching the National Rinderpest eradication program (NREP) in India in 1954. National Rinderpest Eradication program of India provided impetus to the FAO initiative for installing South Asia Rinderpest Eradication campaign (SAREC). Systematic vaccination campaigns were conducted in North, West, Central and East India during 1956–1964. Subsequently, Tissue culture Rinderpest (TCRP) vaccine was introduced as it was safe in all breeds of cattle including exotic European breeds and their crossbreds in which GTV was considered as hot vaccine. This mass vaccination provided sustainability to livestock development programs, especially the cross breeding program in cattle with high milk yielding exotic breeds.

Systematic vaccination decreased the incidence of the disease incidence but did not eliminate the infection in the country. It was with this concern that National Program on Rinderpest Eradication (NPRE) was initiated in 1992 with the financial support from European Union. Under this program, based on epidemiological history of Rinderpest, the country was demarcated into four specific zones (A, B, C and D) for vaccination and follow up action to eradicate Rinderpest. The campaign was finally successful with focused vaccination and zoo sanitary measures with audit on sero monitoring system. About 1600 million doses of vaccination against Rinderpest were carried out between the years 1956–1998.

The last outbreak of Rinderpest occurred in 1995 in India in Tamil Nadu, and vaccination was stopped in 2000. India's sero-surveillance exercise covering 480 million livestock population and involving 86,000 veterinarians and para veterinary staff seems to be the largest of its kind in the world. Though India had a long campaign to conquer Rinderpest, it provided historic evidence to the world community that the stamping out policy as was adopted in Europe and other countries could be avoided. In India, because of the socio religious considerations, slaughtering of animals is considered inhumane and sentiments of Indian population are against the cow slaughter policy. This ethical approach to eradicate rinderpest in India seems to be the greatest achievement in the world history for eradication of a viral disease of food animals. Finally India achieved the freedom both from Rinderpest disease and virus infection in 2004 and 2006, respectively.

Rinderpest eradication could be considered a revolution. The FAO of United nations stated that Rinderpest eradication was instrumental in enabling the green revolution in India. Rinderpest control provided assured draft power for crop production before the introduction and popularization of mechanization of crop farming. Non availability of animal draft power in pre-independence and post-independence period till 1980 resulted in declined planted area leading to smaller harvest. There was enhanced productivity of cattle through cross breeding and artificial breeding. India gained additional food production due to Rinderpest control/eradication from 1965 to 1998 which added up to 289 billion US dollars. This is the greatest contribution of veterinary scientists to crop science and dairy development program in India after Independence.

Eradication of Rinderpest mainly benefitted livestock keepers and enhanced food and nutritional security. Above all there has been argumentation in both meat and milk production. The milk production increased 2.99 times more from the year 1955 to 1995 and 4.796 times by 2006. The meat production increased to 17.99 times from the year 1959 to 1995. Impact of Rinderpest control on values in million dollars milk and meat has been substantial. There has been 102.06 times increase in income from milk and 193.96 times more from meat from 1950–1951 to 2005–2006. Increase in terms of tons for milk production was 72719 (000 tones) from 1955–2006. Increase in terms of meat production was 2671 (000 tons) from 1959–2006. Value in respect of milk is 15563.56 million US dollars and meat comes to 435011 million dollars between the years 1950 to 1996. Rinderpest eradication has also contributed towards conservation of biodiversity as well as species that might be threatened will have one less problem to face. The expenditure on livestock health care programs has also

reduced substantially due to the eradication of Rinderpest. Subsequent to global eradication of Rinderpest as declared by FAO on 28th June, 2011, future generation should be in preparedness for rapid and unequivocal diagnosis in an emergency situation if Rinderpest re-emerges. Updated laboratory facilities for prompt detection of virus and trained manpower in selected places need to be defined and biological material of Rinderpest of value, like vaccine strains, virulent viruses and antisera are capsulated under bio containment facilities with periodical check up by the eminent FAO experts/ national consultants in various countries.

The major viral disease reported in India by IVRI, Mukteshwar after Rinderpest was Ranikhet disease of poultry in the year 1927–1928. Efforts made to attenuate the virus by serial passage in the developing chicken embryos succeeded in 1942 in the form of a modified live virus R2B which provided satisfactory immunity but did not cause disease. However, the R2B strain was hot in imported breeds of poultry. On similar lines attenuated vaccine was developed for Fowl pox by serially passaging the field virus in embryonated chicken embryos. Attenuation of wild virus for vaccine development has also been attempted in primary cell cultures, established cell lines and mice for several diseases including sheep pox, goat pox, orf, camel pox, rabies, African horse sickness. With the introduction of imported high yielding exotic germplasm and their crossbreds, the problem of foot and mouth disease (FMD) took gigantic proportions threatening the dairy industry.

Series of killed vaccines against FMD were developed using different chemicals for inactivation of the cell culture grown virus for large scale vaccine production, both stationary cultures in large flasks and suspension cultures in fermenters have been developed. Presently BEI inactivated, aluminum hydroxide oil adjuvanted trivalent vaccine, having virus types OA and Asia1 is in vogue. Rabbits and chicken embryos have also been used for attenuation of viruses for producing lapinised and avianised vaccines. Alternative passages in cell culture and chicken embryos and unnatural route of infection have also been used for vaccine development. In some diseases, such as ILT, Newcastle disease, Marek's disease, naturally occurring mild/ attenuated viruses have been used successfully. Attenuation of vaccine candidate strains of viruses is both time consuming and uncertain method based on hit and trial method.

African Horse Sickness, a vector born viral disease of equines which is endemic in South Africa, hit India in 1960–1961 for the first time through Rajasthan border. IVRI, Indian Army and National Institute of Virology, Pune jointly work together on war footing to contain the infection and finally succeeded in eliminating clinical cases by 1965 following strict regulations under the law on infectious diseases for collection of samples for diagnosis and disease investigation, destruction and proper disposal of affected animals. The virus isolated was found to be Type IX. Diagnostics along with mouse brain tissue vaccine were developed successfully. India is free from AHS and OIE has given negative status. However, we need to have preparedness for prompt diagnosis of the infection, in case the disease struck again as AHS episodes outside South Africa have occurred after about 30–35 years.

Efforts should be directed to prioritize the existing, emerging and exotic viral diseases of livestock and poultry considering their economic, zoonotic and epizootiological characteristics, and threat perceptions for undertaking appropriate R&D and policy interventions. The diagnostic tests should qualify to OIE requirements which require harmonization, validation and accreditation of all the diagnostic tests in place as a mandatory requirement for health certification for export and import of livestock and livestock products, semen and embryos. Unlike bacterial diseases, viral diseases are difficult to control due to their rapid spread and lack of effective and affordable antiviral drugs. Rapid, specific and precise diagnosis is of utmost importance for timely and effective control of viral diseases. The diagnostic test should be sensitive, specific and at the same time cost effective. Where ever possible, recombinant antigen and monoclonal antibodies should be used in ELISA and other diagnostic assays. Only OIE recommended tests for various diseases, should be used for trade certification purposes. DIVA test along with marker vaccine should be developed for all those diseases where vaccination policy is in vogue, for differentiating the infected animals from the diseased ones. A DIVA has already been developed for FMD at Project Directorate on Foot & Mouth Disease, Mukteswar. An array of diagnostic tests from conventional AGPT, neutralization, CFT, HA-HI and agglutination to the second and third generation series tests, including IFT, IPT, IEM, ELISA, Dot-ELISA, Dip-Strip, EIAs, RIAs, PCR, PCR-ELISAs, LAMP, RT-PCR, Nested PCR, Real Time PCR, Multiplex PCR, nucleic acid hybridization, gene sequencing are now available and many more are under development. Signal (light, electronic) amplification and detection methods are the most exciting ones among these. Now there are tests having sensitivity of detecting as less as 10 virus particles. New innovation in molecular diagnostics include automated PCR, Real-time computerized PCR analysis, second generation PCR kits, DNA amplification finger printing, DNA Chips and Bio Sensors, lab-on-a-chip, micro arrays, nucleic acid sequence based amplification (NASBA), ligase chain reaction (LCR), DNA probe test based on strand displacement amplification (SDA), self-sustained sequence replication reaction (3SR), application of quantum dots and molecular imaging for the diagnosis of respiratory viruses and surface enhanced Raman spectroscopy (SERS) using laser and nanotechnology. From field application point of view, development of Pen-side diagnostic tests is the need of hour as there is limitation of laboratory based diagnosis in rural areas where most of the livestock is available. Work is in progress at IVRI and TANUVS for developing Pen-side diagnostic tests for viral diseases of poultry and other animals.

Ideal vaccines should be potent, capable of imparting lasting immunity, having adequate protective immunogenic mass without other unwanted extraneous antigens, easy to administer, safe for all category of animals including pregnant ones and capable of providing immunity in young animals and after *in-ovo* vaccination of poultry. The vaccine should have good keeping quality at refrigeration temperature as well as at ambient temperature. Development of recombinant bivalent and multivalent vaccines should receive priority of researchers as these vaccines will reduce the cost of vaccination as well as stress to the animals. Immunomodulators including adjuvants and cytokines should be used to enhance the immune response of killed vaccines. The dose of the vaccine should be as small as possible (about 1 to 2 ml for large animals). The vaccine should be able to induce adequate humoral as well as cellular immune responses. The vaccines should be updated periodically to accommodate the antigenic variations in the pathogen. Innovations are also required for devising easier administration of vaccines, such as patch vaccination, pressure vaccination, and vaccine administration through drinking water and feed pallets. This will need to develop thermo tolerant live virus vaccines TANUVAS has developed a thermo stable vaccine against Newcastle disease in feed pellet. Research is in progress at IVRI and other institutions for developing virus like particle (VLPs) vaccines against FMD and Blue tongue viruses devoid of viral genome, through reverse genetics. These vaccines will not have the possibility of reverting back to virulence which is common apprehension against the use of live modified viral vaccines. Modern biology and genetic engineering approaches have made it possible to develop safer and potent vaccines in the form of DNA vaccines, peptide and synthetic vaccines. Understanding of immune mechanism against viral diseases in various host species will help in developing better vaccines utilizing the knowledge of cytokines, histocompatibility complex, interferon ant interference, s-RNAi, TLRs and marker assisted selection for disease resistance breeding. We need to switch over from first and second generation vaccines to third generation vaccines on case to case basis.

Molecular Characterization of Low Pathogenic Avian Influenza Virus Subtype H4N6 Isolated from Ducks and Poultry in West-Bengal, India

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Avian influenza (AI) surveillance was carried out among wild, resident, migratory birds and poultry during 2009–2011 in West-Bengal State which revealed the presence of low-pathogenic AI H4N6 viruses circulating in domestic ducks and backyard chickens. Fifteen out of 299 samples from Murshidabad district were identified as low-pathogenic AI H4N6 viruses by virus isolation, hemagglutination inhibition assay and molecular subtyping. These viruses were characterized by partial sequencing of the Hemagglutinin (HA) and Neuraminidase genes. Herein, we present the complete genetic analysis of the H4N6 isolates by sequencing the HA gene from 15 isolates and full genomes of 7 isolates. The HA gene showed the presence of "PEKASR" motif, a characteristic feature of low pathogenic virus, at the HA cleavage site. The HA receptor binding domain retained Q226 and G228 suggestive of preferential binding to avian specific a 2,3 linked sialic acids. Phylogenetic analysis of all the eight viral genes showed that the H4N6 viruses from two different localities clustered distinctly with the Eurasian lineage. Notably, the isolates from the Nabagram area formed a separate cluster while, the single Golaghat isolate grouped distinctively from the other H4N6 viruses, except in the PB1 gene. Unique mutation in the NA of the Golaghat isolate rendered an additional glycosylation site in it, similarly, several other mutations in HA, NP, NS, PA and PB2 genes of the isolate suggested its genetic diversification. Interestingly, the AI H4N6 viruses isolated in this study also showed close relatedness with other LPAI subtypes like H4N3, H3N6, H7N6, H5N2, predominantly of duck species. This is the first report of molecular characterization of LPAI H4N6 viruses from India indicating genetic diversity of the viruses and suggestive of probable exchange of gene pools among various LPAI viruses circulating in this part of the globe.

Development of an H5N2 DIVA Marker Vaccine Candidate Virus Through Plasmid-Based Reverse Genetics

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Antigenic and genetic analysis of highly pathogenic H5N1 viruses from Indian outbreaks from 2006 to 2010 was carried out for selection of hemagglutinin (HA) gene donor vaccine candidate. On the basis of 3-D antigenic cartography A/chicken/West Bengal/80995/2008 H5N1 virus was found to be the best fit as the HA gene donor virus. For development of reverse genetics based non-pathogenic H5 vaccine strain, the basic amino acid cleavage site RRRKKR*GLF (major genetic character responsible for highly pathogenic nature of H5N1 viruses) in the HA gene of the selected H5N1 strain was modified to IETR*GLF by site directed mutagenesis using reverse-complimentary primers. The mutation in the pHH21-HA plasmid was confirmed by nucleotide sequencing. Using the mutated HA gene in the reverse genetics system of type A influenza virus (WSN/33), a recombinant H5N2 virus was generated from cloned gene segments of influenza virus as a non-pathogenic vaccine candidate for developing DIVA marker vaccine against H5N1 in poultry.

siRNA Mediated Down Regulation of Nonstructural Gene Expression Suppresses Propagation of Influenza A Virus in Mice

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Non-structural protein 1 (NS1) of influenza A viruses counteracts the host immune response against the influenza viruses by not only inhibiting the nuclear export and maturation of host cell messenger RNA (mRNA), but by also blocking the dsRNA-activated protein kinase (PKR) mediated inhibition of viral RNA (vRNA) translation. Down regulation of NS1 gene expression in the host cell may be a potent antiviral strategy to provide protection against the influenza virus infection. We observed the effect of siRNAs, synthesized against NS1 gene, on the inhibition of virus replication in Balb/c mice. The potency of siRNAs was assessed by plaque assay, real time RT-PCR, western blotting and histopathological analysis in mouse lung samples. We also assessed the cytokine levels in the Bronchio-alveolar lavage fluid (BALF) of mice by ELISA. The protective effect of siRNAs was also evaluated by performing animal survival assay. When siRNA was administered in Balb/c mice, 92% reduction in the levels of NS1 gene expression in mice lungs was observed. A significant reduction in the lung virus titers and bronchial inflammation was also detected in the presence of siRNA as compared to the untreated virus control. A decrease in IFN-γ & TNF-α and an increase in IFN-α1, IFN-β & IL-1β was observed in BALF samples of siRNA treated-virus infected mice. The siRNA effectively protected the mice from lethal influenza A virus challenge over a 21 day period. The study was validated by the use of selectively disabled mutants of each set of siRNA. Our findings suggest that siRNA targeted against NS1 gene of influenza A virus can provide considerable protection to the virus infected host cells and may be used as potential candidates for nucleic acid based antiviral therapy for prevention of influenza A virus infection.

Modulation of Cellular Apoptosis by Influenza Viruses During Infection: Role of Matrix 1 (M1) Protein

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Virus infection brings about cell death as a consequence of activation of the host cellular defense mechanism. Since early onset of cell death is detrimental to virus replication, viruses recruit certain proteins to activate cellular survival for counteracting apoptosis. Cell lysates were prepared from A549 and 293T cells infected with InfA/PR8 strain at increasing time points after infection followed by immunoblotting. Role of Matrix protein was assessed by expressing M1 protein in pcDNA6 and using M1 specific siRNA. During influenza A infection viral protein M1 (Matrix 1) activates survival genes at early infection periods but enhance the effects of apoptotic inducers late in the infection. At early infection periods M1 localizes mainly to nucleus and this translocation is cellular kinase mediated phosphorylation dependent. In the nucleus, M1 physically interacts with death domain-associated protein 6 (Daxx) which is a transcriptional repressor of survival genes, especially *Birc2, Birc3, Birc5, c-flip, XIAP*. Daxx interacts and inactivates RelB (NF-κB) transcription factor and recruits DNA methyltransferases, mainly Dnmt1 and Dnmt3a, to methylate RelB responsive promoters and thereby silence gene expression. Thus, M1 prevents Daxx's repressional function

during infection thereby exerting survival role. On the other hand during late infection phases M1 interacts with stress-activated heat shock protein 70 (Hsp70) and shows pro-apoptotic function. Apart from its chaperonine activity it exerts anti-apoptotic function by binding to apoptosis protease-activating factor 1 (Apaf-1) thereby disrupting apoptosome formation. M1/Hsp70 complex formation results in reduced interaction between Hsp70 and Apaf-1 leading to procaspase-9 activation induced by cytochrome c and ATP. Influenza viruses modulate cellular innate immune response such as apoptosis by dual function of its matrix protein different stages of infection.

Mutation Analysis of Haemagglutinin Gene of the Influenza A (H1N1) pdm09 Virus and Its Correlation with Disease Severity

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Influenza A (H1N1) pdm09 was the first influenza pandemic of the 21st century. Though the overall global case fatality rate of the 2009 pandemic H1N1 appears to be low (less than 0.5%), the fatality rate in India was relatively higher (0.86%). Elucidating the viral determinants of disease severity is important for developing better prevention and treatment strategies. Nasal/throat swabs were collected from patients presenting with Influenza like Illness (ILI) and were tested for the presence of (H1N1) pdm09 virus by real-time PCR (CDC Protocol). The positive cases were categorized into mild, moderate or severe group based on the clinical presentation of the patient ($n = 5$ per group). Virus isolation was done using MDCK cell line. The haemagglutinin gene of the isolates was amplified by one step RT PCR using WHO-CDC primers. The expected size products were purified using USB Exosap-IT purification kit and subjected to DNA sequencing using Big Dye terminator V 3.1 cycle sequencing ready reaction kit. The obtained sequences were analysed using BioEditv7.0.9. The viral haemagglutinin gene sequences have been analysed for signature mutations in the three categories of patients with clinical symptoms.

Isolation and Characterization of H11N9 Low Pathogenic Avian Influenza Virus from Live Bird Market of Jharkhand

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Live bird markets in Eastern India comprise of chicken and ducks sold in layered cages. Ducks are known reservoirs of various subtypes of avian influenza virus (AIV) and the chances of interspecies transmission and persistence of AIV in the environments is much higher under these conditions. In India, so far H9N2 and H4N6 subtype of low pathogenic AIV have been isolated from ducks and one more subtype of H11N1 was reported from a migratory bird. Here, we are reporting isolation and characterization of H11N9 subtype of AIV from a duck in the State of Jharkhand from a live bird market for the first time. In November 2011, cloacal and tracheal swab samples were collected from apparently healthy chickens and ducks in a live bird market located at Jamshedpur, Jharkhand. One of the cloacal samples collected from a nondescript duck was positive for AIV by NP gene based RT-PCR. Further subtyping by RT-PCR revealed that the virus belonged to H11N9 subtype. Subsequently, the virus was isolated by inoculation into 9–11 day old embryonated specific pathogen free chicken eggs. The subtype of the virus was confirmed as H11 by hemagglutination inhibition test with reference serum. Sequencing of hemagglutinin (HA), neuraminidase (NA) and non-structural gene (NS) was done and analysis indicated 97% homology of HA and NA genes and 99% homology with those of A/mallard/Czech Republic/13438-29K/2010 (H11N9) virus. This study emphasizes the need for targeted surveillance of live bird markets to understand the various subtypes of AIV that are circulating among Indian poultry.

Assessment of Immunomodulatory and Antiviral Potential of Tulsi (*Ocimum)* Leaves Ethanolic Extract Against Novel Influenza H1N1-2009 Virus

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The recent emergence of a novel swine origin human influenza A virus poses a serious global health threat. The control of this pandemic swine flu by vaccination has become more difficult due to rapid mutation. In absence of effective vaccine, the control is mainly by treating the patient with antiviral drugs especially oseltamivir (Tamiflu). However, the recent 2009 pandemic H1N1 viruses have been found resistant to oseltamivir. This has necessitated to look for more effective and safe alternate antiviral drugs. The anti-flu property of Tulsi (*Ocimum Sanctum)* has been discovered by medical experts across the world quite recently. The present study describes the assessment of antiviral and immunomodulatory potential of ethanolic extract of tulsi leaves against the pandemic 2009 swine flu H1N1 virus. The antiviral activity was demonstrated through in vitro inhibitory potential in MDCK in terms of virus inhibition assay as indicated through CPE, cell viability, hemagglutination, immunofluorescence, real-time reverse transcription-PCR. The immunomodulatory potential of ethanolic extract of *Ocimum* was also assessed in *Balb/c mice* through haematological parameters, mRNA expression of immuno-modulating cytokines & TLR respectively. Further phytochemical characterization of ethanolic extract was accomplished by TLC, HPLC to identify the active constituents responsible for antiviral activity. The virus infectivity was suppressed by ethanolic extract of tulsi leaves in both dose and MOI dependent manner. The time point kinetics of virus inhibition assays indicated that the post treatment was more effective than pretreatment followed by simultaneous treatment. The *in-vivo* immunomodulatory activity in *Balb/c mice* revealed significant increment in the population of defensive W.B.C. through enhanced proliferation of monocytes and neutrophils. The release of immunomodulating cytokines (IL-4, IL-10, IL-15) & TLR-9 was significantly higher compared to control mice. In conclusion, the present study clearly indicated the immunomodulatory as well antiviral potential of tulsi leaves that can offer promising option for supplemental strategy to currently available anti-influenza therapies.

A Comparative Snapshot of Influenza A Virus Infection Among Children of Defense Personnel in Delhi, India

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Influenza A viruses have been widely circulating in human populations and its variants have caused, and continue to cause, substantial

morbidity and mortality worldwide. The objective of this study was to compare the infectivity rates, antigenic variations and the serum cross-protection to other subtypes of influenza A virus among children of defense personnel in Delhi region, from July 2011 to March 2012. The study comprised of 208 patients presenting ILI. All the samples were screened for influenza virus by real-time RT-PCR and the blood samples of virus positive patients were collected to check cross-reactivity of antibody in serum. Of the 208 screened samples, 39 (18.8%) patients were found to be infected with influenza A virus. The PCR typing and sub-typing revealed 3 samples to be positive for H1N1-2009, 13 for seasonal H1N1, 19 for H3N2 and 4 samples were positive for both H3N2 and sH1N1. HAI was performed to analyze the cross reactivity of antibodies against various subtypes of influenza A viruses. It was observed that the previously infected seasonal H1 patients had a good level of cross-reactivity against H3 stain, but the H1N1-2009 infected patients were found to have fair titre of cross-reactivity to sH1N1 strain but drastically less titre against H3 strain. The study revealed that the seasonal H3N2 (48.71%). infection was found to be highest as compared to seasonal H1N1 (33.33%) followed by H1N1-2009 (7.69%). The co-infection of sH1N1 & H3N2 (10.25%) was also observed among the infected patients. The antibody raised by H1N1-2009 gave significant cross-protection against seasonal H1N1 as compared to H3N2.

Development of Semi-Continuous Fermentation Strategy for High Yield Production of H1N1-HA Recombinant Protein Using *Pichia* System

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Swine Origin Influenza A virus belonging to the *Orthomyxoviridae* family is an enveloped virus with segmented negative sense RNA genome surrounded by helical symmetry shell. Current influenza vaccines protect against homologous viruses but are less effective against antigenic variants and provide little protection against a different subtype. New vaccine strategies are therefore needed that can both accelerate production and provide broader spectrum of protection. Subunit vaccines like recombinant HA protein offers an alternative over conventional vaccine strategies that could save several months of manufacturing time. In contrast to conventional approaches there is no need for live influenza virus or large quantities of eggs, and subunit vaccines could be deployed earlier in the pandemic for effective reduction of morbidity and mortality. Moreover, it is also economical to produce these vaccines capable of inducing antibody that can neutralize the circulating strain of influenza virus. As it is very important to produce the antigenic protein in its native soluble form, prokaryotic system like bacteria may not be ideal for making this vaccine protein. With this background, it is practically important to have an alternate heterologous system that can make pandemic influenza HA protein and also can overcome the limitations associated with already reported systems. As Pichia offers several advantages including rapid and economical bulk production of recombinant proteins, an effective influenza HA subunit vaccine can be made with minimal notice for pandemic variants using *Pichiapastoris*. In this study, we have successfully developed the semi-continuous fermentation strategy for bulk production of H1N1-HA recombinant protein using Pichia system. Different fermentation parameters like Dissolved oxygen/aeration, Agitation, pH, Glycerol feed, methanol induction have been optimized for better expression of recombinant HA protein. The expressed proteins have been purified using Ion Exchange chromatography and the authenticity of the purified protein was confirmed using Immunoblotting technique.

Influenza Viral Surveillance in Pune During Period September 2011–2012

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Influenza is a vaccine preventable viral infection that can occasionally cause severe or fatal disease, especially in the elderly, the very young and those with underlying illness. Influenza activity in India is monitored by National Influenza Center at National Institute of Virology (NIV). Clinical samples of suspected patients with influenza like illness were collected from outdoor patient department at local hospitals in Pune (September 2011–2012). Real time RT PCR was used to detect seasonal and pandemic influenza. Virus LIMS software was used for data management. During the study period, total 1307 clinical samples were tested for influenza virus and 10% samples (82-type B, 56-Pdm, 1-H3) were positive for influenza. Type B circulation was observed throughout the study period (except June 2012), however peak activity (32.92%) was observed in October 2011. A spurt in pandemic A (H1N1) cases was observed from February-April with 51.78% positivity and increased activity was observed from August to September during the rainy season in 2012. Pandemic A (H1N1) positivity was highest (46.42%) in age group 15–35 years while lowest (8.9%) in age group above 55. In case of type B, highest positivity (41.46%) was observed in the 5–15 age group while lowest (1.2%) in age group above 55. Overall Male to female ratio for influenza positivity is 1.2:1. Type B and pandemic A (H1N1) virus co circulated; however since February 2012 pandemic A (H1N1) predominated. The spurt of pandemic cases in February–April was unusual. Our findings emphasize the need for continuous virological and epidemiological surveillance of Influenza virus.

DNAzyme Targeted Against the M2 Gene Transcript, Significantly Inhibit the Replication of Influenza A Virus in MDCK Cell Line

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The increasing understanding of the regulatory mechanisms involved in the pathogenesis of influenza is opening up opportunities for new therapeutic intervention. Since currently available treatment options against these viruses are limited owing to genetic drifts, there is a need for development of alternative therapies. DNAzymes (Dz), derived by in vitro selection processes, is one such discovery that has potential for selective gene silencing; thus we aimed to target the M2 gene of influenza A virus to down-regulate its replication in MDCK cells. Several 10–23 DNAzymes were designed and analyzed for their ability to specifically cleave the M2 gene of influenza A virus. The Dz that worked best was further standardized with $MgCl_2$ gradient to achieve the best results. The same concentrations of Dz were also transfected with the whole virus (Influenza A/PR/8/34) to study the inhibition of replication. RT-PCR and Real-time RT-PCR assays followed by western blot analysis were performed to detect the inhibition of the expression of M2 gene. We

observed that the Mg^{2+}-dependent sequence specific cleavage of M2 RNA was achieved up to 75% in a dose-dependent manner. The transfection of the MDCK cells with the Dz also reduced the cytopathic effect caused by A/PR/8/34 (H1N1) and this antiviral effect persisted for almost 48 h. The western blot revealed significant down-regulation in the M2 protein thereby reducing the virus replication in host cells. The designed Dz significantly down-regulated the virus replication in MDCK cells. Since there are very few studies done on applications of DNAzymes against influenza viruses, there is good prospect of its therapeutic use to protect from the lethal effects of influenza A viruses.

Evaluation of Antiviral Activity of *Azadirachta indica* (Neem) Leaf Extracts Against Influenza A virus

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Influenza A virus has been known to cause widespread human epidemics or pandemics with high mortality rates because of its high rate of mutation and rapid transmission by the aerosol route. The prospect of rapid emergence of drug resistance against the currently available antiviral drugs against influenza and the limited use of influenza vaccines against the newly emerging strains have exposed the need for the development of new and better antiviral to counter this ever changing virus. Medicinal plants have proved to be a rich source of alternative therapy against many viruses. Neem plant (*Azadirachta indica*) has been known to contain microbicidal and virucidal properties. In the present study, methanol extract from the leaves neem plant was tested for the cytotoxic activity and inhibitory activity against influenza virus type A. Madin Darby Canine Kidney (MDCK) cell line was used for this study and the antiviral activity of the extract was analyzed by the occurrence of Cytopathic Effect (CPE), Haemagglutination Assay (HA) and real time RT-PCR. The extract showed potent antiviral effect on the propagation of influenza A virus strain (A/Udorn/307/72 (H3N2)), as was evident by the dose dependant reduction in CPE of MDCK cells and HA titer. The 50% cytotoxic concentration (CC50) of the extract on MDCK was 60 μg/ml. The antiviral activity was further validated by real time RT-PCR that showed 40% inhibition in the expression of HA gene of the virus at non toxic concentration. The results of this preliminary study clearly reflect that leaf extract of *Azadirachta indica* is able to inhibit the growth of influenza A virus. Neem leaf extract can be exploited in future for the development of an alternative and effective antiviral therapy against influenza A viruses.

Importance of Microscopic and Molecular Pathology in the Diagnosis of Influenza A (H1N1) 2009 Infection

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The rapid emergence of the novel influenza virus designated pandemic H1N1 2009 caused one of the fastest pandemics of the twentieth century. In India this pandemic peaked between Augusts to October 2009. The fast development of an accurate detection test for this novel virus using real time reverse transcription polymerase chain reaction (rRT-PCR) helped in timely diagnosis. While for majority of the cases rRT-PCR provided reliable confirmation of the virus, it gave negative or indeterminate results in a subset of cases meeting the standard case definition for the pandemic infection and negative for seasonal flu. The rRT-PCR for pH1N1 2009 was the main diagnostic test used on clinical specimens. Microscopic pathology of the lung tissues was done for four fatal H1N1 suspected cases with negative results of rRT-PCR test on clinical specimens. In the present study we examined 4 such fatal cases where microscopic pathology of the lung was consistent with viral bronchopneumonia for the presence of pH1N1 2009 using rRT-PCR on nucleic acid extracted from the paraffin sections that showed presence of viral antigens by immunohistochemistry. In all 4 cases pH1N1 sequences could be identified. Correct diagnosis during pandemic or outbreak situation is very essential for implementation of the appropriate therapeutic as well as preventive measures. These findings therefore emphasize the important role of microscopic pathology techniques in conjunction with molecular tools in the diagnostic confirmation of novel agents during a public health emergency.

Molecular Characterization of Influenza Type B Isolates from Pune During 2010–2012

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Influenza causes annual epidemics worldwide in humans. Influenza B virus evolution was focused on the HA gene. Phylogenetic analysis of the HA1 region suggested that during the mid 1980s HA gene had evolved into two antigenically distinct lineages represented by Yamagata/16/88 and Victoria/2/87 and has co circulated since then. It is well recognized that reassortment between co circulating influenza B viruses is likely to contribute to the evolution of influenza B virus. The study was undertaken to characterize the genetic diversity of the circulating influenza B viruses. Molecular characterization of eighty nine (2010–2012, 2011–2020, 2012–2017) Type B isolates of 2010–2012 periods were studied for. HA1 and partial NA gene sequencing was carried out using gene specific primers. Phylogenetic trees were constructed with Kimura 2-parameter model and neighbor joining algorithm available in MEGA5 software. Phylogenetic analysis of HA gene showed that Victoria lineage viruses were in circulation. All 89 Type B viruses from year 2010 to 2012 were clustered with B/Brisbane/60/2008 (Victoria lineage) which was vaccine strain from 2009–2012. Phylogenetic analysis of NA gene revealed that most of the viruses $n = 85$ were clustered with B/Brisbane/60/2008, while remaining four viruses (3-2010, 1-2012) were clustered with B/Wisconsin/01/2010 (Yamagata lineage) which is the vaccine component for 2012–2013. These findings were suggestive of four viruses having Victoria HA-Yamagata NA reassortant viruses in circulation along with Victoria lineage. All Type B viruses were sensitive for oseltamivir on checking amino acid substitution at E119A, R152K, D198N/E, I222T, H274Y and R371K. During the period of 2010–2012, Type B viruses of Victoria lineage were predominantly in circulation. Reassortment between HA and NA genes highlighted importance of continuous monitoring of type B viruses on genetic basis.

Molecular Surveillance of Pandemic Influenza A (H1N1) in the Post Pandemic Period

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The Influenza A pandemic H1N1 2009 virus appeared in India in May 2009 with considerable morbidity and mortality reported from some parts of the country. On 10th August 2010, WHO declared that

pandemic H1N1 2009 influenza virus moved into the post pandemic period. As part of post pandemic surveillance, National Influenza Center, NIV carried out diagnosis and genetic analysis for pandemic H1N1 virus. 15085 clinical samples with influenza like illness were referred during August 2010–July 2012 to NIV by Maharashtra state health authorities for Influenza diagnosis. Real time RT-PCR was carried for detection of seasonal and pandemic influenza. Isolation of virus was attempted in MDCK cell line. Molecular characterizations of isolates were carried out by sequencing of Hemagglutinin (HA) and Neuraminidase (NA) gene followed by phylogenetic analyses. Out of 15085, 3481 samples were positive for influenza which included 2835 pandemic A/H1N1 and 646 seasonal influenza [355 A (H3N2) and 291 Type B]. 227 pandemic H1N1 positive samples were processed for virus isolation and 100 yielded isolates. Further, 50 representative isolates were characterized. Phylogenetic analysis of HA gene showed that all 50 Pandemic isolates were clustered with A/California/07/2009 which is 2012–2013 vaccine component. Forty nine isolates were of clade 7 and showed S203T clade specific mutation in HA gene. A single isolate of 2010 was clustered with clade 6 with mutation Q293H. Potential changes in the antigenic site were also observed. All Pune isolates possessed D222 along with R223Q in the receptor binding region of the HA1 molecule. Further analysis of NA gene showed that all isolates were sensitive to oseltamivir and had H274 in NA gene. Pandemic H1N1 viruses continued to appear till 2012 along with seasonal influenza. Though potential changes in antigenic sites of HA1 of pandemic H1N1 were observed, all pandemic H1N1 viruses were similar to vaccine component A/California/07/2009. Continued molecular surveillance is important to understand significant evolutionary changes in the pandemic virus.

Immunization with Plasmid DNA Encoding Matrix Epitope Protects Mice Against Influenza A Virus Challenge

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DNA vaccination represents a unique strategy to overcome the limitations of immunization with conventional vaccines which is restricted by the high variability of influenza viruses. We evaluated the protective efficacy of a plasmid DNA, encoding an evolutionarily conserved epitope of viral matrix protein, against the influenza A virus infection. It was found that the mice immunized via the intramuscular route elicited immune response to the peptide encoded by the plasmid DNA, with enhanced level of Th1 cytokines viz. IL-12 and IFNγ production in the stimulated splenocyte supernatant. The T lymphocytes in the spleen of immunized mice significantly lysed the virus infected MDCK cells. A significant decrease in virus replication was also observed in the lungs of immunized mice and 63% of the mice were protected against the lethal challenge of influenza A viruses. These findings suggest that the plasmid DNA expressing a single matrix epitope may serve as a promising vaccine candidate to provide effective immunity in susceptible population.

Inactivation of Avian Influenza (AI) H9N2 Virus Isolated from India for Its Potential Use as a Candidate Vaccine

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Prevalence of avian influenza (AI) H9N2 viruses has been reported in poultry from India. Human infections have been reported from Hong Kong, China, Bangladesh and seroprevalence of AI H9N2 has been shown in humans in India. No commercial H9N2 vaccine is available for animal or human use and various approaches of development of candidate vaccines are in progress. The present study was undertaken to standardize virus inactivation protocols using beta-propiolactone (BPL), ether and heat. Two isolates of H9N2 virus isolated from India were used in the study. The viruses were grown in embryonated chicken eggs and titer was determined by hemagglutination (HA) assay. Virus inactivation was confirmed by in vitro and in-ovo passages of inactivated virus. The immunogenicity of the BPL inactivated virus along with an adjuvant was tested in BALB/c mice. Antibody titers were determined by hemagglutination inhibition (HI) assay. Immunized mice were challenged with live H9N2 virus. Mice were observed for morbidity and mortality. AI H9N2 virus was inactivated by 0.1% BPL, undiluted ether and heat. HA activity after BPL and ether treatment was retained. Virus inactivated by heat only at 55 °C retained HA activity. Two doses of inactivated H9N2 virus with adjuvant induced high titer HI antibodies. Live virus could only be recovered from lungs of unvaccinated mice indicating that vaccinated mice were protected from infection. BPL is an effective inactivating agent as it retained the antigenicity and immunogenicity of the virus after treatment. In the current scenario of emerging influenza viruses, this study provides influenza virus inactivation protocols which can be used for various purposes including preparation of vaccines. Such preliminary studies are an important step towards pandemic preparedness for influenza.

Suitability of Sample Types for Isolation of Avian Influenza Viruses During Surveillance in Poultry

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India experienced the first outbreak of highly pathogenic avian influenza (HPAI) H5N1 virus in poultry in January 2006 in parts of the western states Maharashtra and Gujarat and a central state Madhya Pradesh; since then more than 75 outbreaks have been reported from India. Low pathogenic avian influenza (LPAI) viruses have also been found circulating in India. In view of the outbreaks of HPAI H5N1 virus, its impact on global public health and growing concerns of H9N2 virus infections, AI surveillance in wet poultry markets was conducted in the states of Maharashtra, West Bengal and Jharkhand in India during the period 2009–2012. To analyze suitability of various types of poultry samples for AI virus isolation during AI surveillance in poultry. A total of 2405 samples were collected, which included 1297 tracheal swabs, 1012 cloacal swabs, 79 poultry drinking water samples, and 17 fecal droppings. All samples were processed for virus isolation in 10-day-old embryonated chicken eggs. Hemagglutination (HA) positive samples were identified by hemagglutination inhibition (HI) assay using panel of OIE reference antiserum (H1 to H16). Neuraminidase (NA) subtyping was done by partial sequencing of NA gene. Samples were analyzed for their suitability for AI virus isolation. Overall virus isolation rate was 8.7%, out of which 94.8% were H9N2 viruses. Other viruses isolated were LPAI H4N6 and HPAI H5N1. Virus isolation rate was significantly higher in poultry drinking water sample (22.8%) and tracheal swab samples (12.3%) in comparison with cloacal swabs (2.9%). Number of fecal dropping samples was not adequate for comparative analysis. Poultry drinking water and tracheal swab samples were found suitable over other types of samples for isolation of AI viruses during AI surveillance in poultry.

Development and Characterization of MAbs Against an Indian Isolate of H5N1 AIV

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The highly pathogenic avian influenza (HPAI) H5N1 virus has been causing world-wide outbreaks of disease in avian species and humans since 1997. India is experiencing regular intermittent outbreaks of H5N1 HPAIV in poultry since 2006. An early and prompt diagnosis at field level is essential for initiating control measures. Development of monoclonal antibodies (MAbs) against the AIV H5N1 is an important step towards development of pen-side diagnostic tests. In this study, MAbs were produced using the betapropiolactone inactivated viral antigen of A/chicken/Navapur/Nandurbar/India/7972/2006 (H5N1) AIV and their preliminary characterization was carried out. Fifteen MAbs produced a reactive line at ~70 kDA in western blot with the virus suggesting that it may be against HA protein. Four of these MAbs exhibited Hemagglutinating activity with AIV H5N1. None of these MAbs reacted with H9N2 AIV or NDV indicating its specificity. Two MAbs produced a reactive line at 27 kDa in western blot with the virus suggesting that it may be against M1 protein. The MAbs also reacted with AIV H9N2 and rM1 protein but not with NDV indicating its type-specificity.

Molecular Typing of the Structural Genes of Bovine and Human Group A Rotavirus

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Rotaviruses (RVs) are the leading cause of gastroenteritis, affecting both humans and animals throughout the world. In India, RVs are associated with heavy mortality in bovine calves up to 3 months of age and children up to 5 years of age. The present study describes the distribution of RVs in the bovine and human population. A total of 201 diarrhoeic samples (150 from bovine calves from Uttarakhand and 51 from human infants; 23 from Nagaland and 28 from Uttarakhand) were screened by RNA-PAGE and ELISA with detection of 34 (16.9%) and 42 (20.8%) RVA, respectively. In VP6 gene based RT-PCR assay, 39 (19.4%) samples were found positive for RVA. All positive samples were genotyped by multiplex RT-PCR assay and 24 samples gave desired amplification product of 208 bp fragments in RT-PCR of partial length VP7 gene. For genotyping of VP7 gene, full length VP7 primers and VP7 genotype specific primers were used. The expected amplicon of 1062 bp was obtained in first round. The G3 (23.5%) and G10 (23.5%) were found to be most predominant genotypes in bovine and G1 (28.57%) in human samples. Only one G1 (14.28%) type was detected in the human samples, while G6 was not detected in both bovine and human samples, but was detected as mixed G (41.17%) types in bovine samples and the rest of the samples were non typeable. In RT-PCR of partial length VP4 gene, only 14 samples gave desired amplification product of 863 bp in case of bovine samples and 877 bp in case of human samples. Genotyping of VP4 gene was done using common forward VP4 primers and VP4 genotype specific primers corresponding to bovine and human RVs. Among the P types, P[11] (31.5%) was the most predominant in case of bovine samples and P[8] (75.0%) in case of human samples, followed by P[1] (31.25%) and P[4] (25.0%) in bovine and human samples respectively. Six (37.5%) samples showed mixed P[1]P[11] type in bovine samples, while no samples showed mixed P type in humans and the rest of the samples were untypeable. The most predominant G and P type combination was G3P[11] (25.8%) followed by G10P[11] (22.58%), G10P[1] (22.58%) and G6P[11] (9.67%) in the case of bovine samples, while G3P[8] (37.5%) was found to be the most predominant in case of human samples followed by G1P[8] (25.0%) and G1P [4](12.5%).

Genetic Reassortment Based on Full Length Sequencing of Genome Segments 2&6 of Indian Isolates of BTV 16 and 21

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One of the most important vector borne diseases of ruminants worldwide is caused by bluetongue virus (BTV), an *orbivirus* of the *Reoviridae* family. The virus genome consists of 10 segments of dsRNA which code for 7 structural and 4 non-structural proteins. The outer capsid proteins VP2 (encoded by genome seg-2) and VP5 (encoded by genome seg-6) are the most important proteins responsible for serotype specificity, virus neutralization and haemagglutination. VP5 protein also enhances the protective neutralizing activity of VP2 protein inducing higher serotype specific antibody titre than the VP2 alone. The nucleotide sequence and the phylogenetic analysis of these genes can provide a rapid alternative approach for characterization of circulating strains, and for future molecular epidemiological investigations around the world. Out of the twenty-six BTV serotypes found worldwide, 22 were reported from different states of India. These include serotype 21 which was recently isolated from Andhra Pradesh, and was involved in severe outbreak of bluetongue in Indian native sheep. BTV21 (KMNO-7) and BTV16 were circulating at the same time. This co-circulation, along with the fact that the virus genome is segmented, provides an opportunity for these two isolates of different serotypes to simultaneously infect the same animal, and even the same cell or a same vector with the potential for generation of reassortant viruses. Present study was undertaken to conduct full length nucleotide sequencing of genome seg-2 and seg-6 of two Indian isolates VJW-64 (BTV16) and KMNO-7 (BTV21) which would lead to efficient characterization of the virus, eventually helping in selection of predominant candidate vaccine strains. For the purpose of sequencing, genome seg-2 and seg-6 were divided into several overlapping gene fragments and primer pairs specific to every such fragment were designed. Consensus full length gene sequences thus generated were submitted to GenBank. Nucleotide sequence homology analysis of genome seg-2 and seg-6 of these two Indian isolates revealed an inter-serotypic variation of 29% to 60.1% on the basis of vp2 gene. VJW-64 (BTV16) was found to show a maximum similarity with South African reference strain of BTV3 while Indian isolate KMNO-7 (BTV21) showed a maximum similarity of 68.7% with South African reference strain of BTV14 on the basis of seg-2. On the basis of genome seg-6, VJW-64 (BTV16) was found to show maximum similarity with South African reference strain of BTV21 while Indian isolate KMNO-7 (BTV21) showed maximum similarity with Turkey isolate of BTV16. Intra-serotypic nucleotide sequence homology analysis of genome seg-2 revealed close relationship of Indian isolate VJW-64 (BTV16) with Japan isolates. Indian isolate VJW-64 (BTV16) thus clusters with Japan isolates MZ-1, KSB-31, NS-1, KSB-6 and KSB-7 along with the Greece isolate reflecting there common eastern origin. Detailed phylogenetic analysis revealed thats eg-2 of KMNO-7 isolate of BTV21 shows maximum similarity of 92.3% with japan isolate ON89-1 of BTV21, and thus clusters with BTV21 isolates. However, genome

seg-6 of this isolate clusters with isolates of BTV16 showing maximum nucleotide similarity of 97.6% with TUR/2000/02 isolate of BTV16, which is much more than it shows with any isolate of BTV21. KMNO-7 (BTV21) significantly diverged from original strain of BTV21, and is a reassortant strain having acquired seg-6 from an isolate of BTV16. It was probably because of this reassortment that BTV21 was involved in a severe outbreak of bluetongue in Indian native sheep. In-silico restriction enzyme analysis revealed certain restriction sites which were specific to the Indian isolates of BTV16 (VJW-64) and BTV21 (KMNO-7). In this study full length sequencing of genome seg-2 and seg-6 of Indian isolate VJW-64 (BTV16) was carried out for the first time in India and that of KMNO-7 isolate (BTV21) for the 2nd time in the world. This study in itself is an important initiative towards developing a database of full length genome seg-2 and seg-6 nucleotide sequences of Indian isolates. It also provides some useful insights into the epidemiology of the bluetongue disease in India and undermines serotyping on genome seg-6 basis.

Purification of Infective Bluetongue Virus by Immunoaffinity Chromatography

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An immunoaffinity chromatography for purification of biologically active (infective) bluetongue virus (BTV) has been optimized using polyclonal antibody to core particle of BTV. Anti-core antibody was produced in guinea pig which was further purified by ammonium sulfate precipitation. Immunoaffinity columns were prepared by non-covalent binding of anti-core antibody to cyanogen bromide-activated protein-A Sepharose beads. BTV-infected cell culture supernatant was added to this column, virus was captured by specific antibody, column was washed with buffer and then virus was eluted with a buffer consisting of 4 M $MgCl_2$ and 75 mM HEPES, pH 6.5. The infectivity of eluted BTV was tested on the cell culture. BTV purified by this method retained the antigenicity and infectivity as determined by sandwich ELISA and infectivity assay on BHK-21 cell respectively. There are many reports of occurrence of mixed infection of BTV with other viruses like peste des petitsruminants virus, capripox viruses, orf virus etc. Most of the times, conventional procedure of BTV isolation directly on cell culture from mixedly infected tissue or blood samples leads to isolation of non-BTV. This method will help in selective capture and enrichment of BTV from mixed population of viruses for efficient isolation on cell culture. Further, this method can be used for small scale purification of BTV avoiding ultracentrifugation.

Sequencing of Outer Capsid Protein Gene and Cross-Neutralization of Indian BTV-1 Isolates Indicates Presence of Two Distinct VP2 Phenotypic Variants

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India is enzootic for bluetongue and 21 different serotypes of Bluetongue viruses (BTV) have been reported based on virus isolation and seroprevalence. Bluetongue virus serotype-1 (BTV-1) is most prevalent in the north-western and southern states of India and a good number of viruses have been isolated from different hosts for the past more than 25 years. A study was conducted to understand the antigenic and genetic variations amongst these isolates by VP2 gene sequencing and cross-neutralization. Full length VP2 genes of 20 BTV-1 isolates were sequenced and sequence analyses revealed presence of two distinct geographical clusters of viruses confined to north-western and southern regions of India. Phylogenetically all Indian BTV-1 isolates are very closely related to the Australian isolates and therefore the viruses are 'eastern topotype' of BTV. The viruses of both north-western and southern cluster showed more than 97% sequence identity at nucleotide and amino acid level. In spite of close identity, cross-neutralization studies amongst the Indian BTV-1 isolates revealed co-existence of distinct neutralization variants. One neutralization resistant variant was found amongst the southern cluster of viruses in geographically restricted area of Andhra Pradesh. Antigenic variation was also observed amongst the north-western isolates but no distinct neutralization resistant variant was found. Cross neutralization data suggest that there are at least two distinct VP2 phenotypic variants existing in India and therefore selection of suitable vaccine candidate for BTV-1 should be done very critically to ensure maximum protection against the all the existing neutralization variants.

Investigation on Bluetongue Disease in Small and Large Ruminants of Maharashtra

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Bluetongue (BT) is an economically important viral disease of ruminants in India. Mainly sheep in southern states suffer with heavy mortality due to BT disease than in northern states of the country. According to 18th Livestock census-2007 by Government of India, the country has total livestock population of 529.7 million. Maharashtra state has recorded 2.2% decline in the total livestock population. Regular investigation of the livestock population of the state is required to implement appropriate control and prevention strategies against the disease. Hence, in the present investigation, a total of 378 samples (373 serum samples and 5 samples for BT virus isolation) from small and large ruminants of Maharashtra were collected. Serum samples were collected from Ahmadnagar, Akola, Aurangabad, Jalgaon, Mumbai, Nanded, Pune, Satara and Thane districts. These 373 serum samples consisted of samples from sheep (65), goats (58), cattle (123) and buffaloes (127). Enzyme linked immunosorbent assays (ELISA) kit was procured from Indian Veterinary Research Institute (IVRI) to screen serum samples for BT virus antibodies. Out of 373 serum samples, 47 serum samples have been screened by IVRI ELISA kit. Though, virus isolation was unsuccessful, serum samples of 8 sheep and 18 cattle tested positive to BT virus antibodies. The results indicate prevalence of antibodies to bluetongue virus in small and large ruminants of the Maharashtra state. Presently, vaccination is not being followed in the country, but in near future, as and when vaccine is commercially available, susceptible population of livestock may be immunized against the disease. Further investigations on involvement of culicoides vector in the transmission of the disease at regional level in the state are warranted.

Laboratory Quality Management System as per ISO 17025:2005 in Animal Disease Diagnostic and Research Laboratories in India

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Accurate and reliable laboratory testing is essential to all aspects of health care of human beings as well as animals. The Diagnostic Laboratories that have traditionally directed their efforts toward meeting the needs of Clinician must now also satisfy the needs of society, the greater public health, and the administrators. With increased awareness and as part of global trade requirements the biological laboratories are required to be accreditated as per international standard ISO 17025:2005 by the National Accreditation Board for Testing and Calibration Laboratories (NABL) in India. Central Military veterinary laboratory (CMVL) of Indian Army still remains the only laboratory to achieve this rare feat since the year 2009 when accreditation was awarded for the first time to any animal disease diagnostic laboratory in India. Majority of the animal disease diagnostic laboratories in India are working under the Government or Government aided self financed institutions and performing the dual task of diagnostics and research on animal pathogens. While the consensus is growing by every day to establish international standard in the diagnostic task in the Government owned laboratories, however it's still a matter of debate to bring research work under this ambit due to obvious reasons. Even as in many developed countries worldwide quality systems have been adopted successfully in many key sectors including the research laboratories, research institutes in India are now showing an interest in setting up a system. Establishing the laboratory quality management system as per international standard ISO 17025:2005 is certainly an opportunity for them not only to improve their performance but also to reach a quality label. The success of CMVL in this regard can be taken as role model which is actively engaged in meeting diagnostic as well as research obligations to meet growing needs of Army apart from collaborating research with leading centers' of excellence in the country. The experiences of CMVL are discussed in detail in this paper to show that research institutes can immensely benefit from an international quality standard adjusted and adapted to their peculiar role and needs.

Molecular Characterization of Avian Reovirus

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In recent times Avian Reovirus (ARV) is associated with poor growth, increased feed conversion ratio and mortality in broilers. The present study was undertaken to characterize nine field isolates suspected for proventriculitis/tenosynovitis syndrome of ARV by isolation in SPF eggs, RNA-PAGE, RT-PCR and RFLP. The virus isolates and ARV vaccine (ARVv) taken as virus control were propagated in nine day old specific pathogen free chicken embryos by chorio-allantoic membrane (CAM) route. In eight out of nine samples the chicken embryos showing haemorrhages and yellowish green foci on the liver and death within 64–98 h were considered specific for ARV. RNA was extracted from the CAM of the above isolates by TRIZOL method and subjected to RNA-PAGE after heat treatment at 60 °C in water bath for before loading in the wells. Out of nine isolates, 8 isolates and ARVv control showed profile of 10 segments consisting of large (L1, L2, L3), medium (M1, M2, M3) and small (S1, S2, S3, S4). The S1 and S3 genes of ARV isolates were amplified using gene specific Reo1F, Reo2R and Reo3F, Reo4R primers for S1 gene and P1F, P2R, P3R primers for S3 gene. PCR product sizes of 980 bp, 810 bp and 672 bp, 548 bp respectively were obtained that was specific for S1 and S3 genes of ARV in six of the eight isolates. On RE digestion of the 548 bp product of the S3 gene with enzyme Rsa I, 3 fragments of $\sim$180, $\sim$250 and $\sim$400 bp were observed in 3 of 6 samples and ARVv whereas product digests of $\sim$180 to $\sim$400 bp were obtained in other 3 samples. Based on the variations of the RE digest profiles the latter 3 isolates were designated as field isolates when compared with that of ARVv. To conclude, RNA-PAGE, RT-PCR and RFLP can be employed for detection of ARV and depending upon the similarities and dissimilarities in their RFLP profile the isolates can be identified as either vaccine or field strains of ARV.

Sequence Analysis of Serine Protease Inhibitor 1 (SPI-1) Gene of Buffalopox Virus Isolated from Buffaloes, Cattle and Humans

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Buffalopox is an emerging contagious viral zoonosis of domestic buffaloes (*Bubalus bubalis*) which also infects cattle and humans. The disease is caused by buffalopox virus (BPXV)—a close variant of vaccinia virus (VACV) being recognised as an occupational zoonosis due to the naïve population against orthopoxviruses. Lack of information on host tropism of BPXV, led us to analyse the host range serpin 1 (SPI-1) gene of BPXVs isolated from outbreaks (2010 & 2011) in buffaloes, cattle and human in Maharashtra and Uttar Pradesh. The encoded protein of this gene is expressed in the early stages of infection and acts as anti-apoptosis factor in vaccinia virus. The virus was isolated in Vero cells from infected scabs collected from animals and humans and the extracted viral DNA was subjected to PCR amplification of serpin 1 gene. The amplicons were cloned in pTZ57R/T vector and sequenced commercially. An open reading frame (ORF) nucleotide sequence homology search was carried out using the NCBI BLAST. The nucleotide (nt) and deduced amino acid (aa) sequences were aligned using the CLUSTAL W program and phylogenetic trees were constructed using neighbour-joining method of MEGA5 software. Comparative sequence analysis was done to elucidate variations among BPXV isolates from buffaloes, cattle and human as well as to determine the evolutionary relationship among OPXVs. Sequence analysis revealed that BPXV isolates (buffalo, cattle and human) shared maximum homology (99% at nt and 98.5% at aa level) among themselves as well as with VACV. Furthermore, phylogenetic analysis exhibited closest homology with VACV isolates followed by RPXV, CPXV, HSPV, Cantagalo virus, MPXV and VARV. The high degree of sequence similarity and close evolutionary relationship with VACV and other poxviruses indicates the conserved nature of the gene among Orthopoxviruses which could play a role similar to VACV in viral pathogenesis. This is the first report of genetic analysis of anti-apoptosis gene of buffalopox virus which will be useful in elucidating the host antiviral response.

Sequence Analysis, Expression, Immunogenicity and Functional Activities of Recombinant Major Envelope Protein (B2L) of Orf Virus

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Orf virus (ORFV) infection can give rise to contagious ecthyma in goats, sheep, other ruminants and occasionally humans. ORFV is classified as a member of the genus Parapoxvirus and has a linear double-stranded DNA genome, and known to posses *B2L* gene (ORF011) encoding for major envelope protein ($\sim$42 kDa) which has immune-modulating property. Orf infections are endemic in India, however, limited attempts were made in the past to characterize the ORFV strains as well as its proteins to develop molecular diagnostics/prophylactics to control the disease. The present study was aimed to molecular, serological and biochemical characterization of recombinant B2L protein of ORFV. The full length *B2L* gene ($\sim$1137 bp) of ORFV isolates ($n = 13$) from natural outbreaks in sheep and goats belonging to different geographical regions of India during 2003–2011 were amplified, cloned, sequenced and analyzed with bioinformatics tools. Further, *B2L* gene of ORFV-Muk/05 was amplified, cloned into prokaryotic expression vector (pET32a) and over-expressed in recombinant *E. coli.* The recombinant B2L fusion protein ($\sim$60 kDa) was purified under native condition using affinity chromatography and confirmed by western blotting, peptide finger printing and MALDI-TOF–MS. Functional activities of recombinant B2L was carried out by titration method. The immunogenicity of recombinant B2L protein was assessed in rabbits and guinea pig using Western blot and ELISA. Multiple alignment of amino acid (aa) sequences of B2L showed many substitutions including unique to isolates, dispersed all along the length of the protein. Sequence analysis of the B2L showed the identity among ORFV isolates irrespective species and geographical region; the identity was >97% at nucleotide (nt) and aa level. Sequence analysis has indicated that the presence of HKD-motif at aa position 318–342 of C-terminal of B2L protein indicating it is a member of the phospholipase D (PLD) gene superfamily. Further, phospholipase activity of recombinant protein was confirmed by titration method. Western blot and ELISA assays using sera against the recombinant B2L protein indicated that the rabbits and guinea pig were sero-converted. Hyper-immune serum specific to different related viruses were tested in B2L protein based indirect ELISA and reactivity was specific for only ORFVs. The study reflected *B2L* gene contains epidemiological relevant information in providing an evidence for the close relationship as well as genetic variation among the circulating and movement of ORFV strains. The study also revealed recombinant B2L protein posses phospholipase activity and it is a potential candidate in developing ORFV subunit vaccine, molecular adjuvant and ELISA for sero-surveillance of orf infection in sheep and goats.

Sero-Epidemiological Study of Peste-Des-Petits Ruminants in India

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Peste des petits ruminants (PPR) is an acute, highly contagious, world organization for animal health notifiable and economically important transboundary vdisease of sheep and goats associated with high morbidity and mortality. PPR is enzootic in India and outbreaks are regularly reported throughout the year in most parts of the country. This study describes seroprevalence of PPR in Cattle, Buffalo, Sheep and Goats carried out during the period 2009–2011 using the stratified random samples collected from different parts of Andhra Pradesh, Rajasthan, Maharashtra, Manipur, Jammu and Kashmir and Gujarat states of India. A total of 2100 serum samples [cattle = 1,167, buffaloes = 267, sheep = 176 and goat = 490] were collected by collaborating units of AICRP on PD_ADMAS and submitted to PD_ADMAS for seroepidemiologic studies. These samples were screened for PPRV antibodies by using a PPR monoclonal antibody-based competitive ELISA kit. The report presents the results of PPR virus (PPRV)-specific antibodies in situations where either the subclinical or inapparent or non-lethal infection was there in these livestock species. On analysis out of 2100, 326 serum samples were found positive for PPRV antibodies, which indicates an overall of 15.52% prevalence of PPRV antibody in livestock species with 7.5% in cattle, 23% in buffalo, 46.5% in sheep and 19.3% in goats. The presence of PPRV-specific antibodies demonstrates that animals are exposed to PPR infection naturally, and the transmission mode may be direct or indirect. In general, the percent positivity of the antibodies in sheep and goats indicates enzooticity of the disease in the country, which is attributed to variations in the sheep and goat husbandry practices within different geographical regions, the agro-climatic conditions, the topography of different states, the socio-economic status of individual farmers and the migration of livestock in India. Further, it implies the importance of cattle and buffaloes as subclinical hosts for the virus besides widespread presence of the disease in sheep and goats in India.

Generation of Smooth Virus Like Particles of Infectious Bronchitis by Co-Expression of Membrane and Envelope Proteins in Yeast

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Infectious bronchitis virus (IBV) a threat to the domestic chicken causes acute and highly contagious respiratory disease. IBV is a member of the genus Coronavirus. The genome which is 27.6 kb genome in size is non-segmented, positive sense, single stranded RNA. All coronaviruses maintain a set of essential genes, including those that encode the polymerase (Pol), spike (S), small membrane (E), membrane (M), and nucleocapsid (N) proteins, in the order 5'-Pol-S-E-M-N-3'. The M glycoprotein which is partially exposed at the surface of the virion is a major Type II integral membrane protein and is essential for the production of coronavirus-like particles. Coronavirus have a minor type III envelope (E) protein. Coronavirus M proteins play key roles in virus assembly, through M-M, M-E and M-spike (S) protein interactions. In this study, the M glycoprotein gene (678 bp) and E protein genes (330 bp) were cloned in a yeast expression vector. The recombinant clones were confirmed by restriction enzyme analysis and PCR amplification. Both the genes were co-expressed in *Saccharomyces cerevisiae* resulting in generation of VLPs. The recombinant proteins were confirmed by Western blot analysis and revealed a 14 kDa and 36 kDa band. The virus like particles (VLPs) was purified by ultracentrifugation at 35000 rpm in 15% sucrose cushion gradient. Upon transmission electron microscopy, the VLPs of 100 nm size were visualized. The VLPs developed in this study are smooth in nature as they lack the spike glycoprotein. Smooth VLPs can further be engineered to express spike gene of Infectious Bronchitis virus.

Genetic Analysis of Cytokine Response Modifier B (crmB) Gene of Camelpox Virus (CMLV)

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The camelpox virus (CMLV) causes pox disease mainly in camels and the virus belong to the family Poxviridae, subfamily Chordopoxvirinae, under the genus Orthopoxvirus. Camels, zoonotic CMLV infection has also been reported in the country. This signifies the study of the virus encoded host immune system modifier genes. We report the sequence analysis of crmB gene of CMLVs isolated from outbreaks (2008–2009) in camels in Bikaner, Barmer and Jaisalmer districts of Rajasthan and Delhi. This gene encodes a tumor necrosis factor (TNF) receptor homologue of host cell and plays an important role in inhibiting the TNF-induced apoptosis of the infected cell. Camelpox viruses were isolated in Vero cells from the scab samples collected from outbreaks of CMLV in Delhi and Rajasthan during 2008–2009 in which the Barmer outbreak was associated with human infection. The crmB gene was amplified from genomic DNA purified from the isolated viruses, the amplicons were cloned into pTZ57R/T vector and sequenced. ORFs (1050 bp) were aligned with various Orthopoxvirus sequences available in the database using NCBI BLAST. Current CMLV isolates shared almost 100% sequence identity among themselves as well as with other CMLVs at both nt&aa level and showed maximum homology (95.90% and 92.7% at nt&aa level, respectively) with variola viruses. Furthermore, the phylogenetic analysis based on the nt sequences showed that Indian isolates clustered together and grouped closely with variola virus (VARV). The close similarity of crmB gene sequences of CMLV isolates with VARV implies the similar role in viral pathogenesis. The information on virus encoded host immune system modifier genes will help in studying the molecular mechanisms in adaptation of virus in unnatural hosts.

Development of Recombinant UL-30 Protein of Duck Enteritis Virus

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Duck viral enteritis, which is caused by duck enteritis virus (DEV), is an acute, contagious and lethal disease. DEV is currently classified to belong to the alphaherpesvirinae subfamily of the herpesviridae family. The DNA polymerase, a product of UL30, is of central importance for successful viral replication in alpha herpes virinae group of viruses. The viral enzyme is involved in the initiating events of gene amplification processes. The UL30 protein is highly conserved among herpesviruses at the amino acid sequence level. This is one of the important protein for the replication of virus in the host cell. The DNA polymerase of herpes virus shows a significant similarity with that of eukaryotes. The DNA sequences encoding UL30 was identified from the genomic DNA of DEV. The amplification of the partial gene was carried out, using self designed primers in a polymerase chain reaction. A 1596 bp partial gene product was cloned into pTZ57R/T cloning vector and the UL30 gene was sequenced. The recombinant UL30 protein was expressed by subcloning the UL30 gene from a pET bacterial expression system after induction with 1 mM IPTG. The expressed recombinant protein obtained as a fusion protein with histidine tag was purified by affinity chromatography. The purified recombinant protein was confirmed by SDS-PAGE and western blot analysis using anti DEV sera which detected an 80 kDa protein band in the blot. The recombinant protein was further dialysed and concentrated using Amicon filters and the total yield of the protein was found to be 120 μg/ml.

Rapid Spread of Capripox in Himachal Pradesh: A Hallmark of Emerging & Re-Emerging Infections

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Sheep pox and goat pox are List A diseases according to the Office International des Epizooties meaning that these have potential for rapid spread with serious consequences. These facts have been very well echoed in recent outbreaks of sheep and goat pox in Himachal Pradesh (H.P.). As per the epidemiological data of the Department of Animal Husbandry, Government of Himachal Pradesh, no pox outbreaks were recorded in last decade until in year 2010 when three confirmed capripox disease outbreaks were described. These confirmed capripox outbreaks were in the districts Kangra & Chamba of Himachal Pradesh which share geographical boundaries & migratory tracts with each other and also with adjoining state of Jammu & Kashmir. Ever since the occurrence of these three outbreaks, it has been observed that the capripox virus has been infecting sheep and goat flocks with greater frequency. It has not just remained bound to previously affected areas of Kangra and Chamba but has spread its boundaries to other regions of the state. In year 2011, sheep pox occurred in a mixed herd of unvaccinated sheep & goats and affected only sheep. These animals have migrated to district Kullu from the district of Lahaul & Spiti where disease resembling to that of sheep pox was reported in the previous winter. In the beginning, veterinarians with H.P. state animal husbandry department has undertaken selective vaccination of affected flocks, however, not all shepherds opted for it because of fragmentary reports of lameness like symptoms in animals vaccinated against the disease. Thereafter in year 2011 and 2012; several sheep and goat pox outbreaks have been confirmed using a combination of diagnostic tools (clinical, autopsy and molecular) in districts of Mandi, Kangra, Shimla, Kinnaur, Kullu and Lahaul&Spiti. The rapid increase in temporal and spatial distribution of pox outbreaks points to the fact that disease is emerging or remerging in the state. Since capripox virus is capable of persisting in the environment for long and able to spread fast, and also the fact that the diseases are highly contagious in nature it has become mandatory to take up blanket vaccination programme to induce herd immunity so that losses are prevented.

Molecular Characterization of Canine Parvovirus Isolated from an Organised Breeding Kennel

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Canine Parvo Virus type 2 (CPV-2) is responsible for acute gastroenteritis in pups, with a high rate of morality. CPV is prone to genetic evolution and over the three decades it has undergone several mutations there by resulting in emergence of different strains like CPV-type 2a, 2b, New-2a, New-2b and 2c. In March 2012, an outbreak of acute gastroenteritis occurred in pups of an organised canine breeding kennel located at Meerut, India. The breeding kennel has been stringently following the recommended vaccination schedule against CPV both in pups and in adult canines. During Mar–May 2012, diagnosis of CPV infection was first confirmed by commercial immunochromatographic (IC) test-kit and later confirmed by polymerase chain reaction (PCR). In the present study, isolates were genetically characterized by partial amplification of 611 bp of the gene encoding for capsid protein VP2. These sequences were then aligned and compared with sequences of 03 commercial vaccine stains and with reference CPV strains. Total of 21 faecal samples of pups suspected of CPV were collected during the current outbreak of which, 17 samples were found positive by IC test-kit and 19 samples were found positive by PCR. All the PCR positive samples were subjected for virus isolation in Madin-Darby canine kidney (MDCK) cell line from which 12 isolates were obtained. On the basis of the sequence alignment, these isolates were characterized as New CPV-2a. In all the isolates, at 297 position of VP2 gene, Alanine (Ala) was present instead of Serine (Ser-Ala), while, at 496 position' Asparagine (Asn) was present. At 325 position of VP2 gene, all the isolates showed a point mutation i.e. presence of Isoleucine (Ile) instead of Tyrosine (Tyr). CPV strains present in three different commercial vaccines were also analysed and were characterized as CPV type 2. On the basis of geographic pattern, it has been observed that CPV-type 2b variants are predominant in Northern India while CPV-type 2a variants are prevalent in Southern and Central India. This is the first report establishing the prevalence of New CPV-2a from the Northern India. Further, it is inferred that CPV vaccine strains of most of the commercial vaccines are different from the circulating CPV outbreak strains. This difference raises the concern over the CPV strains contained in most of the commercially available products in India.

Assessment of Novel N-Gene Target for Detection of Peste-Des-Petits Ruminants Virus by RT-PCR

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Peste des petits ruminants is an acute, febrile and infectious viral disease of goats and sheep, characterized by mucopurulent nasal and occular discharges, necrotising and erosive stomatitis, pneumonia and inflammation of gastro-intestinal tract leading to severe diarrhea. The disease is caused by a *Morbillivirus* of the family *Paramyxoviridae*. The present study was aimed to study the incidence of Peste des petitsruminants virus (PPRV) in goats of selected areas of Gujarat by sandwich ELISA and to derive estimates of overall, locationwise, agewise and sexwise incidence and samplewise positivity rates. In addition, the study also involved standardization and application of novel Nucleocapsid (N) gene based RT-PCR, for diagnosis of PPR. A total of 46 clinical samples from 31 goats suspected of PPRV from selected areas of Gujarat were tested by sandwich-ELISA, of which 23 animals were found positive yielding an overall incidence rate of 74.19%. All the 46 clinical samples, a reference vaccine virus (Sungri isolate) and a blood sample from apparently healthy goat were processed for RNA extraction using TRI Reagent®. PCR amplification was done using in silico designed N-gene specific primer pair N1–N2. Reference vaccine virus as well as fifteen (32.61%) of the 46 clinical samples, including fourteen blood samples and one nasal swab, produced the desired amplion of approximately 463 bp with primer pair N1–N2.

Factors Affecting Quantification of Immunogens in 146 s Estimation Test of FMD Vaccine

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Immunogenicity of FMD vaccine is primarily dependent on the presence of intact capsid of the virion. For almost all strains of FMDV the principle immunogenic component is capsular structural proteins include whole virus particles (146S particles) having a sedimentation rate of 146S, amongst them VP1 protein is the most immunogenic structural protein of FMDV. For more than two decades, FMD vaccine manufacturers use the 146S estimation test (quantitative sucrose density gradient analysis) to quantify the virus antigen concentrations and, on that basis, formulate their vaccines. Recent advancement in science has given some alternative tests like double antibody sandwich enzyme-linked immune-sorbent assay (DAS-ELISA) using monoclonal antibodies but due to technical limitations like availability of monoclonal antibodies against several intact immunogens, sensitivity and specificity of the test, 146S estimation test is still preferred to be as a gold standard test for estimation of immunogens in FMD vaccine. Therefore, FMD vaccine manufacturing establishments include the quantification of 146S antigen in the battery of quality control tests during the production of FMD vaccine. Since this test is a complex test which involves indirect measurement of immunogen, there are several possible factors associated with the test which could lead to the variations in the result. Considering the importance of the result obtained from 146S estimation test, it is very important to minimize the variations in result and could quantify the actual value of 146S immunogen to the best possible extent. Therefore, an effort was made to analyze the best suitable combination used in the test which could give the maximum value of 146S immunogen in the sample. An experiment was conducted with the objective of analyzing the effect of various factors like (a.) use of sucrose of various grades from different manufacturers (b.) use of different% of sucrose gradients in the test (c.) sample run at different RPM with different K-factor of ultracentrifugation for a constant time period (d.) sample run at constant RPM with a constant K-factor for different time periods (e.) analysis of sample with different pump speed of DGF unit (f.) suction of the sample by DGF unit through both ways from top to bottom as well as bottom to top (g.) removal of top layer from the sample for eliminating noise in the graph because of impurities (h.) use of different% of sucrose in the blank cuvette during analysis (i.) pre-treatment of the sample with dis-aggregating agent like chloroform & EDTA/sodium lauryl sulphate (SDS) (j.) check on robustness of the test by analyzing sample in different dilutions. It was observed that the several factors included in the test have a significant impact on the value of 146S immunogen of FMD vaccine. Analysis of all individual factors included in the test revealed a best suitable combination under ideal set of conditions.

Analysis of Partial E2 Gene Sequence Reveals Distribution Classical Swine Fever Virus Genotype 1.1, 2.1 and 2.2 in India

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Classical swine fever (CSF), caused by CSF virus, is a highly contagious disease of domestic pigs and wild boar having profound socio-economic impact on piggery husbandry in developing countries like India. CSF is endemic disease in our country and numbers of outbreaks occur every year in many states of the country. Among the three regions of the CSFV genome, targeted for classifying the isolates into different genotypes for deducing the molecular epidemiological information, 190 nucleotide (nt) long major immunogenic protein E2 gene sequence is routinely considered. In order to genetically assort the circulating CSFV isolates in the country, in the present study, we aligned 190 nt of E2 protein of 31 Indian isolates of CSFV belonging to the state of Assam, Andaman and Nicobar Islands, Haryana, Kerala, Meghalaya, Mizoram, UttarKhand, Uttar Pradesh, West Bengal, using ClaustalW and phylogenetic tree was constructed using *MEGA* 5. The phylogenetic grouping using neighbor joining method indicated that these Indian isolates belonged to three genotypic groups viz., genotype 1.1, 2.1 and 2.2. Each group consisted isolates from different states and the isolates from the state of Assam were distributed in all three genotype groupings. The present E2 gene based classification of Indian CSFV isolates confirmed genotypic clustering based on CSFV 5'UTR and NS5B regions in earlier studies (2009 to 2011) from India.

Expression and Purification of Polyhistidine-Tagged Truncated NSP-4 Protein of Rotavirus E2 Genotype in Prokaryotic Expression System

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Rotavirus nonstructural NSP4 protein, first viral enterotoxin known is a trans-membrane endoplasmic reticulum-specific glycoprotein and the key determinant in rotavirus pathogenesis. NSP4 having membrane-destabilizing activity causes an increase in intracellular calcium levels and chloride secretion stimulating endogenous fluid secretory pathway in intestinal cells. In the present study, the truncated region of NSP4 (258–570 nt) of bovine rotavirus E2 genotype excluding the N-terminal toxigenic domain to bacterial cells was amplified from faecal sample by the reverse transcription-polymerase chain reaction (RT-PCR), the amplicon of which was 315 bp. The pET-32a(+) vector, under the transcriptional control of the bacteriophage T7 promoter with lac operator and amplicons were doubly digested by EcoRI and XhoI and cloned into TOP10 competent *E. coli* bacterial cells. The restriction enzyme analysis and colony PCR confirmed cloned plasmid pET-32a-NSP4(T) was transformed into competent expression bacterial host cells i.e. BL21 Tuner (DE3)pLysS. Colony PCR confirmed transformed cells were induced with variables of IPTG, temperature and time combination to get the high level expressed protein. Sample buffer treated lysates of induced cells were loaded directly onto SDS-PAGE, on which approximately 29 kDa fusion protein was observed. The fusion protein was further analyzed using Western blot, which indicated that the protein was reactive with the polyclonal sera against whole rotavirus raised in rabbit. The soluble protein released after lysing the induced cells was purified by Ni–NTA affinity chromatography followed by dialysis and concentration of protein. This recombinant NSP4 protein has future application in the pathogenesis, protection studies and development of diagnostic assay for rotavirus specific antibodies.

Isolation and Identification of Newcastle Disease Virus from Peste-Des-Petits Ruminant Virus Infected Sheep

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Peste des petits ruminants (PPR) is a viral disease of goat and sheep. The disease is characterized by severe oral necrosis, enteritis and pneumonia. PPR virus (PPRV) occurs in goats and sheep but it has been described in captive wild small ruminants. Cattle and pigs appear to be the dead-end hosts, although they develop serum neutralizing antibody following experimental PPR virus infection. No carrier state or persistent infections have been described for the PPRV till date. The disease is caused by PPR virus (PPRV), is a member of family *Paramyxoviridae* and the genus *Morbillivirus*. Incidence of mixed infection of PPRV with other viruses is increasing. PPRV and BTV, PPR and orf virus and PPR and *Pestivirus* infections are now becoming increasingly common in small ruminants. In the present study, three sheep serum samples (S9, S19 and S32) which showed positive reaction for PPRV antigen by sandwich ELISA were subjected for virus isolation in Vero cell. Cytopathic effect (CPE) revealed at passage level two on 2nd days post infection (dpi). Initially rounding and ballooning of the cells (12–24 h post infection; hpi) and later syncytia formation & detachment (48–72 hpi) was noticed. The virus exhibited cytolytic nature after two blind passages. The passaged viruses were subjected for identification for PPR, but interestingly, it did not reacted with PPR and other viruses of small ruminants (viz. Pox, BTV and FMDV) by ELISA/PCR. Further, isolates were screened by microarray and found positive for new castle disease virus (NDV). After indication in microarray, the suspected viruses were further investigated for NDV by haemaglutination (HA) and haemaglutination inhibition (HI) using specific serum against NDV and RT-PCR targeting F-gene of the virus. The tests employed for detection of NDV gave positive reaction and further confirmation of its presence. Present study indicates the presence of atypical virus in sheep, a kind of mixed infection. This could be due to "species jumping" (feature of paramyxoviruses), which may be due to managemental practices (mixed species under same shed) adapted by the farmers.

A Quantitative Comparison of Rabies Virus Using Fat and MICIT Method

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Rabies is one of the most important zoonotic disease of the world which primarily depends on the vaccination to control. Despite of the long history and the remarkable progress in the knowledge, prevention and control of rabies still the disease has maintained its global distribution in animals as well as humans. In the vaccine production the rabies virus is grown on BHK-21 cells followed by further downstream process & finally blended as a vaccine. It is very essential that the growth of the virus is monitored as an in-process control. This can be done by a regular estimation of the infectivity titers. The infectivity titer of rabies can be indirectly measured by an in vivo method known as MICIT—mouse intracerebral inoculation test using weaned inbred

mice (8–10 g) where the titre of virus is expressed in MID50/ml. In this method different dilutions of the test substance is inoculated intracerebrally in groups of 10 mice each and based on the specific mortality due to disease the median lethal dose in 50% of the population (MID50) is estimated using Reed-Muench formula. MICIT test is a time consuming and cumbersome with some inherent biological variations due to certain factors like age, body weight and sex of animal etc. Rabies infectivity can also be quantified using an in vitro method in BHK-21 cells known as Fluorescent antibody test (FAT) using FITC-labelled anti-rabies immunoglobulin, following acetone fixation. The stained cells were washed in buffer and read under blue-light fluorescence microscope to detect the characteristic green fluorescence associated with rabies antigen corpuscles. As the mice test is regarded as a gold standard the present study is undertaken to establish a correlation between the two methods to ensure the reliability of using the method as an estimate and eventually to replace the use of animals in MICIT method. A total of 21 samples of viral harvest from regular production lots of rabies virus were estimated for its respective titers by both the methods. All the samples were tested for its titre as per standard method of MICIT and FAT (OIE2000). A correlation coefficient was found to be 0.667 [$r(19) = 0.667$, $p < 0.01$] which indicates a statistically significant positive correlation between the two different methods of virus titer estimation. Hence it can be proposed to use FAT method alone for the estimation of infectivity titer of virus harvest of rabies replacing MICIT method.

Expression, Immunogenicity and Protective Efficacy Studies of H3L Gene Encoding Major Envelope Protein of Buffalopox Virus, an Emerging and Re-Emerging Zoonotic Agent

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Buffalopox is a highly contagious, zoonotic viral disease affecting buffaloes, cattle and humans and is being reported time-to-time from several states of India. The etiological agent, buffalopox virus (BPXV) is a close variant of vaccinia virus (VACV), the virus used for smallpox vaccination. Considering the zoonotic importance and increased incidence and severity of buffalopox infection in humans in the Indian sub-continent combined with vulnerable population lacking antibodies to smallpox and related orthopoxviruses, studies are warranted to develop recombinant antigen-based prophylactics and diagnostic assays for epidemiological investigations and appropriate control measures. The present study carried out to over-express the major immunogenic protein of BPXV, H3L ($\sim$35 kDa) in prokaryotic system and to evaluate for its diagnostic as well as prophylactic potential. This was the first attempt in the proteomic study of BPXV. In the present study, *H3L* gene of BPXV-Vij/96 was amplified ($\sim$840 bp), cloned into a pET32a expression vector and recombinant H3L over-expressed in *E coli*. Recombinant H3L fusion protein ($\sim$50 kDa) was purified under native condition using affinity chromatography and its specific immunoreactivity was confirmed using anti-orthopoxvirus sera (BPXV and CMLV) in Western blot. Recombinant H3L forms only monomers as analysed by native and denatured PAGE followed by Western blot. Recombinant H3L protein was found to be immunogenic in adult mice and guinea pigs following immunization along with adjuvants as revealed by ELISA and serum neutralization test assays. Further, passive protection studies carried out in suckling mice using hyperimmune sera (HIS) raised against recombinant H3L, showed its prophylactic ability by protecting against virulent BPXV. Purified recombinant H3L protein was used as an antigen in an indirect ELISA format and showed specific reactivity to buffalopox and camelpox sera. There was no cross reactivity with HIS or infected sera against orf, goatpox, sheeppox, PPR, FMD and bluetongue viruses. The study indicated that the recombinant H3L protein is a potential candidate/reagent in the development of prophylactics/diagnostics for buffalopox and camelpox infections.

Cloning and Sequence Analysis of Immunodominant Protein (F1L) of Orf Vaccine Strain (ORFV-Mukteswar/05)

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Contagious pustular dermatitis is a zoonotic, highly contagious skin disease of farmed sheep and goats with a worldwide distribution. It caused by orf virus (ORFV), the prototype of genus Parapoxvirus of the *Poxviridae* family. Orf is endemic in most parts of India, intrinsic immune evasion properties of ORFV coupled with inefficient control measures probably had contributed to increasing number of outbreaks being reported both in sheep and goats. Recently, Pox virus Lab, Division of Virology developed a safe and potent live attenuated Orf vaccine using ORFV-Mukteswar/05 isolate. The key objective of this study was to molecular characterization of virulent and attenuated ORFV-Mukteswar/05 isolate based on the immunodominant protein, F1L. Attenuated ORFV-Muk/05 (passage-50) was revived and passaged in the lamb testis cells. DNA was extracted from scab sample as well as from the cell culture attenuated ORFV-Muk/05 isolate. The full-length *F1L* gene ($\sim$1029 bp) of the ORFV-Muk/05 both from virulent and attenuated was amplified, cloned into pGEMT-Easy vector and sequenced. Comparative sequence analysis of F1L gene/protein among ORFV isolates worldwide with isolates with current study revealed the N-terminal heterogeneity by presence of additions of nucleotides (position at 121–126) (nts) at alanine proline repeating (PAR) motif, presence of putative glycosaminoglycans binding motif and C-terminal transmembrane region. Multiple alignment of aa sequences of F1L of attenuated ORFV-Muk/05 isolate showed three unique aa substitutions (L119P, S124G and L158H) that differed from those of the other virulent ORFV isolates. The results revealed that the F1L of the attenuated and virulent ORFV-Muk/05 isolates showed identities of 99.6 and 98.8%, while with other ORFV isolates 95.4–99.6% and 95.8–98.8% at both nt and aa levels, respectively. Phylogenetic analysis of ORFV isolates shown to form two clusters; ORFV-Muk/05 virulent and attenuated isolates clustered into one group (Cluster-1) separate from other foreign isolates (Cluster-2). We conclude that attenuated ORFV Muk/05 is phylogenetically close to virulent strain of ORFV Muk/05. Three adaptive aa substitution were found in the vaccine virus (ORFV-Muk/05) when compared to virulent viruses may due to passaging of ORFV in cell culture. Further studies are warranted to confirm these unique aa substitution were signature residues for vaccine viruses. This may enable to establish the attenuation markers for vaccine viruses in the ORFV vaccinology.

Development and Evaluation of Real Time PCR Assay for Rapid Diagnosis of Bovine Group A Rotaviruses

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Neonatal diarrhea, of which rotaviruses (RVs) are recognized as the single major viral etiological cause, has been the major cause of death among the newborns creating havoc in the livestock industry. The present study describes the development of a rapid and sensitive diagnostic assay for bovine RVs. In the study, both RNA-PAGE and

VP6 based RT-PCR assay were used to screen a total of 191 diarrhoeic faecal samples from bovine calves (138 from cattle calves and 53 from buffalo calves) collected from different regions of India for the presence of RV and the VP6 based RT-PCR detected 14.1% samples positive for RV compared to 11.5% by RNA-PAGE screening. For the rapid and sensitive diagnostics, aSYBR Green based Real-Time PCR assay was developed with the self-designed primers targeting the conserved region of the NSP4 gene with an amplicon size of 130 bp. For optimization, standard curve was constructed using ten-fold serially diluted plasmid containing the NSP4 gene insert of 130 bp. The standard curve parameters determined were in the optimum range; the slope was −3.336 with a high regression coefficient (R^2) of 0.997. The PCR efficiency was 99.4%. A Ct value of less than 35 and a Tm value of 78.35 °C (± 1.0 °C) were considered as positive. This newly developed qPCR was found 100% specific as no amplification from related enteric viruses was seen. The qPCR assay was compared with available diagnostic assays on 80 clinical samples chosen randomly and its sensitivity in comparison to RNA-PAGE and RT-PCR assay was 4.02 and 3.28 times higher, indicative of suitability of this assay for screening of the samples for bovine RVAs in future. Further validation by screening sufficient more number of field samples is essential for its use at large scale in molecular epidemiological studies of RVs in India.

Determination of P and G Genotypes in Bovine Rotaviruses: A Need to Update the Genotyping Primers

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Rotaviruses (RVs), the single most significant cause of severe gastroenteritis affects a wide range of mammalian and avian species. The present study was carried out to determine the circulating prevalent genotypes in bovines from different regions of the India. A total of 37 rotavirus (RV) positive samples collected from bovine calves from different regions of India were genotyped using multiplex semi-nested PCR assay for determination of P and G genotypes. This was followed by sequencing of VP4 and VP7 genes of randomly chosen 14 samples out of 37 samples to determine genotypes based on sequencing and web based sequence analysis (RotaC). Of the 37 RV positive samples, P[11] (94.6%) and G6 (27%) were most predominant genotypes followed by G3 (10.9%), while 21 (56.8%) samples were having mixed G genotypes. However, sequence based genotyping showed P[1] (64.3%) as the most predominant followed by P[11] (35.7%) while among the G types, G6 (50%) was most predominant, followed by G3 (35.71%). The most predominant G and P type combination as determined by the genotyping PCR assay was G6P[11] (27.8%) while in sequence based genotyping, G3P[1] (28.6%) and G6P[1] (28.6%) were most common combinations. A comparison of the PCR based and sequence based genotyping showed significant discrepancy (as 35 out of 37 samples showed P[11] genotype in PCR typing, while sequence based genotyping of the same samples determined 9 out of 14 samples sequenced, as P[1]) which might be due to rapid evolving rotaviral genome and thus necessitate a need to update the genotyping primers so as to incorporate the new strains with variations in the genotyping regions.

Effect of Lentivirus Expressing Single and Multiple siRNAs on Rabies Virus Multiplication and Protection Against Lethal Challenge

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RNA interference mediated through small interfering RNA (siRNA) mediates degradation of gene transcript in sequence dependent manner. This potential of siRNA has been exploited against many viruses by inhibiting the expression of viral genes that are crucial to viral pathogenesis and virus replication. In this study, the potential of RNAi has been evaluated as antiviral agent against rabies. The siRNAs targeting rabies virus (RV) glycoprotein (G) gene and nucleoprotein (N) gene was designed and evaluated using plasmid co-transfection approach in HEK-293 cells. The potent siRNAs as single and multiple form were delivered using lentiviral vector system. Lentiviruses viz., Lenti-G7, Lenti-N and Lenti-G7 N expressing siRNA targeting RV-genes were constructed. To evaluate the effect of siRNA delivery using lentivirus system, three homogenous cell lines namely, BHK-G7, BHK-N and BHK-G7 N constitutively expressing siRNA were prepared and anti-rabies effect was analysed by challenging these cell lines with RV-PV-11 strain. Forty eight hours post-infection, the reduction in G gene and N gene transcript were quantified and compared with respective controls using real-time PCR. There was significant reduction in RV-G and N transcripts in all the cell lines. Further, the effect of siRNA on RV multiplication was evaluated in RV challenged cell lines by direct florescent antibody technique (dFAT). There was significant reduction in RV multiplication in all the siRNA expressing cell lines compared to control. To evaluate the anti-rabies effect of siRNAs delivered using lentivirus in vivo in mice, the mice were treated intracerebrally with 5×10^5 transduction units of Lenti-G7, Lenti-N and LentiG7N. Five days post-lentivirus treatment the mice were challenged with 20 LD50 of RV-CVS strain using intra-masseter route. The challenged mice were observed for 14 days for rabies specific symptoms and death. There was 66.6% protection in mice treated with Lenti-G7. The mice treated with either Lenti-N or Lenti-G7 N showed complete protection. The control untreated mice died within 9 days post-challenge showing lethal nature of challenge virus. These observations demonstrated the potential of lentiviral delivered siRNAs in inhibition of rabies virus multiplication. This holds the potential of siRNAs as antiviral agent against rabies.

Bluetongue Virus Serotype 9 Isolate from Andhra Pradesh is Phylogenetically Closely Related to Isolates of Mediterranean Origin

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Bluetongue is endemic in native sheep of Andhra Pradesh, causing significant economic losses to the farmers. Attempts have been made to understand the genetic makeup of the bluetongue virus (BTV) isolates recovered from severe outbreaks of the disease in the region. The Bluetongue virus was isolated from sheep blood samples from Mahaboobnagar district of Andhra Pradesh during 2005 (M11/MBN/2005) was propagated in BHK21 cell lines revealed the infectivity titer of 4.5 TCID50/ml. Characteristic migration pattern of bluetongue virus genome was observed in 10% discontinuous polyacrylamide gel. Full length VP2 gene was amplified and sequenced using FLAC method. Phylogenetic studies employing un-rooted Neighbor Joining (NJ) and Unweighted Pair Group Method Arithmetic Mean (UPGMA) revealed that VP2 gene of M11/MBN/2005 isolate grouped with European and Mediterranean isolates of BTV-9 (isolated during 1998–2003, eastern topotype) within Seg-2 nucleotype E showing 98% nucleotide sequence identity. VP2 gene of M11 isolate is

2865 bp encoding 955 amino acids (aa). Four amino acid changes were observed in M11/MBN/2005 isolate VP2 sequences: at position 55 (G to V); 120 (I to N); 286 (I to M) and 536 (N to D). The outbreak of 2005 has transboundary significance as Rambouillet sheep were imported to India from Mediterranean basin in the year 2000 for up gradation of Nellore sheep by private farms of Bangalore.

Molecular Characterization of *Culicoides oxystoma,* Most Predominantly Circulating Vector for Bluetongue Transmission in India

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Bluetongue (BT) is an infectious, non contagious arthropod borne viral disease of wild and domestic ruminants especially sheep which inflicts major losses on subsistence sheep farmers in southern India. Affected sheep may have erosions and ulcerations on the mucous membranes, dyspnea, lameness and inflammation of the coronary band. The disease is caused by bluetongue virus (BTV) the type species of the genus *Orbivirus* and belongs to family *Reoviridae*, transmitted between their ruminant hosts by certain haematophagous *Culicoides* biting midge. It is OIE list 'A' multispecies disease. Till 2008, twenty four distinct serotypes of BTV (BTV-1 to BTV-24) have been isolated and characterised worldwide. Recently, BTV-25 has been reported from Switzerland and BTV-26 from Kuwait. The present study was carried out with the objectives to identify and characterize the culicodes species procured from southern India. A total of sixty seven *Culicoides* DNA samples extracted using a non-destructive DNA extraction method were amplified using mitochondrial gene specific primers. An amplicon of 523 bp was obtained in all the amplified samples. All these positive samples were further targeted for sequencing PCR and the products were purified and dissolved in Hi Di formamide. The samples were sequenced in the DNA analyzer ABI PRISM 3100. The sequencing data obtained was analyzed using computer softwares. A total 48 (71.64%) samples were identified as *C. oxystoma*, 6 (8.95%) as *C. reconditus*, 4 (5.97%) as *C. pseudopalidipennis*, 3 (4.47%) each of as *C. Schultzei* and *C. imicola*, 2 (2.98%) as *C. peregrines* and one (1.49%) as *C. kubenesis*, respectively. The nucleotide sequence based analysis revealed that the majority of the *Culicoides* species belonged *C. oxystoma* and showed their prevalence in India earlier also. The blast analysis revealed that the Indian isolates of *C. Oxystoma* were 90–99% similar with Japanese and Israel isolates. The literature on sequences related to *C. oxystoma* is very scanty. The only sequences belonging to *C. oxystoma* have been of Japanese and Israel origin. The phylogenetic analysis revealed huge genomic diversity among Indian sequences as all the Indian isolates formed more than eight clusters distantly related to Japanese and Israel sequences. The sequences of Israel and Japanese origin grouped together to form one separate cluster. Out of 48 Indian sequences, only one showed closeness to Japanese sequence. The rest of all of the Indian sequences grouped in five major clusters showing identity with each other. However, seven of the Indian sequences depicted distant identity with other Indian sequences and very distantly placed from Israel and Japanese sequences. The observations from the present study indicated that the world literature on *C. oxystoma* sequences is scanty and the information generated in the present study could be useful in molecular characterization and determining the association of this vector with spread of BTV virus depending upon the movement of this vector in various part of the country. The sequencing data generated in this study could form the basis for developing database of *C. oxystoma* in the country.

Segment 6 Based Nucleotypic Variation in Indian Isolate of BTV-9

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Bluetongue virus (BTV) is a prototype species of the genus *Orbivirus* within the family *Reoviridae* which causes Bluetongue disease (BT) in domestic as well as wild ruminants. BTV is non-enveloped virus having 10 segmented dsRNA genome. Segmented 6 encode VP5 protein along with VP2 protein gives serotype specificity to the virus. A pair of vp5 gene specific designed primer generating an amplicon size of 823 bp of Indian isolate of BTV9 was designed. The segment 6 of an unknown Indian isolate of BTV was amplified using this primer, sequenced and analyzed. Nucleotide sequence analysis revealed that Indian BTV9 serotype showed more than 97% identity with other Indian and European BTV9 isolate and more than 90% with Japanese isolates. However, only 68.8% identity was found with South African isolates. More than 97% identity was observed based on deduced amino acids sequences with Indian, Japanese and European isolates and only 73.4% with South African BTV9 isolates. The phylogenetic study based on vp5 gene nucleotide and deduced amino acid sequences revealed that the BTV9 isolate in present study formed a major cluster including European and other Indian isolates of BTV9 indicated the western origin of this BTV9 isolate. The in silico restriction enzyme analysis (REA) with *AflIII, BtrI* and *HindII* showed a common pattern of restriction sites in Indian and European isolates at 1152, 1153 and 591 and lack of any of these restriction sites in African and other Japanese isolates further confirm the western origin of BTV9 Indian isolate. Further, the lack of *BsmAI* restriction site in Indian BTV9 isolate could differentiate it from European, Australian, Japanese and South African isolates. Within a nucleotype the nucleotide and deduced amino acid sequence identities reported were >76% and >86%, respectively. The BTV9 isolate used in present study showed maximum 76–80.2% nucleotide and 88–93.2% amino acids sequence identity with nucleotype 'B' serotypes (serotype 3, 5, 6, 13, 14, 16 and 21). This placed all the Indian BTV9 isolates in nucleotype 'B'. However, the BTV9 isolates from South Africa had shown maximum 76.2–77.5% nucleotide and 87.5–89.4% amino acid identity with nucleotype 'C' serotypes (serotypes 1, 2 and 23). This placed South African BTV9 isolates in nucleotype 'C'. Hence the BTV9 of Indian origin in the current study indicated high probability of reassortment in segment 6 which differentiates it from BTV9 of South African isolates. These observations further suggest occurrence of BTV 9 isolates at global level that can be assigned two different nucleotypes B and C.

VP2 Gene Based Serotyping and Characterization of Indian Isolates of BTV3

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Bluetongue virus (BTV) is a prototype species of the genus *Orbivirus* within the family *Reoviridae* which causes Bluetongue disease (BT) in domestic as well as wild ruminants. BTV is non-enveloped virus having 10 segmented dsRNA genome. In the present study, two BTV isolates of sheep origin, one each from Utter Pradesh and west Bengal were used. The virus isolates were propagated in BHK21 cell line. After appearance of cytopathetic effect in BHK21 cell line the dsRNA of the viruses were extracted using Guanidinium isothiocyanate (GIT) lysis method. The samples were confirmed as bluetongue virus (BTV) based on characteristics cytopathetic effect in BHK 21 cell line and 274 bp amplicon size with group specific ns1 gene RT-PCR. The segment 2 (vp2 gene) specific RT-PCR confirmed the both of the two isolates were BTV serotype 3. The vp2 gene amplicon was sequenced. The nucleotide sequence analysis revealed nucleotide differences in the selected region (592 bp region) of vp2 gene of the two BTV3 isolates. A total of seventeen point mutations/deletions/additions were observed in the partial vp2 gene in region nt1451 to nt2042. Further, the vp2 gene sequence based analysis revealed 4 to 11% genetic diversity among BTV3 isolates of Indian and global origin.

Isolation of a Bluetongue Virus (BTV) Serotype-1 from an Asymptomatic Goat and Its Neutralization with a Panel of Anti BTV-1 Sera

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In the autumn of 2011, blood samples ($n = 51$) were collected from goats in Pithoragarh area of Uttarakhand (India). About 64% of these samples were tested positive for BTV antigen (by a sandwich ELISA) including a number of samples being strong positive. From a strong antigen-positive blood sample, a BTV was isolated (named as PTG-13) on cell culture, which was confirmed as BTV-1 by RT-PCR coupled with partial sequencing of genome segment-2. The cytopathic effects, typical of BTV, appeared as early as on 18 h post infection and virus titer was measured at third passage on BHK-21 cells which was found to be ($10^{8.7}$ $TCID_{50}$/ml). The neutralization behavior of PTG-13 was studied by virus neutralization with hyperimmune sera (HIS) prepared against a panel of eleven BTV-1 isolates collected from different parts of the country over a period of more than two decades. PTG-13 was completely neutralized between 1:16 and 1:32 dilution of HIS against two of the south Indian BTV-1 isolates. HIS against six north Indian isolates neutralized the PTG-13 virus at 1:4 dilution. However, HIS against three of the south Indian isolates could not neutralize the PTG-13 virus. One-way neutralization data suggest that PTG-13 has closer antigenic relation with two south Indian isolates than with the north Indian isolates. Neutralization resistance of PTG-13 with HIS against three south Indian isolates suggests that PTG-13 could be a VP2 phenotypic variant of BTV-1 co-existing in southern India. Detailed genetic analyses and cross-neutralization of the Indian BTV-1 isolates will provide better understanding of the level of genetic and antigenic relatedness between the isolates.

Dual infection of Goats with Bluetongue Virus Serotype 1 and 23 as Evidenced by Virus Isolation and Detection of Neutralizing Antibody

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Bluetongue is a disease of sheep caused by the bluetongue virus (BTV). The virus can infect almost all domestic and wild ruminants. The disease is subclinical in cattle and goats, and these animals can act as reservoir hosts for the virus. In the autumn of 2011, blood samples ($n = 51$) were collected from goats in Pithoragarh area of Uttarakhand (India). About 64% of these samples were tested positive for BTV antigen (by a sandwich ELISA) including a number of samples being strong positive. From a strong antigen-positive blood sample, a BTV was isolated (named as PTG-13) on cell culture, which was confirmed as BTV-1 by RT-PCR and partial sequencing of genome segment-2. The goat plasma samples were found to contain high titer of neutralizing antibody against BTV-23, however, the virus could not be isolated. Interestingly, no neutralizing antibody was detected against PTG-13 or other BTV-1 isolate, which suggests that sampling was done probably before the development of neutralizing antibody against PTG-13 virus in the host. Isolation of BTV-1 (PTG-13) and presence of BTV-23 neutralizing antibody in blood samples indicate that goats were naturally infected with BTV-1 and 23. The dual infection in goats with two BTV serotypes has potential for intertypic genetic reassortment leading to evolution of more virulent virus strain.

Detection and Characterization of Group A and D Rotaviruses Isolated from Enteritis Cases of Poultry from Uttarakhand

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Rotaviruses are a major cause of acute gastroenteritis in the case of several mammalian and avian species. According to WHO, the rate of infection and fatality due to rotavirus is more in developing countries. Rotavirus causes runting and stunting syndrome is a major cause of great economic impact among the poultry rearers. The virus belongs to the family *Reoviridae* and has a double stranded RNA as its genome. The virion is non-enveloped and genome is distributed among 11 segments of dsRNA. The outer layer of the virus is made up of VP4 and VP7 proteins and intermediate layer made up of VP6. The VP6 proteins are group specific protein which helps to classify rotavirus into different groups. Electrophoretic pattern of rotaviruses on poly acrylamide is also specific and according to all these seven groups of virus (A-G) are identified till now. Among these groups group A rotaviruses affect both mammals and birds and groups D, F, G are identified only from the avian species. During the current study samples collected from enteritis cases in poultry from foot hills of the Uttarakhand were screened for the presences of different groups of rotaviruses. Enteric samples were collected as fecal samples, intestinal contents, peeled off intestinal mucosa etc. all the samples were processed and a 10% PBS suspension of the samples were made. RNA was isolated using standard TRIZOL method. Initial screening was done using conventional RNA-PAGE. The cDNAs were prepared from the isolated RNA using standard procedures and group specific VP6 gene based RT-PCR was used for detection of group A and D rotaviruses. Out of the seventy samples screened 21 were found positive for group A rotavirus and 6 were found positive for group D rotavirus. One of the samples was having mixed infection of both group A and D. The results of present study confirms circulation of avian group A and D rotavirus in this region and warrants further studies to characterize the viruses in depth so as to develop a good vaccine for preventing the infection in poultry.

Amplification and Cloning of Glycoprotein E (gE) Gene of Duck Enteritis Virus

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Ducks, placed under the class-Aves, order-Anseriformes, and family-Anatidae also include geese and swans. Ducks are mainly reared for egg and meat production having a high nutritional value. Duck farming and production, however, invariably suffers from some of the dreadful disease causing significant mortality and morbidity. Two such acute and highly contagious viral diseases are Duck Virus Hepatitis and Duck virus enteritis (Duck Plague). Definitive diagnosis of DVE requires isolation of the virus, neutralization with specific antiserum in the infected systems, fluorescent antibody test and PCR using DVE virus specific primers. DVE is caused by Duck enteritis virus which belongs to the Alphaherpesvirinae subfamily of the family Herpesviridae. The genome of DEV, a linear and double stranded DNA is about 150 kb. The enveloped glycoprotein E (gE) in Herpesviridae is important for the expression of virulence of the virus. In the present study amplification of the gE gene from the isolated genomic DNA available in the Recombinant DNA laboratory of the Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar was performed. The 1.472 kb gene was amplified using the forward (F) and reverse (R) primers. The amplicon was checked by using 1.2 percent (w/v) agarose gel electrophoresis. The gE gene was successfully cloned into InsT/A clone PCR product cloning vector system (pTZ57R). The insert release obtained after digestion with restriction enzyme *BamH1* had a size of 1325 bp when run in 1.2% agarose gel electrophoresis against a mass express ruler which confirmed successful cloning of gE gene into the vector.

Characterization of Cell Penetrating Rath Peptides Analogues by Their Ideal Delivery in Cancerous Cell Lines

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Cell penetrating peptides (CPPs) enter the cell by transversing the cell membrane and localize to different cellular components depending on their amino acid composition and perform different roles. A novel Rath peptide originating from the C-terminus VP5 protein of avian Infectious Bursal Disease virus has already been described for efficient delivery of proteins and nucleic acids (Bais et al., 2008). We designed & synthesized four Rath peptides analogues *i.e.* Rath-navigator, Rath (Phe-Leu), Rath-navigator with spacer, Rath (Phe–Phe). The cell penetrating ability of these peptides were assessed by transfecting different concentrations of FITC labelled peptides, and it was found that cell penetrating ability of Rath (Phe–Phe) was maximum with transfection ability of around 59.46% followed by Rath (Phe-Leu) having 43.93%, Rath Navigator 28.6% and Rath navigator with spacer 25.18% penetrating ability. The Cargo (FITC labelled oligo DNA) delivery properties of these peptides were also assessed and it was observed that these peptides delivered DNA into 40–60% of cells. The Rath (Phe–Phe) had maximum DNA delivery ability (46.29%) followed by Rath (Phe-Leu) with 32.67%. Several CPPs (Rath, Penetratin etc.) have proven ability of delivery of cargo (Bais et al, 2008; Derossi et al, 1994). Our data demonstrate that Rath peptides analogues are the novel CPPs that could be used to translocate different cargoes inside cells efficiently.

CPV-2 Induced Apoptosis of MDCK Cells is Caspase and p53 Dependent

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Canine parvovirus-2 (CPV-2), of the genus Parvovirus of family *Parvoviridae*, is an important pathogen of dogs which causes acute enteritis, myocarditis and lymphopenia, especially in pups. CPV-2 induced pathogenesis in animals is attributed to apoptosis in infected cells. Caspases and p53 play an important role in mediating apoptosis. In this study we confirmed that the CPV-2 induced apoptosis of MDCK cells is mediated by extrinsic, intrinsic/mitochondrial and, endoplasmic reticulum stress mediated pathways and is p53 dependent. MDCK cells infected with CPV-2 at 30–40% confluency with 0.01 *m.o.i.* were harvested at 24 h, 48 h and 72 h post infection. The total RNA was isolated from the harvested cells and the cDNA synthesized using random hexamer primers and MMLV-RT. Real time PCR was carried out to evaluate the expression of caspase-3 (Effector caspase), Caspase-8 (initiator caspase in extrinsic pathway), Caspase-9 (initiator caspase in intrinsic pathway), Caspase-12 (initiator caspase in endoplasmic reticulum stress mediated pathway) and p53. The expression of the above caspases and p53 was found to be upregulated at all time points in comparison to control(s) and increased with time, indicating the involvement of all these caspases. The expression of caspase 3, caspase 8, caspase 9, caspase 12 and p53 at 72 h post infection was found to 9.80 ± 0.85; 5.10 ± 0.55; 3.98 ± 0.55; 2.82 ± 0.30 and 4.27 ± 0.62, folds higher than control(s), respectively. The real time results were also confirmed by IFAT which showed bright fluorescence as compared to controls with caspase-3, 8, 9, 12 at 48 h post infection. The efflux of cytochrome C and increase in percentage of caspase-9 positive cells detected by flowcytometry at all time points with respect to control further confirmed the involvement of intrinsic pathway.

Viral Metagenomics: A Vital Approach in Modern Virological Research

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Viral metagenomics, coupled with high-throughput sequencing and bio-informatic analysis of mammoth genomic libraries, has recently emerged as a potential tool to characterize the unculturable viruses from the environment as well as animal samples. Animal disease outbreaks which seem to be multi-factorial and complex in nature due to involvement of multiple microbes, with genetic variations at species, subspecies, strains level coupled with non-isolation of etiological agent, pose greater challenge to the researchers. In such scenario, metagenomic approach provides a novel way to unravel the mysteries of disease outbreak as well as better understanding of microbial characteristics, host-pathogen interaction and insights into design and development of novel diagnostics/therapeutics. In India, although, the realm of viral metagenomics is at infancy, the approach seems to grow exponentially in the days ahead as the infrastructure and technique employed become largely applicable in most veterinary research laboratories with a greater focus to safeguard the health scenario of livestock population.

Molecular Characterization of σB Encoding Gene of Indigenous Avian Reovirus Isolate

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Avian reoviruses (ARVs) are the cause of an important emerging disease of the commercial poultry birds in India. Serological screening has revealed its wide spread distribution in poultry population and there is a report about the high economic losses due to stunting syndrome caused by ARVs on several broiler farms in southern parts of the country. The ARV isolate used in the present study was obtained from the Avian Diseases Section, Division of Pathology, IVRI, Izatnagar. In the present study, σB encoding gene of ARV field strain was amplified, cloned into pTZ57R/T vector, sequenced and finally expressed. Phylogenetic analysis of σB encoding gene showed nearly complete homology with American, Canadian and Chinese isolates except Taiwanian isolates of ARV, muscovy duck reovirus strain 89026 and two turkey reovirus strains NC98 and Tx99. The presence of a large no of antigenic regions in σB protein was determined using PROTEAN programme of DNA STAR software. The σB encoding gene was successfully expressed from a pET bacterial expression system. The recombinant protein was confirmed by SDS-PAGE and western blot analysis using hyperimmune sera against ARV raised in rabbit. This protein can be further purified and can be used in σB ELISA for diagnosis of ARV infection and to better predict the immune status of dam for successful vaccination strategy.

Cloning and Sequencing of Nucleocapsid (N) Gene of an Indian Isolate of Infectious Bronchitis Virus

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Infectious bronchitis (IB) is an economically important disease of poultry and it affects chickens of all ages causing respiratory and nephrotic syndromes in broilers, reduced egg production in layers and breeders. Rapid diagnosis and immune status determination are critical to controlling outbreaks of infectious bronchitis virus (IBV). The nucleocapsid (N) protein, a major structural protein of IBV, is the preferred protein for use in development of group specific serologic assays. It has highly conserved sequences, which share 91 to 96.5% identity among various strains, is produced abundantly during infection, and has high immunogenicity, readily inducing antibodies as well as cytotoxic T lymphocyte immunity in chickens. In the present study, propagation of an Indian isolate of IBV was done by chorioallantoic method in 10 days-old specific pathogen free embryonated chicken eggs. Characteristic curling and dwarfing of embryos was noticed in all embryos at sixth day post inoculation. Viral RNA was isolated from allantoic fluid by TRIzol method and transcribed into cDNA by gene specific primers. About 1.2 kb nucleocapsid gene was amplified by RT-PCR. The amplified product was cloned and the nucleotide sequence of the N gene was determined. The Indian IBV isolate exhibited more than 90% homology with the M41 vaccine strain. Efforts are now being made to clone and express the N protein in prokaryotic expression system for development of recombinant N protein based ELISA which will be useful for detecting IBV specific antibody.

Prevalence of Canine Parvovirus 2a in Diarrhoeic Canines: A Genetic Shift from CPV2b

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Canine parvovirus (CPV) is a causative agent for acute hemorrhagic enteritis and myocarditis in dogs. The virus belongs to the family *Parvoviridae* and is a non-enveloped, single-stranded DNA virus of approximately 5 kb. The viral genome encodes two structural (VP1 and VP2) and two non structural (NS1 and NS2) proteins. It was emerged in late 1970s and original form of CPV-2 is completely replaced by antigenic variants CPV-2a, CPV-2b and CPV-2c. Our study aimed at detection of CPV by nested polymerase chain reaction (PCR) using VP2 region based primers. These were further sequenced and genotyped to characterize it on the basis of strains. A total of 101 faecal samples from parvovirus suspected cases were collected during the year 2011 and dispensed in PBS. Viral DNA was extracted using DNAzol method and nested PCR was carried where 18 samples (17.82%) were found to be positive for canine parvovirus with the amplified region of 747 bp. Out of 18 positive samples, 3 were vaccinated and 6 were unvaccinated while status of 9 was not known. Among the 18 positive samples, 4 cases were in range of 2–4 months while 5 were ranged from one and half month to 6 months category while status of 9 was not known. Genotyping was done using genotype specific primers such as P2abS, P2abAS for both CPV2a and CPV2b. The primers P2bS, P2abs were used for detecting CPV2b. A total of 12 samples (11.88%) were genotyped as CPV2a with a specific product of 681 bp while 6 samples (5.9%) were found to be positive for CPV2b genotype and the amplified product was of 427 bp. A sum of 14 CPV positive samples was sequenced and the sequencing data was used for phylogenetic analysis using MEGA 4.0 software. In previous studies in the same area under study showed prevalence of CPV2b, whereas the recent studies (January 2011 to September, 2011) depicted change in trend of circulation of virus from CPV2b to CPV2a. The study is crucial in epidemiological studies for future prevention and control of the disease and indicates for an urgent need to develop a new multivalent vaccine including local field strains.

Recombinant UL30 Antigen-Based Single Serum Dilution ELISA for Detection of Duck Viral Enteritis

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A recombinant UL30 antigen-based single serum dilution enzyme linked immuno sorbent assay (ELISA) was developed to measure specific antibody in the sera of ducks against duck enteritis virus (DEV). The partial UL30 gene of DEV was cloned, expressed, purified and tested for its diagnostic use by designing a single serum dilution enzyme linked immuno-sorbent assay (ELISA). A total of 226 duck sera samples were tested using the assay. A linear relationship was found between the predicted antibody titres at a single working dilution of 1:100 and the corresponding serum titres observed as determined by the standard serial dilution method. Regression analysis was used to determine a standard curve from which an equation was derived which demonstrated this correlation. The equation was then used to convert the corrected absorbance readings of the single working dilution directly into the predicted ELISA antibody titres. The assay proved to be specific, sensitive and accurate as compared to the virus neutralization test with a specificity, sensitivity and accuracy being 96%, 95% and 95% respectively.

Genetic Characterization of Innate Immune Gene (TLR-7) of Changthangi Goat from Cold Desert of Leh, J&K, India

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Toll-like receptors (TLRs), sentinels of innate immunity are membrane-spanning and non-catalytic receptors which recognize structurally conserved molecules i.e. Pathogen-Associated Molecular Patterns (PAMPs). TLR-7, the key innate immunity receptor against single stranded viruses induce the expression of Type I interferons and chemokines through a number of signaling pathways. Different TLRs gene have been studied from different breads of goats all over the world but high quality wool producing Changthangi or Pashmina goat still remain untouched. In the present study, blood was collected from Changthangi goats reared in cold desert of Leh-Ladakh and genomic DNA was extracted using QIAampDNA Blood Mini Kit. The TLR-7 gene was amplified with the self-designed primers for LRR region (lucine rich repeat) with specific amplicon size of 1,053 bps. The amplicon after purification by GeneJET Gel Extraction Kit was cloned into pJET 1.2/blunt cloning vector and confirmed by colony PCR and plasmid restriction digestion with Bgl II. Phylogenetic analysis of sequenced TLR-7 gene by Neighbor-joining method revealed close sequence homology (99%) with caprine TLR-7 gene followed by 98.4% and 97.7% with ovine and bovine, respectively, while least homology (86.4%) was seen with human TLR-7 gene at nucleotide level. Similar amino acid sequence homology pattern was observed with 99.1% identity with caprine, 98.3% and 97.5% with ovine and bovine; respectively while least homology (87.4%) with human TLR-7. All other innate immune receptors of Changthangi goat needs to further characterized for better understanding of TLR-mediated response and increase our range of weapons to treat infectious and immune diseases.

Molecular Differentiation of Indian Isolates and Vaccine Strain of Infectious Bursal Disease Virus by RT-PCR/RFLP

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Infectious bursal disease virus (IBDV), the causative agent of Infectious bursal disease (IBD) of poultry belonging to *Avibirnavirus* of *Birnaviridae*, is a small non enveloped RNA virus having bi-segmented double stranded RNA. Simple techniques to differentiate very virulent (vv) and vaccine strains of IBDV is very useful for monitoring infection and vaccination in poultry and also to ensure authenticity of vaccine lots. Reverse Transcription-Polymerase Chain Reaction/Restriction Fragment Length Polymorphism (RT-PCR/RFLP) analysis of the 248 bp (804–1051 nt) of vp2 gene which is a portion of hyper variable region allows the differentiation of vaccine strains, vvIBDV strains, classic strains and variant strains into thirteen different RFLP patterns. The selected gene fragment of very virulent Indian field isolates and vaccine strain Georgia were amplified by RT-PCR and subjected to Restriction Enzyme (RE) digestion with *Taq*I, *Sty*I, *Ssp*I, *Sac*I, *Mva*I, *Dra*I enzymes. Single restriction site has been observed in vvIBDV strains for REs *Taq*I, *Sty*I, *Ssp*I but no restriction site has been observed in vaccine strain. Similarly, single restriction site has been observed in vaccine strain for REs *Sac*I, *Mva*I but no restriction site has been observed in vvIBDV strains. However no restriction site has been observed for both vvIBDV strains and vaccine strain for RE *Dra*I. Hence RT-PCR/RFLP analysis can differentiate very virulent Indian isolates and vaccine strain of IBDV.

Growth Potential of FAV-4 in Chicken Embryo Kidney Cells (CEK) *vis a vis* BHK-21 Cells and Detection by Different Serological Methods

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Fowl adenoviruses are known to grow on cell systems of homologous species in vitro. The plan of the study was to observe the growth potential of FAV-4 (causative agent of hydropericardium syndrome) on cell cultures of avian kidney and compare it with growth on cell cultures of mammalian kidney and their subsequent detection by employing different serological methods. CEK and baby hamster kidney-21 (BHK-21) cell cultures were infected with FAV-4 isolate. A total of five passages were given in CEK cultures and eight in BHK-21 cell lines. Cytopathic effects (c.p.e) first appeared at the 2nd passage level in CEK cultures. The c.p.e was characterized by retraction and loss of cells subsequently leading to extensive destruction of the monolayer by 120 h. p.i. Additional passages of the isolates in CEK decreased the time interval in initiating c.p.e. By the start of the fifth passage however, the retraction of cells from the monolayer was marked leading to complete detachment and degeneration of the monolayer by 72 h. p.i. Eight blind passages were given in BHK-21 cells to check adaptation of the virus to these cells but without success. Virus detection from infected cell cultures was done with the help of AGID, CIE, Dot ELISA and FAT. Detection of the virus from CEK cells was possible from the 3rd to 5th passage level employing AGID and CIE. Dot ELISA and FAT however could detect the virus in the infected CEK cell cultures as early as the 2nd passage level. Virus detection however was not possible from cell culture supernatants from BHK-21 infected cell lines by any of the serological methods. Serologically, Dot ELISA and FAT were found to be more sensitive in detecting virus than either AGID or CIE. The virus infectivity titer after the 5th passage level in CEK were found to be higher than the initial level showing that the virus adapted well and grew to high titres in CEK cells. It is concluded that CEK cell cultures can be used for rapid growth and multiplication of FAV-4 while BHK-21 cells are not conducive for the growth of this virus.

An Improved Non-Enzymatic DNA Extraction Method for Detection of Poxviruses by Polymerase Chain Reaction

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Classical DNA extraction procedures such as Phenol: Chloroform: Isoamyl alcohol and other commercial column-based commercial kits are expensive, time consuming, and require multiple extraction and purification steps in addition to proteinase K digestion. A rapid non-enzymatic procedure for extracting poxvirus DNA from clinical samples (scab material) and tissue culture was developed to overcome some of the limitations of the available DNA extraction techniques. The procedure requires few mg of tissue and produces highly purified DNA [OD260/OD280 ratios is >1.8] with concentrations ranging from 13 to 17 μg/mL. The DNA was extracted from samples suspected/known tissue culture of sheeppox, goatpox, and buffalopox viruses. The suitability of the DNA for PCR assay was assessed using poxvirus specific primers. We have also compared the new DNA extraction method with DNA extracted using commercial QIAamp DNA Mini Kit. We observed that the sensitivity of new non-

enzymatic protocol is more sensitive than commercial kit for the detection of poxviruses. We have also successfully applied this non-enzymatic protocol for the detection of infectious bovine rhinotracheitis (BoHV1). This result indicates that the simple non-enzymatic DNA extraction protocol can also be used for the detection of other viruses.

Detection of Classical Swine Fever Virus from the Backyard Pig of Uttarakhand

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Classical swine fever (CSF) is an economically important and highly contagious viral disease of swine worldwide. It is one of the most important viral diseases of pig with serious socio-economic impact on Indian economy. The causative agent is Classical Swine Fever Virus (CSFV) belongs to the family *Flaviviridae,* genus *Pestivirus,* and has one serotype divided into three major genotypes and ten subtypes. CSFV is a small enveloped virus encompassing a single-stranded RNA genome of positive polarity with a length of about 12.3 kb. The viral genome contains one large open reading frame (ORF) which encodes a polyprotein of 3898 amino acids (aa). This polyproteins is both co and post translationally processed to yield four structural (C, E^{rns}, E1 and E2) and 7–8 nonstructural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) viral proteins. CSF in Uttarakhand is mostly unnoticed due to lack of awareness of the disease amongst the pig raiser. The incidence of the disease is very scanty and confused with other swine diseases. In the present study, a total of 204 CSF suspected tissue samples were collected from different parts of Uttarakhand viz. Bhowali, Rudrapur, Lalkuan, Haldwani, Dehradun, Uttarkashi and Haridwar. The suspected field samples were processed and stored in DBT-CSF National Repository, Division of Virology, IVRI, Mukteswar. Total RNA of all suspected tissue samples were isolated following modified guanidium-phenol–chloroform procedure. The quality and concentration of RNA was checked by Nanovieue spectrophotometer followed by synthesis of cDNA. Further, PCR was carried out for the confirmation of CSFV antigen targeting three different genes viz 5′NTR (422 bp), E2 (273 bp), NS5B (449 bp). PCR amplification revealed the presence of CSFV antigen in the suspected samples. Out of 204 tissue samples, 50, 18 and 22 samples were found positive with NS5B, E2 and 5′NTR genes respectively. Finding indicates the presence of CSF virus in the backyard pigs, which could be the major source and cause of death (generally unnoticed), of the pigs in Uttarakhand.

Adaptation of Cytopathic Classical Swine Fever Virus (CSFV) in RK-13 Cell Line

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Classical swine fever (CSF) also known as hog cholera, is an economically important contagious viral disease of swine. The disease is caused by Classical swine fever virus (CSFV). The virus is small (40–60 nm) enveloped ribonucleic acid (RNA) with a single stranded RNA genome with positive polarity. CSFV belongs to the *Pestivirus* genus of the *Flaviviridae* family. The adaptation of CSFV was reported in both homologous as well as heterologous cell line. In case of homologous cell line, Swine Kidney-6 (SK-6) and Porcine Kidney (PK-15) have been used for adaptation of the CSFV vaccine C-strain. In order to adapt Madin-Derby canine kidney (MDCK) adapted cytopathic isolate in rabbit kidney (RK-13) cell line, five consecutive passages have been done. Eagle's minimum essential medium (EMEM) with supplements was used for culturing RK-13 cells and maintaining CSFV in RK-13 cells. During first and second passage virus didn't produced any evidence of characteristic cytopathic effect (CPE), but in third passage morphology of cell monolayer was changed. During fourth and fifth passages, characteristic CPE was observed within 48 to 60 h post infection (hpi). The CPE was characterized by rounding of infected cells in focal areas. Rounding progressed and led to clumping of cells followed by detachment of cell. The virus titer after passage five was estimated to be $10^{-3.06}TCID_{50}$. Further, genetic characterization of the CSFV based on NS5B gene (449 bp) revealed presence of the virus in the RK-13 cells. Study confirms the adaptation of cytopathic CSFV in Heterologous cell lines. This will helps in easy visualization and characterization of the virus as compared to homologous cell line; where it doesn't produce CPE.

Nanobiosensors in Viral Disease Diagnosis

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From the start of 21st century, researchers are interested in the field of nanotechnology. Nanotechnology based diagnostics and therapeutics have started budding through scientific research. Nanobiosensors are one among them, used for detection of chemicals or biological materials. Nanometer refers to a unit which is one billionth of a meter. Biosensors are analytical devices incorporating a biological material which is intimately associated with a physicochemical transducer or transducing microsystem. Nanobiosensors are known for their high sensitivity, specificity, rapidity and portability. There are various types of nanobiosensors based on various principles namely, electrochemical biosensors, voltammetric and amperometric sensors, impedance sensors, optical fiber based sensors, surface plasmon resonance based biosensors, quartz crystal microbalance and atomic force microscopy based nanobiosensors. Nanobiosensors have been designed for diagnosis of dreadful human viral diseases like HIV, hepatitis B, Hepatitis C, Ebola virus etc. Nanobiosensors have also been designed for diagnosis of animal diseases like avian influenza virus, infectious bovine rhinotracheitis, rabies, bovine leukemia virus. In future, nanobiosensors may replace the current viral disease diagnostics as they are time consuming and expensive.

Development of a Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid, Sensitive and User Friendly Detection of Infectious Bovine Rhinotracheitis (IBR) Virus in Bovine Semen

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Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV) is an OIE notifiable disease affecting cattle of all breeds

and age groups and is a major cause of direct economical loss to the cattle industry. The disease is widely distributed around the world and is endemic in India, with outbreaks reported from almost all the states. Once the infection is established, the virus remains in latency and persists lifelong. It is the most common viral pathogen found in bovine semen and is one of the major hurdle in implementation of government run breeding and artificial insemination (AI) programmes. To avoid risk of spreading infection, regular testing of semen at semen collection centers is necessary. Therefore, a rapid, sensitive and user-friendly test that can be adapted at semen collection centers for routine screening IBR virus in bovine semen is always in demand. A LAMP based visual detection assay was developed for detection of IBR viral genome. The reaction includes three pairs of primers specific to IBR viral genome which gives unique specificity to the assay. The test was carried out using a simple heat block. The developed assay could detect as low as 10 fg of viral DNA per reaction, which was 10-times more sensitive when compared to conventional PCR. Further, the developed assay was adapted for rapid and sensitive detection of the IBR virus in bovine semen in a user-friendly manner by differentiating positive and negative reaction by visual colour development. The whole LAMP assay, including DNA isolation, isothermal amplification and visualization of results with naked eye could be completed in 90 min. The assay had an analytical sensitivity of 0.215 $TCID_{50}$ or 0.4 infective virus particles per reaction when spiked into IBRV negative semen. The LAMP assay was validated for specificity, sensitivity and repeatability using spiked bovine semen. The test has been evaluated on 198 semen samples collected from bulls to detect IBR virus. The rapidity and ease of performing the test makes it an attractive option to be employed in government run IBR control programme as well as breeding and artificial insemination programmes.

Characterization of the Double Gene Construct Containing in Canine Parvovirus NS1 Gene and Reporter Gene GFP in Eukaryotic Expression Vector pVIVO2-mcs

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The use of chemo and radiotherapy for treatment of cancer is limited due to genotoxic side effects on healthy cells, involvement of anti-apoptotic signal transduction pathways that prevent cell death and requirement of functional p53 for induction of apoptosis in cancerous cells. Efforts are beings made worldwide towards the development of new anticancer therapies as an alternative to chemotherapy. Viral gene therapy is one of the most potent therapeutics that is being ventured into worldwide. Canine parvo Virus-2 (CPV-2) is one of the viruses with an inherent oncolytic property. The Non-Structural protein-1 (NS1) protein of CPV-2 plays a major role in parvoviral cytotoxicity and pathogenicity in permissive cells. The oncolytic potential of CPV2-NS1 was established in vitro. Prior to taking up the in vivo studies, the present study was undertaken, to clone Canine Parvovirus Ns1 gene and reporter gene GFP in eukaryotic expression vector pVIVO2mcs and characterize the double construct in mammalian cells. The genes were successfully cloned in pVIVO2-mcs and characterized for their expression as demonstrated by fluorescence microscopy and immunofluorescence. This characterized double gene construct will be used to evaluate the oncolytic potential of CPV-2 NS1 in experimentally induced in vivo tumour model.

Comparison of IL-2 mRNA Levels in Natural Outbreak of Foot-and-Mouth Disease and Vaccinated Cattle

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Having successfully eradicated Rinderpest, greater thrust is being made to untangle mysteries related to Foot-and-Mouth Disease (FMD). FMD is caused by Foot-and-Mouth Disease virus (FMDV), an *Apthovirus* under family *Picornaviridae*. FMD causes critical economic losses to small or large animal holders. Investigation on cytokine responses after natural Foot-and-Mouth disease outbreak and vaccination will reveal significant information. In the present investigation, blood samples from 7 adult Holstein–Friesian crossbred breeding bulls (*Bos taurus* × *Bos indicus*) of Germplasm center, Indian Veterinary Research Institute (IVRI), Izatnagar and 4 vaccinated Holstein–Friesian crossbred cattle were collected in RNAprotect® Animal blood tubes (Qiagen, Germany). To immunize cattle (cowsof Dairy Section, IVRI, Mukteshwar and young calves at experimental animal shed of PD-FMD) trivalent inactivated vaccine (FMDV; Type 'O', Type A' and Type 'Asia 1') was used. After incubation at room temperature for 2 h, these RNAprotect® Animal blood tubes were stored at −80 °C. Purified total RNA was extracted as per manufacturer's instructions (RNeasy® protect Animal blood kit, Qiagen, Germany). One blood sample of Mithun (*Bos gaurus*) showing clinical FMDV infection was also processed. Cytokine, IL-2 mRNA expression level in the total RNA was detected in one-step real-time polymerase chain reaction (PCR) (7500 real-time PCR, AB Applied Biosystems, USA) using comparative CT (ΔΔ) type of experiment (QuantiTect® SYBR® Green RT-PCR kit, Qiagen, Germany). Melt curve analysis was performed. Expression levels of IL-2 mRNA were depicted in the form of Relative Quantitation (RQ) values vs Target/Sample. Odd sample of Mithun showed lower level of IL-2 mRNA expression. Increased IL-2 mRNA expression levels were found in FMDV infected breeding bulls than the vaccinated cattle.

V and W Genes of NDV are not Apoptotic in HeLa Cells

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NDV, an avian virus is classified in the genus *Avulavirus* of *Paramyxoviridae* family. It causes economically significant disease in birds of various species. The NDV genome consists of six genes arranged in the order 3′-NP-P-M-F-HN-L-5′ which encodes for six structural and two non-structural proteins. The two additional non structural proteins, V and W are formed by the RNA editing process during P gene transcription. In this study, V and W genes of NDV amplified and cloned in eukaryotic expression vector pcDNA3.1 (+), were assessed for their apoptotic potential in human cervical cancer cell line (HeLa). The HeLa cells at around 70% confluency were transfected with pcDNA.ndv.v and pcDNA.ndv.w. The transfected HeLa cells were harvested at 24 h, 48 h and 72 h post transfection and analyzed for induction of apoptosis by DNA fragmentation, PI staining and Annexin v binding assays. No inter-nucleosomal cleavage was observed in the transfected cells even at 72 h post transfection indicating that these two genes did not play role in

induction of apoptosis by NDV. It was further confirmed the study of phosphatidylserine translocation in transfected cells using Annexin V binding by flow cytometry. The pro-apoptotic role of these genes (proteins) were also studied by staining transfected cells with propium iodide where flowcytometric analysis revealed slight decrease in sub-G1 peak (hypodiploid cell population) as compared with even vehicle and vector control, indicating that this protein is not involved in causing apoptosis rather it has anti-apoptotic role. In conclusion our study indicated that V and W lacks pro-apoptotic behaviour and seems to be play antiapoptotic role in HeLa cells.

Efficacy of Indigenous Sheeppox Vaccine (Srinagar Strain-38/00) with In-Use Commercial Vaccines: A Comparative Study

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Sheeppox, is a highly contagious viral disease of sheep and notifiable to OIE. Sheeppox is caused by sheeppox virus (SPPV), which belongs to the genus Capripoxvirus, subfamily *Chordopoxvirinae* of *Poxviridae* family. Vaccination is considered as the cheapest and best alternative to control the incidence of the disease and its ultimate eradication. Sheeppox live attenuated vaccines are highly immunogenic but their usefulness is limited because they stimulate a pock reaction and/or lead to the death of some of the vaccinated animals. Therefore, there is a need for the development and selection of efficacious and safe attenuated live indigenous vaccine to combat the disease. In the present study, sheeppox vaccine made from indigenous sheeppox virus-Srinagar, 38/00 (SPPV-Srin) attenuated in Vero cells at Division of Virology, IVRI, Mukteswar was compared in terms of their safety, potency, sero-conversion and protective dose with the commercial in-use vaccines, Roumanian Fanar (SPPV-RF), a foreign strain and Ranipet (SPPV-R), an indigenous strain adapted in primary lamb testes cells and calf thyroid cells respectively. Identity of SPPV vaccine strains were confirmed by PCR, PCR–RFLP and sequencing. Animal experiments were carried out in sero-negative sheep as per the OIE recommendations. The safety test indicated that the SPPV (Sri and RF) vaccines were safe while SPPV-R was found to be "hot"; not completely attenuated and caused excessive adverse reactions at the passage level tested. The immunized animals showed delayed hyper sensitivity reaction and resisted virulent SPPV challenge, while control animals developed disease. SPPV could be detected in the controls and animals immunized with lower dilutions of vaccines after challenge but not in any of the sheep immunized with 1 and 100 doses of each vaccine by PCR and real time PCR. All vaccines were found potent and the PD_{50} was highest for SPPV (Srin and R) followed by RF. The immunized animals were sero-converted following vaccination with sustained antibody responses after challenge was assessed by ELISA and serum neutralization test. In conclusion, indigenous SPPV-Srin vaccine was found to be as efficacious as SPPV-R and SPPV-RF vaccines, in addition to better safety. Thus, there is potential benefit in use of indigenous SPPV-Srin vaccine for control and eradication of sheeppox in India.

HSP70 Mediated Multi-Epitope DNA as Vaccine Candidate for Newcastle Disease of Poultry

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Newcastle disease virus is an infectious disease of poultry which causes severe economic losses in domestic poultry. New generation vaccines are currently in demand to avert deficiencies of vaccines currently in market. Among these, rational design of vaccine candidate is a promising approach. Multi-epitopic DNA vaccine construct containing fusion and haemagglutinin epitopes were designed with additional elements like endoplasmic reticulum secretory signal sequence, poly histidine and gene optimization to improve immunogenicity. Mycobacterium tuberculosis HSP70 was used as a genetic adjuvant to increase innate and adaptive immune responses. Transient expression of the construct was confirmed with an immunofluorescent assay in Vero cells. The constructs were injected subcutaneously into 15 days-old specific pathogen free chickens. Immune responses were studied with HI, ELISA, LTT and FACS assays. Protection was assessed by challenging with a virulent virus. HSP mediated multi epitopes improved the immune responses and conferred protection against Newcastle disease. The results indicate that a rationally designed DNA vaccine construct can improve vaccine strategies involving nucleic acid immunizations.

Ex vivo Effect of *Jatropha curcas* Leaf Extract on Replication Kinetics of Infectious Bursal Disease Virus

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Use of medicinal plant/herb extracts for viral infection has raised future of phytoantiviral therapeutic agent. *Jatropha curcas* Linn is a multipurpose, drought resistance, perennial plant belonging to Euphorbiaceae family. The present study was undertaken to observe the effect of *Jatropha curcas* leaf extract on replication kinetics of infectious bursal disease virus (IBDV) and also the mechanism of antiviral action was explored. Chick embryo fibroblast (CEF) adapted IBDV was treated with 50% methanolic extract of *Jatropha curcas* leaves and the viral replication kinetics was assessed by virus titration (TCID50), reverse transcription polymerase chain reaction (RT-PCR) and quantitative real time PCR. The antiviral action of extract was determined by observing the effect of extract on virion binding to host cell, RNA entry and damage to nucleic acid. The results indicated that 50% methanolic extract of *Jatropha curcas* leaves was found to inhibit viral replication possibly through direct antiviral effect of one or more of the constituents of the extract. The mechanism by which the extract of *J. curcas* inhibits viral replication was explored. The results showed that the extract inhibits viral RNA entry into host cell, when added simultaneously at the time of infection, whereas no effect on virion attachment to host cell as well as no apparent damage to RNA genome was observed. Thus these experiments demonstrated that antiviral activity of *J. curcas* leaves extract, the activity may be due to direct action of one or more of phytochemical present in extract which in part inhibiting viral RNA entry into host cell.

Bovine Picobirnaviruses: Detection, Phylogenetic Analysis and Development of RdRp Gene Based RT-PCR Diagnostic Assay

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Among the infectious agents associated with gastroenteritis, rotaviruses (RVs) are the major ones, while picobirnaviruses (PBVs) are

emerging viruses affecting number of mammalian and avian species. The present study describes the development of a diagnostic assay for the detection of bovine PBVs. A new RT-PCR assay was developed with the self-designed primers targeting RdRp gene (segment-2) with detection limit of 4.45×10^2 copies/μl of plasmid having specific 272 bps amplicon. This RT-PCR diagnostic assay was used to screen a total of 228 faecal samples collected from diarrhoeic bovine calves (176 from cattle and 52 from buffaloes) from different regions of the Indiaand the assay detected 5.26% samples for PBVs compared to 2.63% by RNA-PAGE. Further genotyping of PBVs presented the predominance of genogroup I while one sample, the first of its kind in the world revealed mixed infection of genogroup I and II in bovines. The sequence analysis of positive amplicons of bovine PBVs unveiled the highly diverse nature irrespective of their place of isolation. Grippingly, three clones from a single sample showed significant sequence divergence. These findings advocated the circulation of more than one strain of PBVs within a single host. Since the inadequate availability of PBVs sequences in public genome databases, our bovine PBVs isolates makes a separate cluster on phylogenetic analysis and some of the isolates displayed higher sequence homology with human and porcine isolates showing probability of interspecies transmission of the virus.

Loop Mediated Isothermal Amplification (LAMP) Assay for Rapid and Sensitive Detection of Capripox Virus

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A loop-mediated isothermal amplification (LAMP) assay was developed and evaluated for rapid, specific and highly sensitive detection of Capripox virus (CaPV) genome. The assay was optimized using purified viral genomic DNA and the reliable amplification was observed at 63 °C for 60 min using a set of four primers targeting *DNA polymerase gene of* CaPV. The amplified LAMP products was identified by gel electrophoresis and direct observation by naked-eye for presence of turbidity and colorimetric detection of the amplified products following the addition of SYBR Green I and Hydroxy Naphthol Blue (HNB) dyes. The specificity of the LAMP assay was assessed by amplifying 9 different strains of CaPV (5 sheep pox and 4 goat pox viruses) isolated in different geographical areas. No cross-reactivity with other related viruses, including orf virus (ORFV), buffalpox virus (BPXV) and camelpox virus (CMLV), was detected. The detection limit of LAMP is found to be 10 copies/μl of standard plasmid and 10 fold higher to standard gel based PCR (100 copies/μl). The assay was evaluated for detection of CaPV in field clinical samples ($n = 200$) and cell culture isolates ($n = 9$) and compared with conventional PCR and TaqMan probe based QPCR. As a field diagnostic test, the stability of LAMP reagents including primers and enzyme after exposure at 37 °C is checked and found to be stable for 5 days without affecting diagnostic efficacy of the assay. The study proves that the LAMP assay is a simple and cost-effective on-site diagnostic tool for rapid clinical diagnosis of capripox in sheep and goats suitable in resource limited field laboratories.

Selection of Amino Acid Substitution on the Capsid of FMD Virus Serotype O Propagated in Vitro Under Vaccinal Serum Antibody Pressure

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Foot and mouth disease (FMD) virus is constantly evolving under neutralizing antibody pressure in either naturally infected or vaccinated animal population of disease endemic India. So to understand the dynamics of evolution of antigenic sites this simulation study was carried out. Isolation and characterization of neutralizing antibody-resistant populations of 3 strains of FMD virus serotype O (INDR2/1975, IND 120/2002, IND271/2001) was carried out by serial propagation on BHK21 cells in the presence of subneutralizing level of bovine vaccinal sera (BVS). The partial neutralization escape variants showed many characteristic changes like increased resistance to neutralization by BVS, reduction in plaque size, selection of amino acid substitutions on loops and termini of capsid protein. A minimum of one and a maximum of four residue substitution were observed in any of the neutralization resistant virus population. Fixation of aa substitutions were observed at critical residues of all established antigenic sites of type O [144 (site 1), 45 and 48 (site 3), 72 and 132 (Site 2)] except site 4 and 5. Besides, substitutions were also observed in proximity to the identified residues within antigenic sites of serotype O or other serotypes which could be significant in terms of neutralizing antibody binding and immune escape [41 and 51 (B–C loop), 133, 140 and 143 (G-H loop), 201, 204 and 209 (C termini) of VP1, 71 and 75 (B–C loop), 131 (EF loop), 174 and 179 (G-H loop) and 219 (C termini) of VP3]. In majority of the virus-serum regimens site 3 was found to be substituted in the populations. Variants could be identified with substitutions at site 2 or 3 only but site 1 variants were always accompanied by substitutions elsewhere on the capsid. Changes observed in VP3 region were always associated with substitutions at VP1 suggesting a minor role for such residues which act in synergy with other sites towards the neutralization escape phenotype. Presence of substitutions at the same locations as identified in this study in the Indian field isolates supports the importance of these sites.

A Single Amino Acid Substitution Preceding RGD-Motif in the VP1 βG-βH Loop Confers Resistance to Antibody-Mediated Neutralization of Serotype A FMD Virus Infectivity in Vitro

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Emergence of antigenic variants during the course of evolution of foot-and-mouth disease virus (FMDV) under immune pressure exerted in the host population of an endemic region jeopardizes the prospect of vaccination-based control programmes. Here, the Indian serotype A FMD vaccine strain A IND 40/2000 (P0) was serially propagated 50 times (P50) in BHK-21 cells in presence of an increasing concentration of homologous anti-A IND 40/2000 bovine convalescent serum (BCS) to isolate and characterize neutralizing antibody-resistant virus populations. The majority capsid coding region sequence at every fifth passage was derived from PCR amplicons for both virus populations grown in presence and absence of antibody pressure. A single amino acid substitution with a neutral-positive charge shift $\{Thr_{143}\ (AC_{427}A) \rightarrow Lys(AA_{427}A)\}$ was observed at -1 position before the RGD-tripeptide motif in the VP1 βG-βH loop in a region analogous to the antigenic site 1 of serotype O virus at P_{20} and remained fixed till P_{50}. In the entire control passage regimen without antibody pressure, the position remained unchanged as T_{143}. Similar amino acid footprints have been mapped in the monoclonal antibody neutralization resistant mutants of type A_{10} ($R_{142} \rightarrow G$), A_{22} ($R_{144} \rightarrow S/G$) and A_{24} ($R_{143} \rightarrow G$) viruses. When the P1 sequence dataset of serotype A Indian field outbreak viruses were aligned, VP1 143 position revealed as many as 5 amino acids (T,

R, G, A, V). Interestingly, a positively charged residue 'R' was seen exclusively in genotype 16 and 10, while genotype 18 including the vaccine strain showed T_{143}. When the P50 genetic variants along with its parent population (P0) were subjected to two dimensional micro-neutralization test and plaque reduction neutralization (PRN) assay using the BCS, a considerable reduction in the SN_{50} titre ($\sim$0.47 $\log_{10}$ titre) and PRN_{70} titre ($\sim$0.32 $\log_{10}$ titre) was noticed. Hence, it is presumed that amino acid substitution at VP1 143 position might have played a role in luxuriant growth of the virus in presence of neutralizing antibody and only reiterates the significance of this position in the formation of the neutralizing antigenic site.

Association of *BoLA DRB3* Alleles with Variability in Immune Response Among the Crossbred Cattle Vaccinated for Foot-and-Mouth Disease (FMD)

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Polymorphism of bovine leukocyte antigen (*BoLA*) *DRB3* gene is being intensively investigated for potential association with economically important diseases of cattle. Accordingly, we investigated the association of *DRB3 Exon 2* polymorphism as evidenced by the variation in the binding pockets with variability in immune response to inactivated trivalent (O, A and Asia1) Foot and Mouth Disease virus (FMDV) vaccine in a closed population of crossbred cattle. Antibody titer of $\geq$1.8 was set as the cut off value to distinguish the protected ($\geq$1.8) and unprotected (<1.8) animals. Eleven different alleles of over 3% frequency were detected in the population. We found that *DRB3* alleles *0201, *0801 and *1501 always ranked high for protective immune response whereas alleles *0701, *1103 and *1101 consistently ranked low for unprotected immune response for all the three serotypes. Rank correlation of *DRB3* alleles among the three serotypes was positive, high in magnitude and statistically significant ($p < 0.05$). Logistic regression analysis revealed that odds of protection from the vaccine were highest for all the three serotypes if allele *1501 was present and strengthened the results of allele ranking. Predicted amino acid substitution in the peptide binding pockets revealed that all the important sites had high Wu-Kabat index. Similarly, specific residues in pockets were crucial for immune response to FMD vaccine. There were specific substitutions in un-protected alleles such as absence of acidic amino acids substituted by basic amino acid at β71, presence of non-polar cysteine or basic histidine at β30 and presence of polar tyrosine at β37. From the observations, we hypothesize that the substitutions lead to unique conformational changes in the protein products of the studied alleles that would associate with the protective or unprotective antibody response to FMDV vaccine. The knowledge has potential implications in future selection programs if integrated with the complete *BoLA* haplotype details and production traits of the herd.

Analysis of Capsid Coding Region of Foot-and-Mouth Disease Virus Serotype Asia 1 Field Isolates

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Foot and mouth disease virus serotype Asia1 accounts for about 12% of the FMD outbreaks recorded in India. Five serotype Asia1 isolates collected from different place were analyzed at capsid coding region. For three isolates (PD509/2010, PD18/2011 and PD155/2011), sequences were generated directly from clinical materials. For two isolates (PD508/2010 and IND327/2009), sequences were generated from cell culture supernatants. The integrin binding ligand 'RGD' tripeptide motif was conserved in all the five isolates. The leucine residue at position +4 and alanine at position +2 were 100% conserved in all isolates compared. But the leucine at +1 position revealed frequent variations. Methionine was found conserved in all the four isolates except PD155/2010 in which it was L. R56 in VP3 and R135 in VP2 thought to be critical for heparan sulfate binding were 100% conserved in all the isolates. High degree of conservation of these residues indicates that this virus can utilize cell surface heparan sulfate to gain entry into the cells. The S73 euroasiatic signature in VP4, residues predicted to be important in 1A-1B cleavage and histidine residues at positions 21, 87, 145, 157 and 174 in VP2 that mediate H-bonding at 1B/1C interphase were conserved. The cleavage sites for FMDV type Asia1 isolates from this analysis are ↓GAG for L/P1, ALLA↓DKK(R)T for 1A/1B,PSKE↓GIVP for 1B/1C, ARR(Q)E(Q)↓TTT(A)T for 1C/1D, PEKQ↓ for 1D/2A. The residues critical for antigenic sites were fully conserved. At least one residue in VP2 (56) and six amino acid sites in VP1 (35, 45, 47, 119, 132 AND 169) was found to be under positive selection. In similarity plot and boot scan analysis, no evidence of recombination was detected. In Maximum Likelihood tree all the isolates were clustered in Lineage C which has been in circulation in India since 2005. No discrepancy in topology between VP1 and P1 was observed.

Intrinsic as Well as Extrinsic Thermo-Stability Evaluation of a Vero Cell Attenuated Camelpox Vaccine

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Camelpox is the contagious skin disease of camelids caused by camelpox virus (CMLV) and the disease causes economic impact in terms of morbidity, mortality, loss of weight and reduced milk yield. Both inactivated and live attenuated vaccines are available in few countries. Considering the disadvantages with inactivated vaccine, a live vaccine is the best choice as a long-term solution towards control of camelpox. With the right earnest, a Vero cell attenuated live vaccine from an Indian isolate of CMLV (named CMLV-1) has been developed recently at IVRI, Mukteswar and it was found safe, potent and efficacious. But a study with respect to thermo stability has not been undertaken. Here a research was carried out on evaluation of thermo stability (with and without extrinsic stabilizers) of the vaccine virus. Though the pox viruses are thermo-stable in general, almost reports on the stability of camelpox vaccine virus are found to be nil. On evaluation of intrinsic thermo-stability, the current indigenous isolate was found to have an expiry period of only 9 days and 4 days at 37 °C and 45 °C, respectively. Hence, a study was carried out to analyse the stability of CMLV vaccine with extrinsic stabilizer combinations to use it on the field for a long period. A total of three stabilizers were used for freeze-drying the vaccine and stability of both freeze-dried and reconstituted vaccine was tested at different temperatures. It revealed that the camelpox vaccine lyophilized with TAA stabilizer (Trehalose dehydrate with amino acids and divalent cations) appeared relatively superior with an expiry period of 44 months at 4 °C, 215 days at 25 °C, 22 days at 37 °C and 20 days

at 45 °C. Among the three stabilizers, BUGS stabilizer appeared relatively inferior to others at all temperatures. Further, among four diluents viz. PBS, 0.85% NaCl, distilled water and 1 M $MgSO_4$ used for reconstitution of the vaccine, PBS appeared better followed by 0.85% NaCl for reconstitution of the vaccine.

Bacteriophage Therapy: Back to the Past

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Bacteriophages are viruses which infect bacteria. Even though phages were recognized anti-bacterial agents in the 1920s, the advent of broad spectrum antibiotics overwhelmed it. But with the start of development of antimicrobial resistance and destruction of commensal microbes by broad spectrum antibiotics, it is time to close the chapter of "antibiotics" and start a new era with bacteriophages. Compared with antibiotics, phages are able to replicate and make themselves available in the target site, able to mutate and counteract bacterial mutations, very specific towards target species and very low concentrations are necessary for action. In veterinary field, phages are used against bovine mastitis, enteric infections in calves and poultry.

Serological Evidence of Foot-and-Mouth Disease Virus Circulation in an Organized Sheep Farm at Bikaner, Rajasthan, India

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This work reflects the serological evidence of foot and mouth disease virus (FMDV) circulation in an organized sheep farm at Arid Region Campus, Central Sheep and Wool Research Institute, Beechwal, Bikaner, Rajasthan. A total of 524 (175 Magra and 349 Marwari breed) sheep serum samples (125 in February and 399 in August 2011) were collected and subjected to 3AB non-structural protein (NSP) ELISA. A high, but comparable percentage of NSP antibody prevalence {(44/125 (35.20%) and 122/399 (30.57%)} was found at both time points 6 months apart indicating an exposure of animals to FMD virus before first collection. In past there had been procurement of sheep into the farm from local sources. The serum samples were also tested in liquid phase blocking ELISA (LPB ELISA) to assess the level of structural protein (SP) antibody titre against FMDV serotypes O, A and Asia 1. The overall herd immunity ($\log_{10}$ titer of $\geq$1.8 for all three serotypes) in the farm was found to be 0% (0/125) and 29.32% (117/399) for the samples collected at two different points of time as mentioned. The farm was under the practice of 'once annual' vaccination against FMD, which was done in the month of May, 2011. Declining herd immunity as evident from low SP antibody titre might have made the farm susceptible to FMDV exposure. Hence, a 'biannual' vaccination schedule may be more suitable to build up optimum level of protective antibody against FMD.

Adverse Effect of Foot-and-Mouth (FMD) Vaccination on Ejaculates Characteristics of Holstein–Friesian Crossbred Bulls

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Present investigation was conducted on eight Karan-Fries bulls maintained at A.B. Complex, NDRI, Karnal, India from October, 2011 to December, 2011 to study the effect of FMD vaccination on semen quality in KF bulls. A total of 48 ejaculates were taken before vaccination while a total of 112 ejaculates collected after vaccination to study the effect of vaccination stress on semen quality parameters. There was significant increase in rectal temperature during post vaccination up to one week. FMD vaccination had no significant ($p = 0.101$) effect on ejaculate volume and CMA_3 positive spermatozoa, which represents chromatin integrity. After FMD vaccination there was significant decrease in mass activity, sperm motility, live spermatozoa, sperm concentration, HOST positive and acrosomal integrity, which represents the structural defect in spermatozoa during epididymal maturation stage. After three weeks post-vaccination more than 50% bulls have produced satisfactory acceptable fresh semen quality. There was no correlation between antibody titre to different serotype FMD vaccine with fresh. When the bulls are classified into sire bred HF ($n = 3$, Group I) and sire half bred ($n = 5$, Group II) on the basis of exotic genetic level the data revealed that vaccination had more detrimental effect on fresh and frozen semen of Group I comparison to Group II. The testosterone concentration was higher in all the bulls during 1st week after vaccination compared to the pre-vaccination period, which may be due to stress immediately after vaccination and reduced to normal level after one week. No significant correlation was found between testosterone concentrations and semen characteristics.

Viral Infections in Fish: The Role of Toll-Like Receptor 3 (TLR3) in Sensing Viral Double Stranded RNA and Innate Immunity

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Many RNA viruses, viz., viral hemorrhagic septicemia virus (VHSV), grass carp reovirus (GCRV) and infectious hematopoietic necrosis virus (IHNV) infect a wide variety of fish species, and their ds (double stranded) RNAs are expected to be recognized by TLR3. We investigated molecular interaction between viral dsRNA and TLR3 inrohu fish (Labeorohita) following full-length TLR3-cDNA cloning by rapid amplification of cDNA ends (RACE). The domain architecture in rohu TLR3 (rTLR3) was analyzed by SMART, Pfam and SignalP3.0, and the leucine rich repeat regions (LRR) were manually identified by locating "LxxLxLxxNxL" motifs. The 3D-structure of TLR3 was created by homology modeling and molecular dynamics (MD) simulation was carried out in GROMACS 4.0.3 program. In rTLR3, poly I: C (synthetic dsRNA) binding regions were predicted in AutoDock4.0 and GOLD4.1, and the dsRNA of GCRV, VHSV and IHNV binding regions were identified by HADDOCK. Analysis of dsRNA-mediated TLR3-signaling cascade was carried out by intravenous injection of poly I: Cinrohu fingerlings, and quantitative real-time PCR analysis of type I IFN, Mx, TNF-α and IL-1β genes expressions. The full-length rTLR3-cDNA comprised of 873 amino acid (aa) residues with a signal peptide of 22 aa. The mature rTLR3 comprised of ectodomain (ECD), trans-membrane (TM) domain, and TIR domain with 706, 23 and 117 aa respectively. The horseshoe-shaped ECD of rTLR3 consisted of 27 LRRs, and structurally resembled human TLR3-ECD. In rLTR3-ECD, the peptides at LRR 4–6, 13–14 and 20–22 were predicted as poly I: C binding sites, and LRR 8–15 and LRR17–24 were identified as GCRV, VHSV and IHNV-dsRNA binding sites. Injection of polyI:Cactivated TLR3-signaling resulting in significant ($p < 0.05$) up-regulation type I

IFN, Mx,TNF-α and IL-1β genes expressions. These data together highlight the conserved function of TLR3 in recognizing viral infections and innate immunity from lower to higher eukaryotes.

Expression of Interferon and Interferon-Induced Genes in Clown Fish Cell Line

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Role of innate immune system is crucial in the non specific first line antiviral defence in fish, which co-ordinate with adaptive immunity for the control of infection. Betanodavirus infection in fish is a very serious neuropathological viral disease causing large scale mortalities in marine fishes. We have developed cell lines from marine finfish Amphiprionsebae (Clown fish) and used for finding out the effect of viral infection in the expression of type I interferon and interferon stimulated genes following pre-treatment with different synthetic TLR ligands such as poly I:C and CpG ODNs. Among the different genes investigated (Type I Interferon (IFN), ISGs (Interferon stimulated genes) such as Mx, ISG-15, IRF-3 and viperin), interferon stimulation was more or less uniform in all the treatments, while virus infection and poly I:Ctransfection had higher immune gene expression compared to CpG ODN. Induction of ISG-15 was higher in poly I:C while virus infection alone had higher expression of viperin (Vig-1). Poly I:C and CpG pre-treated cells upon virus infection showed high induction of all except the ISG-15 genes tested in poly I:C treatment with highest expression of 25.36 fold for Vig-1 gene followed by 11.94 fold for ISG-15. CpG pre-treatment also showed similar upward trend of gene expression (13.59 fold) for Vig-1 gene and 12.75 fold increase for ISG-15 gene. Other immune genes exhibited lesser induction compared to Vig-1 and ISG-15. The study indicated that poly I:C and CpG ODNs could induce increased innate immune response in clownfish against betanodavirus. The increased expression of immune genes would help enhance the antiviral defence in live fish and could act as potential immunostimulatory compounds and also as adjuvants in vaccination trials.

Emerging Viral Diseases in Brackishwater Finfish Aquaculture in India

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Viral nervous necrosis (VNN, also known as viral encephalopathy and retinopathy-VER) is one of the emerging viral diseases of finfishes caused by Betanodavirus belonging to the family Nodaviridae. Betanodavirusis one of the two genera making up the family Nodaviridae infecting fish along with Alphanodavirus infecting insects. This RNA virus is icosahedral (25–34 nm) infects a large range of host species, at least 40 species of marine and freshwater fish world-wide and the known host range, continues to expand as new species of fish are used for aquaculture. Of further interest is the potential of wild fish to become sub-clinical carriers as virus-contaminated water spreads from aquaculture enterprises into the marine environment particularly for those countries with large mariculture industries. In India, the virus has been reported from Asian seabass and freshwater ornamentals. A study revealed that 18.5% ($n = 243$) of the fish samples from wild, hatchery and farmed samples of fish species from different geographical locations were positive for the virus of the genotype RGNNV. The cultured fish species (seabass, mullet and milkfish etc.) revealed 8% infection in the form of sub-clinical infection. Characterization such as the morphology, growth study on cell line, temperature sensitivity, EM, partial nucleotide sequencing etc. has been undertaken and will be discussed. Iridovirus is a large double-stranded DNA virus with icosahedral symmetry, and ranges in size from 120–200 nm in diameter. Family Iridoviridae comprises three piscine iridoviruses, including the genera Lymphocystivirus, Ranavirus, and Megalocytivirus, all of which are known causative agent of fish iridoviral diseases. Among them, Ranavirus and Megalocytivirus are always associated with mass mortalities. The genus Ranavirus is having a broad host and geographic range and it causes Epizootic haematopoietic necrosis in fishes. Another group of emerging iridoviral disease, Red sea bream iridoviral disease causes considerable mortality in both farmed as well cultured marine fish of around 30 species. The most characteristic histopathological change in target organs viz., liver, spleen and kidney include the presence of basophilic, hypertrophied cells, often in large numbers. The iridoviral diseases are mainly diagnosed by gross lesions, histopathology, immunohisto-chemistry, PCR and virus isolation in cell culture. Lymphocystis is a common, chronic and benign infection caused by an iridovirus (Lymphocystivirus) affecting many species of teleosts world wide including India. They results in uniquely hypertrophied paraffin like nodules in the skin and fins. Mixed infections of iridovirus and betanodavirus are also in literature and found in Indian waters also. Co-infection of two different species of viruses affecting same fish host from sub-clinically affected seabass and milk fish under culture has been observed rarely. However, persistent and latent infections is the feature in field until adverse environmental conditions, such as overcrowding, temperature or poor water quality trigger the onset of disease in aquaculture sector.

Detection of Exotic Mourilyan Virus in Indian Shrimp Culture Farms

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Mourilyan virus (MoV), a virus which is closely related to Bunyaviruses was first detected in Penaeusmonodon shrimp from Eastern Australia. MoV is an enveloped negative-sense ssRNA virus containing four genome segments which includes the L RNA segment encoding the RdRp, the M segment encoding two transmembrane glycoproteins (G1 and G2), and two small segments (S1 and S2) the nucleoprotein (N) and a small non-structural protein (NSs2). In the present study, we confirm for the first time the natural prevalence of MoV in India. The shrimp (*P. monodon*) from the culture ponds located at East coast of India were found positive for MoV using gene specific primers by RT-PCR. The shrimp collected from these farm appeared healthy with no visible signs of any disease. The sequence analysis of the G2 virion envelope glycoprotein gene PCR product of MoV showed close similarity to the Australian isolate of MoV. The viral gene isolated from *P. monodon* exhibited 76% nucleotide and 81% amino acid sequence identity on comparison. The amino acid sequence obtained for Indian MoV isolate revealed 39 amino acid replacement on comparison with Australian MoV isolate. Virus presence could be detected in various shrimp tissues such as gills, gut, hepatopancreas, muscle and haemocytes. However, due to the sequence variations present in the Indian isolate of MoV, it may be necessary to design internal primers based on the G2 virion envelope glycoprotein gene sequence specific to Indian isolate of MoV to detect low level of MoV infection in shrimp.

Characterization and Expression Analysis of Toll Like Receptors 3 (TLR-3) and Its Role in Recognizing Viral PAMPs in *Barilius bendelisis*

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Induction of innate immune pathways is critical for early host defense, but there is limited understanding of how teleost fishes recognize pathogen molecules and activate these pathways. Toll-like receptors (TLRs) have emerged as crucial sensors of invading microbes through recognition of pathogen-associated molecular patterns (PAMPs) in viruses, bacteria, fungi and protozoa. TLR3 is known to recognize double stranded RNA in humans, mice, pigs and fishes, which is a viral PAMP. In addition it is induced by bacterial as well as single stranded RNA viruses in fishes, indicating its role in recognition of bacteria and certain viruses. *Bariliusbendelisis,* is a coldwater fish that inhabits the streams and rivers and is a member of family*Cyprinidae*. In order to find out the homology between the TLR3 of this fish with other members of *Cyprinidae*, we designed a set of primers and amplified a fragment of around 1 kb. This amplicon was cloned in pTZ57R/T vector and sequenced. Analysis of the sequence revealed that it was 85% identical with TLR3 of *Daniorario.* Further to characterise this gene, the complete nucleotide sequence has to be worked out. This would enable the characterization of its agonists, its expression analysis before and after induction. The outcomes of the study will reveal the possible role of TLR3 in viral recognition in *Bariliusbendelisis*.

Homology Modelling of Infectious Hematopoietic Necrosis Virus Glycoprotein

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Computational biology is an important tool in deciphering the structure of a target protein when X-ray structure is unknown. Infectious hematopoietic necrosis virus (IHNV) causes a serious disease in salmonids fish like trout and salmon. The glycoprotein of this virus plays an important role in attachment and fusion. However the X-ray structure of this glycoprotein is not yet known. Study shows that glycoproteins of viruses are involved in entry and eliciting protective immunity. Therefore, the understanding the structure besides function of this proteins is important in order to find the specific sites on viral proteins which binds to precise receptors on cell surface. The*in silico* 3D model can help to understand the structure of this glycoprotein so that we could get more information about its active sites. The physiochemical characteristics of these glycoproteins were determined to predict protein structural and functional classes and performance. Using ExPASy program, the signal peptide and transmembrane regions of the proteins were identified. The structure was obtained through homology modelling using MODELLER 9.11 and SWISS-MODEL. Model optimization, quality assessment and visualization was done using combination of several in silico approaches like GROMOS96[10] force field in Swiss-Pdb Viewer, What IF Web Interface, ERRAT.

Molecular Cloning and Characterization of Important Antimicrobial Peptide Genes of Golden Mahseer *(Tor putitora)*

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Infectious diseases are the bottleneck in development of aquaculture industries worldwide. Innate immune system is the major host defence system of fish and antimicrobial peptides (AMPs) are the unique defence peptides of this defence system. They are around 30 residues in length and cationic in nature. AMPs have broad spectrum activity and are generated within few minutes of microbial infections. As the AMPs are generated within the host, these natural products act as alternative endogenous antibiotics to combat infectious diseases. Conventional antibiotics are associated with development of antibiotics resistant pathogens and side effects but it has been observed that AMPs are not associated with such limitations. Therefore, AMPs can be used as alternatives to antibiotics in aquaculture for the treatment of infectious diseases as they have no such limitations. AMPs have immunomodulatory and anti-inflammatory activities. In response to viral infections, AMPs are known to stimulate the expression of TLR3 thus generate antiviral state in fish.

Japanese Encephalitis Virus Induced Altered Expression of Host Cell Genes: An Orchestrated Interplay of Cell–Virus Interaction

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Massive neuronal destruction in a Japanese encephalitis (JE) infected individual results in neuro-cognitive malfunction. This could be caused by direct hit of neurons or alterations in the infected host cell physiology. The present study was undertaken to investigate JE virus mediated alteration in functional gene expression profiles in mouse neuroblastoma cells (Neuro2A). Microarray analysis was performed on total RNA extracted from JEV infected Neuro2a cells. TUNEL assay and Real-time PCR were employed to validate microarray results. An upregulation of 660 and downregulation of 949 genes was observed in JEV infected Neuro2a cells in the microarray analysis. Genes involved in apoptosis, oncogenesis, metabolism, neurodegeneration and immunological functions were found to be differentially expressed. Pro-apoptotic genes (p53, VEGF, Gadd45) were upregulated while anti-apoptotic gene bcl-2 was downregulated. Similarly, alteration of expression of genes associated with neurodegeneration was observed. Upregulation of Hsp5, Enah, Btg2, Rassf and Apo e while downregulation of Casp6, Aplp1, Atp1a, Rtn4 and Nde1 noticed was validated in terms of fold change using threshold cycle number obtained from real-time PCR. TUNEL assay, DNA fragmentation and Real time PCR confirmed apoptosis in infected cells. Downregulation of expression and subsequent functional pathway of genes associated with neuronal regulation may result in improper neurogenesis, synaptogenesis, prevent neuritic growth and promote neurodegeneration.

Development of SYBR Green I Based Real-Time RT-PCR for Chikungunya Virus and Assessment of Replication Kinetics in *Aedes aegypti*

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Global expansion of CHIKV endemic areas in recent years can be ascribed to increasing vector population, adaptability of virus to new vector and new geographical areas. Here, we developed a SYBR Green I based quantitative real time RT-PCR assay and applied for vector surveillance and to study the virus vector interactions. In the present study, SYBR Green I based quantitative real time RT-PCR assay was developed and validated with field caught mosquitoes. The detection efficiency of this assay was confirmed in mosquito pools of different pool sizes. Cross reactivity studies were done with

related alphaviruses and flaviviruses. This assay was further adopted to assess the replication kinetics of CHIKV at different anatomical sites in *Ae. aegypti* at different days of post infection (pi). The detection efficiency of the assay was impervious to mosquitoes of different pool sizes. Vector surveillance has resulted in detection of CHIKV RNA in *Ae. aegypti* pools, confirming its vectorial potential for CHIKV in northern India. The assessment of the assay was further carried out by studying the competence of Indian *Aedes aegypti* for CHIKV, which revealed a cent percent infection rate and dissemination rate with 60% transmission rate. The replication kinetics of CHIKV in different anatomical sites of *Aedes aegypti* revealed the highest mean titre of CHIKV RNA at day 6 pi in midgut and at day 10 pi in saliva, legs and wings. The implementation of the assay in detecting lower viral load makes it a remarkable tool for surveillance of virus activity in mosquitoes with higher sensitivity and specificity. The identification of infected mosquito is considered as a potential indicator of an impending outbreak. Since, there is a paucity of vaccine or antiviral drugs for chikungunya virus, early and sensitive detection will help in proper management and will arrest spread of virus among human population.

Evolutionary Dynamics of West Nile Viruses: Molecular Clock and Selection Pressure Analyses of Different Genes

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Flaviviruses are a useful model for studying the evolution of vector-borne diseases as their evolutionary characteristics are believed to have been determined through a combination of constraints imposed by the arthropod vector and the vertebrate hosts. It is not fully known which genes of these viruses have evolved for adaptability in different hosts and what is the extent of selection pressure. In this study, we determined the evolutionary timescales of West Nile (WN) viruses and compared all the genes of WNVs to identify patterns of selection pressure. The analyses was carried out using full genomes, E–gene as well as structural and non-structural regions separately, considering 39 representative sequences of all WNV lineages, 1 to 5. Molecular clock analysis was done using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST and selection pressure analysis was performed by using the methods available in the Datamonkey web server. The rate of nucleotide substitution ($\sim$6–8 $\times$ 10^{-4} subs/site/year) and mean root age of all WNV lineages was comparable in case of E-gene, structural and non-structural region. Based on the E-gene, the estimate for the mean age of dominant lineage 1a and lineage 5 which includes Indian isolates (1957, 1980) was found to be 1911 and 1927 respectively. No evident selection pressure was noted in the structural region while selection pressure was noted in the non structural region at NS3-249 and NS4A-85. Mapping of known T-cell epitopes on the NS3 structure showed that the positively selected site NS3-249P/T/A was not associated with the known epitopes. However, it has been reported that this site is responsible for avian virulence in WNV. Overall, molecular clock as well as selection pressure analyses collectively showed that the WNV evolution is constrained by purifying selection. Similar studies in the context of additional Indian isolates need to be carried out in future.

Identification of Human Protein Disulphideisomerase (PDI) as a Dengue Virus Non-Structural 1 (NS1) Interacting Protein

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Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are severe forms of dengue fever caused by dengue virus, which is an enveloped, positive sense, single stranded RNA virus having 3 structural and 7 non-structural proteins. So far, no vaccine or treatment is available to combat the virus replication. In our previous study we observed increased expression of an endoplasmic reticulum resident chaperone protein, PDI which helps the virus to multiply in the cell. Identification of interaction/s between PDI and structural and non-structural proteins of DENV to understand how the virus uses host machinery to replicate and persist within the cell. DENV type 2, New Guinea C (NGC) strain infection was established in human monocytic cell line THP-1. Optimum dose for productive viral replication was standardized at multiplicity of infection (MOI) 3, 48 h post infection (p.i), which was confirmed by Image flow cytometry, FACS analysis and western blotting for DENV envelope and NS1 proteins. Host protein PDI and viral protein interactions were screened by co-immunoprecipitation using PDI antibody to pull down interacting proteins from the whole cell lysate. Apoptotic death in DENV infected cells increased in a dose and time dependent manner, and was maximum at MOI-3, 72 hrp.i. We found that PDI expression in association with lipid rafts on the cell membrane was increased during DENV infection. Treatment with cholesterol inhibitor (Methyl Beta cyclodextrin) led to decreased expression of lipid rafts and surface PDI in DENV infected cells. Furthermore, interaction of viral proteins with PDI was screened and it was found that host protein PDI interacts with viral NS1 protein. The present study suggests that PDI interacts with viral NS1 protein hence, inhibition of PDI and NS1 interaction could lead to decreased DENV load and it could be an important therapeutic target for the management of DHF and DSS.

A Study of Concurrent Outbreaks of Dengue and Chikungunya in Delhi

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Dengue is the most notable mosquito-borne viral disease in India, the incidence of which has increased several folds in the past few decades. Many states in this region are endemic for Dengue viruses (DENV). Recently, several states in the country reported large scale Chikungunya virus (CHIKV) outbreaks. Simultaneous CHIKV and DENV outbreaks have rarely been observed. This study investigated the twin outbreaks of dengue and Chikungunya in Delhi. In 2010, Delhi witnessed a large Dengue outbreak. During the same period, large number of patients reported to health centers with acute fever and arthralgia identified as due to CHIKV. Materials & Methods: This study was conducted in the virology laboratory of Maulana Azad Medical College and associated Lok Nayak Hospital, Delhi from July 2010 to February 2011. A total of 2038 serum samples collected from suspected febrile dengue patients were tested for the presence of anti-dengue IgM antibody while serum samples of 324 patients presenting with fever, arthralgia, myalgia, rash were tested for anti Chikungunya IgM antibody. These tests were conducted using IgM capture ELISA kits provided by NIV Pune. Conclusion: 746/2038 (31.2%) were positive for anti Dengue IgM antibody while 148/324 (45.6%) samples tested positive for anti Chikungunya IgM. Distinct differences were observed in the two outbreaks. Dengue affected more males M:F ratio being 1.7:1. Chikungunya affected more females, M:F being 1: 1.34. Characteristically, maximum Dengue cases (34.2%) occurred in the paediatric age group ($\leq$15 yrs.) followed by 21–30 years olds. Pediatric age group was least affected in Chikungunya. There was an

overlap of clinical symptomatology in both infections. This report confirms emergence and establishment of CHIKV in northern region of India, which is also endemic for dengue viruses. In future, this region could witness more of such outbreaks with serious social and economic implications thus emphasizing the need for an early detection and containment measures.

Characterization of West Nile Virus Prevailing in Assam, India: Insight into Genetic and Antigenic Properties of the Circulating Strain

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West Nile Virus (WNV) is a mosquito-borne flavivirus causing subclinical/encephalitis symptoms. It can be classified into 8 putative genetic lineages which differ considerably in virulence and neuroinvasiveness. Since WNV was first serologically detected in Assam during the year 2006, it has spread to newer areas within northeast India bordering South East Asia. The purpose of this study was to establish the circulating strain/lineage of the WNV and to analyse its antigenic properties. Attempt was made to isolate WNV from cerebrospinal fluid (CSF) of acute encephalitis syndrome (AES) patients. Genotyping was done based on partial gene region encompassing 921 nucleotides (C-prM-E) of the WNV genome. To study antigenic properties, cross neutralization was performed by employing plaque reduction neutralization test (PRNT) with polyclonal sera raised against respective WNV. Swiss albino mice models were used to assess the pathogenicity of the isolates. Two isolates were obtained and confirmed to be WNV by genotyping. The mean genetic distance within the study isolates (JQ037832 and HQ246154) was 0.02% at the nucleotide level. Derived amino acid sequence alignment showed substitution in A81T and A84P of the capsid region in HQ246154. Phylogenetic analysis placed both the study isolates under lineage 5 along with other Indian strains isolated till 1980s; however, it forms a distinct sub-clade showing a variant. Cross neutralization suggests antigenic variation between the isolates. Pathogenicity analysis in mice showed the circulating WNV to be neuroinvasive and pathogenic in nature. The study established the circulating WNV in eastern India and demonstrates circulation of two distinct WNV variants in Assam. In will be interesting to observe whether Lineage 5 of WNV remains confined to the Indian belt or overtime spills over to bordering South East Asian countries.

Entomological Investigation of Chikungunya Virus Outbreak in Jamshedpur City, Jharkhand

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Entomological studies were conducted during an outbreak caused by Chikungunya virus at Jamshedpur city, Jharkhand during 2011. Breeding of *Aedes aegypti* mosquitoes was encountered in all the affected areas. Drinking water containers were comparatively free from mosquito breeding. Immature stages of the vector were mainly found in non potable water containers kept outdoors. Large number of larvae and pupae were observed in those containers which have been dysfunctional and stacked in the backyards of houses. Presence of *Ae. aegyptipupae* in the indoor and outdoor containers, high density of adult female mosquitoes and the crowded housing settlements seems to have added to the risk of virus transmission and spread. Numerous tyre vulcanizing shops on the streets have added to the *Aeaegypti* breeding spots where mosquito breeding and biting activity was observed. Adult mosquito catch from Jamshedpur was processed in the laboratory for virus isolation in C6/36 cell line, but virus could not be isolated. A colony of *Aedes aegypti* population from Jamshedpur was established in the laboratory and F1 generation adult female mosquitoes were infected with chikungunya virus by membrane feeding technique. After an incubation period of 8 days, the mosquitoes were screened by immunofluorescent antibody test. Percent positivity was found to be 58%. Insecticide susceptibility of Jamshedpur the mosquitoes was also carried out by dose–response assay on 3rd–4th stage larvae. Insecticide tolerance was observed in Jamshedpur strain to DDT, malathion and delta-methrin when compared with Pune strain of *Ae. aegypti* (Probit analysis). Approx 1–2 fold increase in LD50 values was observed in Jamshedpur strain as compared to Pune strain.

Chikungunya Virus Structural Polyprotein Expressed in Yeast Forms Virus Like Particles

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Chikungunaya virus (CHIKV) is the prototype of the alphavirus genus of the family *Togaviridae* that gets transmitted by *Aedes albopictus* and *Aedes aegypti* mosquitoes. CHIKV is an enveloped particle with single-stranded RNA genome of positive polarity and has a genome size of approximately 12 Kb. In India, the outbreak of CHIKV disease caused more than 1.3 million cases in 13 states during 2005–2007 after 32 year absence. The severity of the disease and spread of this epidemic virus present a serious public health threat in the absence of vaccines or anti-viral therapies. Amongst several types of new generation viral vaccines, Virus Like Particle (VLP) vaccines are known to be highly immunogenic and elicit higher titer neutralizing antibody response. Such VLPs authentically present viral spikes and other surface components similar to native viral infection. We have developed recombinant yeast (*Pichiapastoris*) that produces Chikungunya virus structural polyprotein which consist of capsid (C) and envelope proteins (E1, E2, E3 and 6K). The C-E3-E2-6K-E1 genes expressing the native polypeptide were cloned into yeast transfer vector (pPIC9K) under the control of Alcohol oxidase I (AOX I) promoter. The expression cassette having N-terminal alpha secretory fusion tag was integrated into Pichia genome via electroporation. The selected recombinant pichia transformant was analyzed for the expression of C-E3-E2-6K-E1 proteins through SDS-PAGE and western blotting. VLPs were purified via density gradient sedimentation using ultra centrifuge. The assembly of the expressed recombinant proteins into VLPs was further confirmed through transmission electron microscopy. The morphology of VLPs was validated in comparison to the authentic Chikungunya virions. In conclusion, we have shown that the selective expression of Chikungunya viral structural proteins using *Pichiapastoris* gives rise to VLPs that resemble the native virus. Our findings suggest the possibility of using Pichia as an attractive alternate for making correctly folded and processed Chikungunya VLPs that may be further used as candidate vaccine.

Hepatitis C Virus: An Emerging Threat

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Hepatitis C virus (HCV) infection is a serious global health problem that affects 180 million people worldwide and it is estimated that 3–4 million people are newly infected each year. HCV infection is an emerging threat and result in chronic liver disease that often progresses to liver cirrhosis and hepatocellular carcinoma. HCV is a small enveloped virus belonging to the *Hepacivirus* genus in the *Flaviviridae* family. It possesses a positive-sense, single-stranded RNA genome encoding a polyprotein that is processed by cellular and viral proteases into 10 different proteins, including structural proteins (core, E1, and E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B NS5A, and NS5B). Based on nucleotide sequences, HCV is grouped into six genotypes and more than 80 subtypes so far, that differ from each other by 31 to 33%. HCV-1b is the most common genotype in the worldwide while HCV-3 is the most prevalent in Indian subcontinent. Currently, there is no vaccine available for prevention of HCV infection due to high degree of strain variation. The current treatment of care, pegylated interferon α in combination with ribavirin is costly, has significant side effects and fails to cure about half of all infections. So, the prevention through vaccination remains the only method for its control. One possible vaccination strategy that can be used against viral infection is to produce artificial virus-like particles (VLPs). These VLPs, corresponding to empty virions, are therefore, non-pathogenic and generally highly immunogenic, the major two characteristics required for the development of an effective vaccine. In this approach, in order to develop a vaccine against HCV, the gene encoding the small envelope protein or the non-structural proteins is cloned into a BAC-EBV virus vector and is expressed in epithelial cells. BamHI region of EBV genome (149116 bp-154747 bp) was targeted for homologous recombination. Here, we tried with inserting a RFP cassette into BAC EBV as a trial approach. A DNA fragment was generated by PCR amplification of a RFP/AMP cassette with a BamHI site. The fragment was electroporated into *E. coli* EL350 carrying EBV Bacmid and expressing recombinase to allow homologous recombination. After recombination the BamHI site splits into two fragments compared to earlier Bacmid which was visualized in 0.65% agarose gel. BamHI fragment was used as a probe for Southern blot analysis which showed the 5.6 bp fragment from Bacmid and two BAC RFP-EBV fragments, 5 kb and 4.3 kb. Further, RFP-inserted BAC-EBV vector was stably transfected into HEK293-LP and confocal microscopic study shows expression of RFP in EBV vector.

Isolation and Molecular Characterization of Enteroviruses from Acute Gastroenteritis Patients

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Acute gastroenteritis (AGE) is one of the most common diseases in humans, worldwide. Though rotavirus is the leading viral etiological agent of this disease, enteroviruses (EVs) have also been reported to be associated with AGE. Isolation of EVs detected in patients with AGE by culture adaptation and their characterization can be useful in creation of virus bank which can then be utilized to produce diagnostic reagents for detection of enterovirus infections. The present study was conducted to create a virus bank and characterize EVs associated with sporadic infections of AGE. In a surveillance study carried out at NIV, Pune for EVs and their diversity in children aged $\leq$5 years with AGE (2006–2008), multiple serotypes were detected. A total of 46 (33 typeable and 13 nontypeable) EV positive fecal specimens from this study were inoculated in RD cell culture for virus isolation. Culture isolates were confirmed for the presence of EVs by RT-PCR using 5′NCR specific primers and genotyped on the basis of VP1 or VP1-2A gene sequences. Phylogenetic analysis of these sequences was carried out using Kimura-2 parameter algorithm and Neighbour-joining method, MEGA 4.0 software. Twenty two of 33 (66.6%) typeable and 3 of 13 (23.1%) nontypeable culture isolates showed cytopathic effect and tested positive for EV RNA while 22 (20 typeable and 2 nontypeable) of 25 (88%) EV positive isolates were genogrouped and genotyped. HEV-B (86.3%) {Echo-13, Echo-6, Echo-7, Echo-1, CB-2, HEV-B, Echo-27, Echo-29, Echo-11, CA-9, EV-75} was found to be predominant genogroup that was followed by HEV-A (4.5%) {EV-90} and HEV-C (4.5%) {CA-22}. Among the serotypes isolated in study, echovirus-13 was found to be predominant. Isolation of novel serotypes EV-75 and EV-90 and dual EV types (Echo-14/EV-76) from a single specimen was also achieved in this study.

Prevalence of HCV Infection in Blood Donors of Kumaon Region of Uttarakhand

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HCV Infection is an evolving public health problem globally, infecting approximately 3% of the world population and placing approximately 170 million people at risk for developing chronic liver disease. The natural history of HCV infection is characterized by an often clinically unapparent acute phase, followed by chronic infection in over half of those infected. Epidemiological assessment of the magnitude of the problem is essential for public health planning for preventing the HCV infection. The present study was aimed to know the prevalence and yearly trend of HCV infection among blood donors of Kumaon region of Uttarakhand. Blood donation records over 5 years (Oct 2007 to Sept 2012)from blood bank of Soben Singh Jeena Base hospital, Haldwani were reviewed retrospectively for seroprevalence and yearly trend of HCV infection. Total prevalence of HCV infection was 0.64%, higher rates were found among Sikh (3.28%), followed by Muslim (1.8%) and Hindu population (0.31%) respectively. Maximum antiHCV prevalence was seen in Udham Singh Nagar District (3.25%) where as prevalence in Nainital District was only 0.23%. Statistically significant higher seroprevalence was seen in replacement donors in comparison to voluntary donors. Increasing yearly trend of HCV infection was seen from 2007 to 2012 among blood donors. The study highlights the need for more stringent screening in blood donors. The replacement donors should be discouraged. All this is not possible without increased public awareness of the magnitude and implications of this infection and its mode of spread. Health authorities have to include hepatitis C on their radar as a disease which can result in significant morbidity and mortality in the years to come. HCV positive donors should be informed about their disease, counselled and referred to hepatologist, and permanently deferred for future donations.

Vector Competence of Two Populations (Kerala and Pune) of *Culexquin quefasciatus* Say Mosquitoes to West Nile Virus

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On the outset of West Nile virus (WNV) isolation from patient serum in Kerala, a study was carried out to determine vector competence of local population of *Culexquinque fasciatus* in comparison to

laboratory strain (Pune). Growth kinetics of WNV in the two populations demonstrated an identical growth pattern though the virus intake by the Kerala strain was low compared to Pune strain. Maximum virus yield in Kerala and Pune populations was observed on 17th day post infection (PI) yielding 7.15 and 7.48 $\log_{10}$ PFU/ml respectively. Both the strains replicated the virus to >5 $\log_{10}$ PFU/ml within 5 days of feeding on the virus blood mixture and maintained the titer in ascending order throughout the study period. A rapid increase in virus titer from 2.15 $\log_{10}$ on 1st day PI to 5.36 $\log_{10}$ PFU/ml on 5th day PI was observed in the Kerala strain while the Pune strain demonstrated a gradual increase. Virus dissemination to legs was observed on 7th day PI in both the strains. Presence of WNV in saliva, which is a marker for vector competence, was detected on 7th day PI yielding 2.67 $\log_{10}$ and 2.15 $\log_{10}$ PFU/ml by the Kerala and Pune strains respectively. The high replication potential of WNV by the mosquitoes, rapid dissemination salivary glands, ability to feed on humans and birds and its abundance makes the Kerala strain of *Cxquinque fasciatus* a competent vector for the transmission of WNV in Kerala.

A Comparison of the Role of Selection Pressure in the Evolution of Dengue Viruses (Type 1 and 2)

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Epidemics of dengue virus (DENV) have been recorded from almost all over India at different times since 1940s. Evolutionary dynamics of both DENV-1/2 in India have been restricted to analyses based on the Envelope (E) gene sequences and have shown a change of genotypes over the time period. The establishment of the Cosmopolitan genotype was noted in India in both cases since the 1980s. It is thus important to investigate the role of selection pressure in the different genes of the DEN viruses of this genotype. Whole genomes of representative DENV-1 isolates ($n = 128$) and DENV-2 ($n = 138$) as well as a sub-dataset including only the Cosmopolitan genotype were used in this study. For the E gene, larger datasets ($n = 267/289$ respectively for DENV-1/-2) were used. The selection pressure analysis was performed by using the SLAC, FEL and REL methods available at the Datamonkey web server. Selection pressure analyses of DENV-1 sequences, showed one positively selected codon site in the capsid protein (112) and two positively selected sites in the non-structural proteins at NS1-94, NS3-288. For DENV-2, one positively selected site (360) in the E-protein and 4 sites in the non-structural proteins at NS1-714, 715, NS2A-433 and NS3-255 were noted. Residue 360 was also the only positively selected site within the Cosmopolitan genotype of DENV-2. This site is found to be located near the domain I/III interface involved during conformational changes of the E-protein in association with virus entry and the putative receptor-binding loop in domain III. Overall, It was noted that DENV-1/2 evolution process is mainly constrained by purifying selection. The positively selected site in the E-protein of DENV-2 may have a role in membrane fusion efficiency and virus-cell interactions. NS3 that has been reported to be the most immunogenic antigen in dengue, showed evidence of positively selection for both DENV-1/2 and thus needs further investigation.

Recombinant Expression of Properly Folded Dengue NS1 Protein in *E. coli* for Diagnostic and Prophylactic Applications

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The Dengue non structural protein (NS1) is known to be protective antigen and also has immense application for serodiagnosis. Several serodiagnostic assays available for dengue viral infection are dependent on tissue culture-grown viral proteins. This task is unsafe, laborious, more expensive that makes it unsuitable for routine large-scale production. Although bacterial expression is relatively simple and easy for recombinant protein expression, it is more challenging to make NS1 protein with native structural and immunological features using bacterial expression system. We have successfully developed a method leading to the purification and refolding of recombinant dengue virus type 3 (DENV3) NS1. The NS1 gene was amplified from cell culture infected viral RNA using reverse transcription polymerase chain reaction. The amplified gene was further cloned into pET28a (+) vector under the control of T7 RNA polymerase promoter. In order to increase the purity level of the recombinant protein during purification, the transgene was engineered to carry 6X-Histidine tags at both N-terminal and C-terminal ends. The recombinant construct (pETNS1) was transformed into *E. coli* rosetta gamicells. The selected bacterial transformant known to express the recombinant NS1 protein was further used to optimize the expression conditions viz IPTG concentration, media type, temperature and harvest time. With optimized conditions, a good expression of NS1 was noticed as early as 0.5 h of post induction. The size of the expressed protein was found to be $\sim$43 KDa and the authenticity of the expressed protein was confirmed using anti-His and anti-NS1 monoclonal antibodies. The recombinant protein was found to be partly soluble and major portion was found in inclusion bodies. The NS1 protein was purified from inclusion bodies, refolded and dialyzed against PBS. Presence of double fusion tag facilitated the recombinant NS1 protein to withstand stringent purification steps that resulted in improved purity.

Isolation of Viruses from Cerebrospinal Fluid of Acute Encephalitis Syndrome Cases of Eastern UP

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Acute Encephalitis Syndrome (AES) is defined as clinically, as a person of any age, at any time of year with the acute onset of fever and a change in mental status. Encephalitis is a significant cause of morbidity and mortality in children each year in Uttar Pradesh. The cause of encephalitis varies depending on the season, the area of the country, and the exposure of the child. Viruses are the leading cause of encephalitis. These include enteroviruses, Japanese Encephalitis virus, West Nile virus, Herpes simplex virus, Measles and Mumps. In this study we screened more than 300 Cerebrospinal fluid (CSF) samples from AES cases admitted in BRD Medical College, Gorakhpur during June to September 2012. For screening large no of CSF samples 24 well cell culture plate method was adopted. All the samples were passaged in Rhabdomyosarcoma (RD) cell line for minimum of three passages. Cytopathic effect (CPE) was observed for 5–7 days in each passage and recorded. All the tissue culture fluid was subjected to PCR for Enteroviruses, Parecho, Flavi, and Herpes virus. CPE positive samples were also screened with antigen capture ELISA and IFA for enterovirus. PCR negative and CPE positive samples were further subjected for electron microscopy for identification. The outcome of this study will help in knowing the cause of disease. The significance of these findings will also be helpful in future for deciding proper line of treatment and in planning the control strategy of AES cases.

Early Diagnosis of Dengue by Combining the use of NS-1 Antigen and IgM Antibody ELISA

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Dengue is a most important emerging arboviral infection transmitted through mosquito. Dengue, a positive stranded RNA virus belongs to the family Flaviviridae genus Flavivirus. There are four distinct serotypes of dengue virus (DEN-1, 2, 3 & 4) each capable of producing a wide spectrum of signs and symptoms varying from sub clinical disease to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), that characterize dengue infection. The timely diagnosis of dengue is essential not only to prevent the complications such as dengue hemorrhagic fever and dengue shock syndrome but also to prevent the spread and containment of the disease. This study was conducted in Virology laboratory, Department of Microbiology, Maulana Azad Medical College New Delhi. 400 patients with acute febrile illness of clinically suspected dengue cases attending the outpatient department, emergency or wards of Lok Nayak Hospitals from January to September 2012 were included in the study. Serum samples from these patients were tested for both IgM ELISA (NIV PUNE) and NS1 antigen ELISA (Pan Bio) by commercially available kits as per the manufacturer's instructions. Out of the 400 patient samples tested; 54 were positive for either NS1 antigen or IgM antibody by Capture ELISA. Dengue NS1 antigen capture ELISA gave an overall positivity rate of 66.6% (36/54) and IgM ELISA gave an overall positivity rate of 46.29% (25/54). Only NS1 antigen can be used to test during the first four days of fever. IgM ELISA begins to show positive by fifth day of illness and gradually its positivity increases. The sensitivity of IgM antibody detection by capture ELISA is insufficient in the early phase of the infection and it would be beneficial to identify early cases so as to prevent its complications. Therefore NS1 antigen assay along with IgM antibody detection assays is a useful tool for early detection of dengue infection.

Co-Circulation of Multiple Dengue Serotypes During an Outbreak in Delhi

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Dengue an emerging arboviral disease because of its rapidly increasing incidence and expanding geographic range causes frequent outbreaks in tropical and subtropical regions with significant morbidity and mortality. It is classified antigenically into four serotypes; since these serotypes vary in their virulence their detection and analysis of spatial and temporal transition are essential. Sera were collected from 400 patients of clinically suspected dengue cases attending the out/in-patient department of Lok Nayak Hospital, Delhi from July 2012 to September 2012. Dengue infection was confirmed by serology, NS-1 antigen and RT-PCR. Acute phase sera from patients were tested for the presence of dengue virus RNA by RT-PCR assay. Of the 400 samples tested for dengue virus RNA, 48 (12%) were found to be positive. Multiple dengue virus serotypes were found to be co-circulating in this outbreak with DENV-3 and DENV-1 being the predominant serotype. This study indicates hyperendemicity of dengue in the region with the presence of multiple serotypes and high rates of co-infection and local genomic evolution of the viral strains involved in this outbreak which emphasize the need to prevent and control dengue infection. However the association of concurrent infections with severe forms of disease (DHF/DSS) needs further studies as different serotypes vary in their virulence and this should be a point of concern for health care agencies. Health authorities should consider strengthening surveillance for dengue infection, given the potential for future outbreaks with increased severity.

Prevalence of Dengue Virus Serotypes at Jaipur in 2011

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Dengue fever and Dengue haemorrhagic fever (DF/DHF) continues to be a major public health problem globally especially in tropical countries like India. The risk of DHF and severe disease increases in a secondary heterotypic infection. The existence of waning neutralizing antibodies to one serotype may promote the enhancement of infection upon subsequent infection with another serotype. There is a lack of information on prevalent Dengue virus serotypes at Jaipur. This study was conducted to detect the prevalent serotypes in Jaipur and nearby areas. The blood samples of Dengue suspected patients attending SMS Medical College and attached group of hospitals were collected during the period of October 2011 to December 2011. Fifty nine samples (either NS1 Antigen positive or Dengue IgM antibodies positive) were processed for the Dengue Virus serotyping. cDNA was prepared using random hexamer and MuLV Reverse transcriptase. Serotyping was done by heminested PCR by using primers developed by Lanciotti et al., (D1, D2, TS1, TS2 and TS3) and later modified by Harris et al. (DEN 4). Out of 59 samples, 54 (91.52%) samples were found to be serotype 3, three (5.08%) samples were serotype 1, one (1.69%) sample each of serotype 2 and 4. All four serotypes were found to be circulating in our area in 2011 and serotype 3 was the predominant type (92.4%). Circulation of multiple serotypes in an area increases the risk of severe disease. The information on prevalent serotypes in an area is important for surveillance and understanding the epidemiology of the virus for initiating appropriate treatment and control measures.

Microbial Profile in Acute Meningoencephalitis Cases at Jaipur

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Acute meningoencephalitis is one of the major cause of morbidity and mortality throughout the world especially in developing countries. Majority of acute encephalitis cases have been reported due to viral pathogens. Rapid identification of the specific agent is important for proper management of the infection. 150 CSF and 50 blood samples were collected between Oct 2011 and Sep 2012 from SMS and attached hospitals in Jaipur. Nucleic acid was extracted from CSF on Nuclisens EasyMag automated nucleic acid extractor. Viral DNA/RNA of *Epstein Barr virus* (EBV), *Herpes simplex virus* (HSV), *Varicella zoster* (VZV), *Cytomegalo virus* (CMV), *Mycobacterium tuberculosis* (MTB), *Japanese encephalitis* (JE) was done on Roche's LC 480 by using virus specific primers and probes and Roche master mix. Virus specific IgM antibodies against *West Nile virus* (WN), *Dengue* and Chikungunya (CHIKV) were detected by ELISA using commercial kits. Out of 150 CSF samples, 30 (20%), 20 (13.3%), 12 (8%) & 9 (6%) were found positive for EBV, HSV, VZV & CMV

respectively and none were found positive for JE and forty two (28%) patients were positive for MTB by real time PCR. While 2 (4%) each were positive for JE & Dengue IgM ELISA respectively. None of the samples were found positive for WN and CHIKV IgM. Herpes group of viruses and MTB were found to be the commonest causes of meningoencephalitis in Jaipur. Rajasthan is supposed to be JE non-endemic area but 2 patients were found to be positive for JE IgM is a significant finding but its important to rule out cross reacting antibodies. However steps should be taken to carry out active surveillance for presence of JE in our area as it causes outbreaks and epidemics with high mortality but is a vaccine preventable disease.

Transmission Dynamics of Japanese Encephalitis Virus in Assam

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The incidence of Japanese encephalitis (JE) in recent times has shown an increasing trend in India and has become a major public health problem. There is no treatment for JE. The present study aims at identifying the temporal transmission pattern of JE virus (JEV) infection by tracking the sentinel pigs, mosquito vectors and human JE incidence in Assam. This information will provide baseline data to design and implement JE prevention programmes in endemic areas of the country. The suspected human cases were confirmed by ELISA and Viral neutralization test. JE antibody seroconversion in sentinel pigs was detected by Haemagglutination inhibition test. Per man hour density and minimum infection rate in collected adult mosquitoes were determined by standard formula. Data were analysed by using SPSS software. Sentinel pig seroconversions were significantly associated with human cases 4 weeks before their occurrence; highly correlated during the same time and till 2 weeks before case occurrence and remained significantly correlated up to 2 weeks after human case occurrence. JEV was detected in the same month in pigs and mosquitoes; peaks of pig seroconversion were preceded by JEV infection peaks in vectors by 1–2 months. Kaplan–Meier analysis indicated that detection of JEV positive mosquitoes was significantly associated with the median time to occurrence of seroconversion in pigs. This study reveals the temporal relationship of transmission of JE in an Indian setting which may depict identical scenarios in other pig-rearing areas of South East Asia with a similar ecological niche and potential mosquito vectors. This will not only help in predicting JEV activity in a particular JE endemic area but also accelerate the initiation of timely vector control measures and animal vaccination programmes to reduce the risk of JEV infection. This will also alert the local health authority and the administrator for management of any future JE outbreak in areas under their jurisdiction.

Effect of Antibiotics Upon Typical Japanese Encephalitis Virus Infection Induced Pathogenic Mechanism in In Vitro

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Japanese encephalitis (JE) infection is caused by Japanese encephalitis virus (JEV), a RNA virus belonging to the genus *Flavivirus*, family—Flaviviridae. Currently, no therapeutic regimen is present to treat JE. 'Antibiotics' compounds tend to work against bacterial microbes but not against viruses. Recently, efficiency of certain antibiotics against Flaviviruses has been documented in experimental models. A study was conducted to evaluate 8 antibiotics (Tetracycline and Aminoglycoside group compounds) against JEV strain P20778 in Baby Hamster Kidney 21 (BHK21) cell line. CC_{50} for drugs were calculated based on Optical density (OD) readings by MTT assay at 490 nm. Antiviral activity of drugs at non toxic concentrations was evaluated by Cytopathic Effect Inhibition (CPEI) assay. Virus yield reduction assay (VYRA) was done to compare infectious drug titers of infected cultures treated at different concentrations. Effect of drugs upon kinetics of JEV induced pathogenicity was studied. Plaque reduction assay at non cytotoxic concentration of drugs were carried out. CC_{50} of drugs ranged from 7–520 μg/ml as assessed by MTT assay. CPEI assay with non toxic concentration of drugs showed that all eight antibiotic compounds showed antiviral efficacy inhibiting JEV induced CPE. VYRA revealed decline of virus titer ($TCID_{50}$) at varied drug concentrations. One drug induced decline in plaques numbers with increase of the drug concentration. It can be concluded that at non toxic concentration, antibiotics have inhibited JEV induced typical CPE, reduction in $TCID_{50}$ of drug treated compounds and reduction in JEV induced plaques treated with antibiotics. These observations showed that the antibiotics (Tetracyclines and Aminoglycosides groups) affect JEV induced pathogenicity in in vitro. However, mode of drug efficacy is to be studied, the study is in progress.

Mechanism of EBV Latency Control by Inflammation

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Epstein Barr Virus (EBV) is a ubiquitous gamma-herpesvirus which predominantly targets B cells and epithelial cells. This virus is found to be associated with a large number of human cancers like Burkitt's lymphoma, nasopharyngeal cancer, Hodgkin's disease as well as a number of B cell lymphomas in ADDS patients and transplant associated immunoblastic lymphomas, and also with invasive breast cancer. The in vitro infection of B cells with EBV gives rise to lymphoblastiod cell lines (LCLs), which expresses a subset of 12 latent viral transcripts. Chronic inflammation at various steps is associated closely with progression of tumorigenesis including cellular transformation, survival, proliferation, invasion and angiogenesis leading to cancer. Various stimuli like cytokines, hormones, and mitogens can cause induction of Cyclooxygenase-2 (Cox-2). Cox-2 have been reported to be expressed in EBV associated cancer. EBV transformed lymphoblastiod cell lines were used as model for latent infection. LPS was used to up-regulate COX-2 expression and EBV was detected in cell free supernatant using PCR targeting Bam W region of EBV genome. We found that inducing LCLs with LPS, unregulated Cox-2 expression, which is also responsible for inducing Lytic reactivation of EBV in latently infected cells. Cox-2 is a multifunctional moderator protein and is capable of inducing lytic reactivation of EBV in latently infected cells. Cox-2 mediated pathway play a critical role in regulation of EBV life cycle and EBV mediated malignancies. Our studies suggest a direct link between Cox-2 upregulation, chronic inflammation and EBV associated malignancies.

Surveillance of Norovirus Genetic Diversity in Pune, Western India (2005–2010)

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Acute gastroenteritis is one of the most frequent cause of infectious disease, worldwide and norovirus (NoV) has been found to be most common cause of viral gastroenteritis. A 6-year (2005–2010) NoV surveillance study was conducted in the city of Pune, India to find out trends and temporal variations in the circulation of NoV strains. A total of 1424 fecal specimens collected from children ($\leq$ 5 year) with acute gastroenteritis were tested by RT-PCR for detection and characterization of NoV by using primers targeting RdRp and VP1 (capsid) regions. PCR products were sequenced and analyzed phylogenetically. NoV positivity was 7.8% (111/1424) and varied between 5.3–11.6% in 6 years of study. The rate of infection was higher (55.8%) in children with $\leq$1 year of age than in children >1 $\leq$2 year of age (34.2%). Majority of the patients (56.7%) suffered from severe disease. NoV associated gastroenteritis was observed throughout the year, however in 2007 summer month seasonality supported NoV infections. GII.4 (45%) predominated throughout the period of study except in 2005 and 2007 when recombinant strains GII.b/GII.21 (9.9%) and GII.b/GII.3 (15.3%) were found to prevail, respectively. Nearly 30% of NoV infections were contributed by different NoV strains. Out of 111 specimens detected positive for NoV RNA, 96 (86.5%) were genotyped. Phylogenetic analysis of partial RNA polymerase and VP1 (capsid) gene sequences identified, 7 GII genotypes (GII (2–4), (6–8), and GII.14), 1 GI genotype (GI.2) and twelve different combinations (GII.b/GII.21, GII.b/GII.3, GII.b/GII.4, GII.d/GII.3, GII.e/GII.13, GII.1/GII.12, GII.7/GII.9, GII.17/GII.13, GII.m/GII.1, GII.m/GII.4, GII.17/GII.4,GI.b/GI.6) of RdRp and VP1 genes. The genetic diversity and temporal variations in the NoV strains circulating in Pune emphasize continuous monitoring for better understanding of evolutionary relationship among NoVs and development of suitable diagnostic assays or vaccines.

Isolation of Bacteriophages Against Food Borne Pathogens *Salmonella sp.,* and *E.coli* Associated with Meat Products

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In recent years the rate of food borne outbreaks increases throughout the world. It is estimated that there are between 81 million cases of food-borne illness each year and approximately 50% of these cases are associated with meat and poultry. Microbial contamination of raw meat and meat products has been the predominant cause of food related illness. Major food borne pathogens are *E.coli*, and *Salmonella* which are the common flora associated with the meat products. Customers concern towards the use of chemical preservatives has increased and hence biological preservative is opted to be the best Phage based therapies have a great potential for a variety of antibacterial application. Bacteriophages are the viruses that are capable of infecting bacteria and lyse the cells. Phages have been proved to be safe for consumption and FDA approvals have also been given for the application of phages for bioprocessing of food materials. Based on the above objective an attempt has been made to isolate Bacteriophages against major food borne pathogens. Salmonella and *E.coli* cultures were isolated from the fresh and packed meat products. A total of 10 salmonella cultures and 5 E.coli cultures were isolated. Salmonella cultures were identified by using specific primers *inv*A139 (5′-GT GAAATTATCGCCACGTTCGGGCAA-3′) and *inv*A141 (5′-TCAT CGCACCGTCAAAGGAACC-3′). Bacteriophages were isolated from the meat samples by soft agar overlay method. A total of 6 Bacteriophages were isolated. Among the six phages BPMR2 phage showed ambivalent activity against the test strains of *Salmonella* and *E.coli*. Stability of the phage against wide range of pH and chlorine was checked. The phage BPMR2 was capable of surviving in pH range between 4–9. But at pH 2 the phage activity cannot be detected. Chlorine inhibited the phage activity completely. Significance of the current work is that Bacteriophages are mostly very specific to their host, but here bacteriophage BPMR2 is capable of lysing two food borne pathogens, Salmonella and *E.coli* which makes it an ideal agent that can be used in bioprocessing of the meat products.

Molecular Characterization of Human Bocavirus 1, 2, 3, and 4 in Children with Acute Gastroenteritis in India

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Acute Gastroenteritis (AGE) is one of the leading causes of morbidity and mortality in children, worldwide. Among viruses, Rotavirus is the most important etiological agent of this disease. Recently, Human Bocavirus (HBoV) has been detected in faecal samples from AGE patients suggesting its etiological role in AGE. HBoVs have been classified into four genotypes; HBoV1–HBoV4, all of which are associated with AGE. The purpose of this study was to investigate the presence of HBoVs in paediatric patients with AGE and determine their genetic diversity. Faecal samples collected from children <5 years of age ($n = 372$), hospitalized for AGE in Pune, India, during Jan 2009-December 2011, along with age matched controls ($n = 187$) were included in the study. HBoV was detected in faecal samples by PCR amplification of VP1/VP2 region and positive amplicons were characterized by sequencing. Phylogenetic analysis was performed using MEGA 5 computational tool. HBoV was detected in 21/372 (5.6%) test samples but none of the control samples. Co-infection with Rotavirus was observed in 3 (14.3%) cases. Nucleotide sequence analysis of partial VP1/VP2 region of the 21 strains illustrated the predominance of HBoV1 (57.1%). HBoV2, HBoV3 and HBoV4 were detected at 19.1%, 9.5% and 14.3%, respectively. Phylogenetic analysis showed that the study strains shared 92–99% nucleotide identity with their respective prototype strains. HBoV positivity was observed in children $\leq$1 year of age throughout the year, with peak HBoV activity occurring in July and December. Severity assessment of AGE revealed moderate infection in 7 and severe in 14 of the HBoV positive cases. The study reports detection of HBoV in paediatric population with AGE for the first time in India and circulation of all 4 genotypes in the study region. Although our study corroborates association of HBoVs with AGE, further epidemiological studies are warranted to establish a causal relationship between HBoV and AGE.

Diagnostic Utility of Dried Blood Spots as a Useful Alternative for Hepatitis E Virus Serology in an Outbreak Setting

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Viral Hepatitis E, an important disease of public health concern, conventionally relies on anti-HEV IgM serology. In an outbreak situation, collection of sample by venipuncture for laboratory confirmation is often difficult. Thus testing the specimens of dried blood spots (DBS) on filter papers can proof to be an easy, simple and reliable alternative. The present study was designed to evaluate the

applicability of anti-HEV IgM detection from DBS samples and to determine the stability of anti-HEV IgM detection at varied time interval and at various storage temperatures. Paired blood and DBS sample were collected from 44 jaundice patients and 8 healthy controls during an outbreak of hepatitis E. These samples were tested for anti-HEV IgM antibodies using commercially available ELISA kit. The DBS were tested by the HEV IgM ELISA kit with in-house modification accommodating varying disc diameter, incubation time of disc elution and sample diluents. Out of 44 samples, 21 samples were stored at 4 °C and 37 °C and tested for a period of 210 days and 65 days respectively. Phosphate Buffer saline with 0.5% fetal bovine serum was found to be the best sample diluents. The optimum diameter and incubation time for elution was found to be 6 mm and 4 h respectively. Three cut offs (CO_1: kit cut off, CO_2: mean of negative controls above 3SD and CO_3: area under Receiver operating Curve) were determined. The sensitivity of anti-HEV IgM detection in the DBS sample ranged from 86–91% using the various cut off values. The maximum sensitivity of 91% (40/44) and specificity of 100% was obtained using CO_3. Maximum stability of anti-HEV IgM antibodies (100%) was observed till 65 days at 4 °C. By 210 days, 23.8% (5/21) samples became negative. Storage at 37 °C indicated a significant reduction in the anti-HEV IgM positivity, wherein 57.89% (11/19) sample became negative at 45 days. Conclusions: DBS was found to have good sensitivity and specificity for detecting anti-HEV IgM antibodies and may be considered as an alternate to serum sample in field setting. Further, anti-HEV IgM stability was better at 4 °C making the DBS a preferred method for storage as well as transportation of the sample to reference lab.

Circulation of Two Mump Virus Genotypes in Unimmunized Population from India

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In February and March 2012, outbreaks of fever with parotitis were reported from Apsinga and Pimpla villages in Osmanabad district (Maharashtra State). The investigation was focused on serological, molecular and epidemiological studies. Blood, throat swabs, oral swabs and urine were collected from patients with fever and parotitis. Suspected cases ($n = 62$) and few convalescent ($n = 19$) serum samples were tested for mumps specific IgM and or IgG antibodies. The presence of mumps RNA in throat swabs, oral swabs and urine specimens were confirmed by SH gene RT-PCR and sequencing. Virus isolation was attempted using Vero cells. The presence of mumps specific IgM antibody was noted in 44 of 62 serum samples (71%). Of 19 sera from convalescent patients, 16 had IgM antibody after 5–6 weeks. Of 27 throat swabs from suspected cases, 23 showed presence of mumps RNA. Sequencing revealed circulation of genotype C ($n = 18$) and G ($n = 5$) in Apsinga and Pimpla villages respectively. Two mumps viruses were isolated in Vero cells. This report showed co-circulation of mumps genotype C and G in unimmunized village population in India at a similar time-point. Additional studies are needed to understand the circulating mumps genotypes in various parts of India.

Cervical Smear Abnormalities in Female Sex workers in Chandigarh

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Female sex workers (FSWs) are at increased risk of cervical cancer due to the multiple sexual partners. The conventional screening test for cervical cancer is Pap smear which has limited sensitivity. Recently, Liquid based cytology (LBC) techniques has demonstrated better results. Another alternative is screening for high risk HPV types. The aim of the present study was to screen the FSWs in Chandigarh for the presence of cervical abnormalities by various screening methods. The study subjects included 101 FSWs and 99 age-matched healthy females (HC) residing in the same locality. Cervical pap samples were collected and subjected to conventional as well as SurePathTM LBC examination. A total of 30 samples from each group, were further processed for HPV16/18 genotyping using PCR based technique. Out of 200 samples collected, unsatisfactory smear was observed only in 1.5% smears. Cervical smear abnormalities in the form of high-grade intra-epithelial lesion/squamous cell carcinoma (SCC) were identified in 1.9% FSWs while none of the controls showed any evidence of cancer cervix. The rate of unsatisfactory smears reduced from 11% in conventional smears to 1.5% in LBC samples. Pap smear was inflammatory in 49.5% FSWs vs 34.3% in HC. Bacterial vaginosis was reported on the smear in 45.5% FSWs vs 29.3% in HC. hrHPV types were detected in 12/30 (40%) FSWs as compared to 3/30 (10%) of HC. FSWs with SCC showed the presence of HPV 16, however HSIL did not show the presence of HPV 16/18. hrHPV positive cases were reviewed for cytologic abnormality; however were normal on Pap test except one case. LBC samples showed better cytologic and nuclear details. Inflammation and epithelial cell abnormality was easier to appreciate in LBC. FSWs are at higher risk for cervical cancer and need periodic screening for early detection of cervical abnormalities. Presence of hrHPV alone should be interpreted with caution and used in conjunction with PAP smears for further evaluation.

Non-Infectious Neuropsychiatric Disorders in HIV-Infected Individuals at NIMHANS: A Retrospective Analysis

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A wide variety of neurological disorders are seen in individuals infected with HIV. Although a majority of these are due to opportunistic pathogens, a number of HIV associated neuropsychiatric illnesses other than opportunistic infections have been reported. Medical records of all HIV infected individuals admitted in neurological/neurosurgical/psychiatric wards at NIMHANS between January 2009 and August 2012 were examined for neuropsychiatric disorders, confirmed in the laboratory or at autopsy for a retrospective analysis of HIV associated neuropsychiatric disorders. The medical records of 340 HIV-infected individuals were analyzed; 155 (45.6%) had non-infectious neuropsychiatric disorders of the CNS associated with HIV disease. Of these, 20% presented with psychiatric manifestations, 14.2% had stroke, Guillian Barre Syndrome (GBS) was seen in 11% of the patients and 5.8% had HIV associated encephalopathy. Substance abuse was also seen in 7.1% of the patients. Other conditions included peripheral neuropathy (4.5%), cervical myelopathy (4.5%), infarcts (3.9%), tumors (2.5%), Motor Neuron Disease (2%), polyradiculopathy (2%) and metabolic encephalopathy (2%). 185 (54.4%) patients had infections of the CNS including most commonly neurotuberculosis, Cryptococcal Meningitis, Toxoplasma Encephalitis, pyogenic meningitis, Herpes simplex, Encephalitis, Leucoenc, Neurosyphilis and progressive multi functional. Although

HIV-associated dementia has not been widely reported in India, other non-infectious neuropsychiatric disorders formed a significant proportion of cases in the retrospective analysis.

Loop Mediated Isothermal Amplification Assay for Rapid and Sensitive Detection of Zoonotic Orthopoxviruses: Buffalopox and Camelpox Virus, the Emerging Form of Infections

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Camelpox and buffalopox are considered as emerging and reemerging viral diseases. In this study, Loop-mediated isothermal amplification (LAMP) assay targeting A-type inclusion body protein gene (*ATI*) of orthopox viruses (OPV) namely buffalopox virus (BPXV) and camelpox viruses (CMLV), for specific, sensitive and rapid detection of both of these zoonotic viruses. The assay was optimized using purified viral genomic DNA from density gradient purified CMLV and BPXV vaccine viruses and standard reagents namely LAMP primers, Betaine, $MgSO_4$ and *Bst*DNA polymerase and it resulted in reliable specific amplification at 63 °C for 45 min. The amplified LAMP products was identified by agarose gel electrophoresis and subsequent direct visualization under UV light or observation by naked-eye for presence of turbidity and color change following the addition of SYBR Green I and Hydroxyl naphthol blue (HNB) dyes. The analytical specificity of the LAMP and conventional PCR assays was evaluated using other related poxviruses (Goatpox, Sheeppox, and Orf virus), which revealed that, specific amplification only for OPVs. LAMP assay had shown 100 fold higher sensitivity compared to conventional PCR when tested using purified viral DNA and standard plasmid construct. Further, the developed assay was evaluated using cell culture isolates {CMLV ($n = 10$) and BPXV ($n = 12$)} and clinical samples ($n = 100$) of animals and humans. These results prove that the developed OPV-LAMP is a simple and cost-effective diagnostic tool for rapid, highly sensitive and specific detection of OPVs from clinical samples of both animals and humans without the need of high precision tools like PCR/real-time PCR cyclers in field diagnostic laboratories.

Detection, Genotyping and Phylogenetic Analysis of Circulating Genotypes of Hepatitis-C Virus in Uttarakhand State, India

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Hepatitis C virus (HCV), is the one of the major causes of parenterally transmitted non-A and B hepatitis, affecting more than 170 million people worldwide. HCV genotypes have shown geographical linkages and the genotyping is known to play a critical role in the outcome of the HCV infection, progression of disease, and response to interferon therapy. In India, approximately 1.8% to 2.5% of the population is infected with HCV and at present there is not a single report on the confirmation of detection, and genotypic distribution of HCV in Uttarakhand region of North India. We describe here the detection and distribution pattern of prevalent genotypes of Hepatitis C virus (HCV) in Uttarakhand state of India. In the study, 18 serum samples from healthy blood donors and 21 serum samples from chronic hepatitis patient, were screened for anti-HCV antibodies by using 4th Generation TRI-DOT Immunoassay followed by 5′UTR region PCR-restriction fragment length polymorphism (RFLP), sequencing and phylogenetic analysis. Twelve out of eighteen healthy blood donors noted a very high prevalence of anti-HCV antibodies (66.67%) and all the 21 patients suffering from chronic hepatitis were detected positive for both anti-HCV antibodies and PCR assay. The restriction patterns obtained clearly indicated the presence of genotype 3 in all the clinical samples but patterns were found to be flawed when subtyping was considered. The sequence based HCV genotyping and subtyping revealed predominance of HCV genotype 3a (77.8%) followed by 3b (22.2%) in the state. Phylogenetic analysis clustered seven HCV-3a and three HCV-3b Uttarakhand isolates with the Canadian HCV-3a and 3b isolates. HCV genotype 3a remains the dominant genotype in Northern India but also indicates circulation of 3b type in Uttarakhand state. The close association between Indian and Canadian isolates may suggest the origin of current circulating strains in Uttarakhand region back to Canada but needs more elaborative investigations to confirm this assumption. Stringent screening of blood collected from blood donors is in high demand in India.

Cytomegalovirus Infection and Hearing Loss in a Highly Seropositive Rural Population

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The cytomegalovirus seropositivity rate is very high in developing countries, including India. This study was carried out to determine the incidence of congenital cytomegalovirus infection, and its association with sensorineural deafness, against a backdrop of high CMV seropositivity. At the Comprehensive Rural Health Center (CHRC) of the Center of Community Medicine, All India Institute of Medical Sciences (AIIMS) in Ballabgarh, Haryana, the CMV seropositivity was previously estimated to be 99% (*Dar* et al. *Pediatr. Infect. Dis. J. 2008; 29: 871–873*). Saliva samples were collected from 1582 neonates delivered at CHRC, within a day of birth. Simultaneously, their hearing was screened using portable otoacoustic emission (OAE) equipment (Bio-Logic, U.S.A.). At the virology laboratory at AIIMS, a polymerase chain reaction was performed for cytomegalovirus (CMV) gB gene DNA. Nineteen (1.2%) saliva samples were PCR positive at birth, indicating congenital CMV infection. Hearing screening was possible in 1536 babies, 24 of whom failed the test in one or both ears. Of the 43 neonates who either failed the hearing test (24) or were CMV positive (18) or both (1), 25 have been followed up at 3–6 months of age and their hearing further tested by brainstem-evoked response audiometry (BERA), revealing unilateral or bilateral hearing loss in 3 of 13 babies positive for congenital CMV infection, as well as in 3 of 12 babies who were negative for CMV at birth but failed the hearing screening. The results show that congenital cytomegalovirus (reactivation or reinfection) not only occurs in a CMV seropositive population, but is also a significantly cause of hearing loss. This indicates a need for neonatal screening for both cytomegalovirus and hearing in India.

Epstein Barr Virus Nuclear Antigen 3C Interfere with the TAp63α Mediated Apoptosis in Human B Cells

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p63, a gene at chromosome 3q27-29 has significant homology to the tumor suppressor p53. The expression of p63 is regulated by two different promoter resulting in the production of full length transcriptionally active TAp63 and amino deleted dominant negative ΔNp63 isoforms. Role of p63 in the process of development and differentiation has been studied extensively but its importance during apoptosis in tumor cell remains elusive. Epstein Barr virus (EBV) is a tumor virus which infects more than 90% of the human population worldwide. This virus maintains latency in B cell and is associated with most of the B cell lymphomas. Epstein Barr Virus nuclear antigen 3C (EBNA3C) is a latent protein express during latency III stage of the virus life cycle interfere with the apoptosis in B cells and help during its transformation to lymphoblastoid cell lines (LCLs). This study is aimed to obtain new insight into the inhibitory effect of EBNA3C during the process of TAp63α induced apoptosis in B cell. Human B cells and EBV transformed lymphoblastoid cells were cultured in vitro. Expression vectors containing EBNA3C, p63 isoforms and their truncations were transfected in these cell lines and further assays were performed. Apoptosis were detected by annexin-V staining using flowcytometry. Mitochondrial membrane potential assay was performed by DiOC6 staining and cytochrome c release by western blot. Interaction between EBNA3C and p63 isoforms were checked by immunoprecipitation and co-immunoprecipitation. Downstream signaling of extrinsic and intrinsic pathways of apoptosis was traced by using ligand, inhibitors and siRNA studies. TAp63α expression in B cells induces apoptosis which involves activation of caspases and death receptors. TAp63α also induces mitochondria mediated apoptotic pathway. EBNA3C expression reduces the rate of apoptosis in these cells by interfering the downstream targets of TAp63α. TAp63α induces and recruits genes that play roles in different steps of apoptosis program which is inhibited by EBNA3C. These findings explain one possible mechanism by which EBV blocks the p63 mediated apoptosis especially in EBV transformed LCLs.

Acute Respiratory Viral Infections Among Pediatric Patients at Jaipur: A Preliminary Approach

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Acute respiratory infections (ARI) are one of the major causes of morbidity and mortality in young children throughout the world especially in developing countries. WHO estimated an approximate 3.9 million deaths annually. Majority of acute lower respiratory tract infections in developed countries have often been reported due to viral pathogens of which most common are *Respiratory Syncytial Virus* (RSV), *Influenza virus*, *Adenovirus* (Adv), *Human Coronavirus* (hCoV) and *Bocavirus* (hBoV) etc. The present study was conducted to detect *Influenza A 2009 H1N1* (Swine Flu), *Influenza A virus* and RSV among the hospitalized children with ARI in and around Jaipur, Rajasthan. Nasopharyngeal swabs from pediatric patients of 0–5 years of age were collected from the hospitals in and around Jaipur. The swabs were transported in viral transport medium. RNA was extracted on Nuclisens EasyMag automatic extractor. Real time RT-PCR was performed employing standardized protocols using virus specific primers. A total of 93 samples were screened for the occurrence of ARI infections. Out of these twenty three (24.7%) patients were found infected with *Influenza A virus* (seasonal flu), 8 (8.6%) for *Influenza A 2009 H1 N1* (Swine Flu) and 9 (9.6%) patients were found positive for RSV. Two peaks were seen in the occurrence of these viruses one during March–April and other in August-October. The study being preliminary in the study area has demonstrated occurrence of RSV Influenza A pandemic and seasonal Influenza in significant number of cases as a major cause of childhood acute respiratory infection in the area. The data addresses the need for more studies on virus associated respiratory tract infection.

Invasion and Replication of Chandipura Virus in Nervous Tissues Induces Pathogenesis in Experimentally Infected Mice

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Chandipura virus produces encephalitis/encephalopathy in naturally infected young children and experimentally infected young susceptible mice. Neuro-tropism is major feature in many viral infections. Neurotropic viruses use nervous tissues for replication and also take a route to central nervous system (CNS). This study was undertaken to find out the neuro-invasive behaviour of Chandipura virus in young susceptible mice. Young mice less than 14 days old are susceptible to Chandipura virus infection and adults are refractive. The susceptible mice were infected with virus via foot pad injection. Different nervous tissues including sciatic, spinal cord, brain and other tissues were collected at 24 h intervals up to 72 h post infection (PI). The tissues were processed for virus quantification and histology. Adult mice were infected through different routes including intra-cerebral route to determine the requirement of nervous tissues for virus replication. In virus infected susceptible mice it was observed that time dependent increase in viral RNA copies in different nervous tissues. Other tissues like spleen, lung, kidney etc. the peak virus titre was noticed at 24 h PI and it was later declined. Perivascular cuffing and other signs of inflammation were noticed in lower, upper spinal cord and brain. TUNEL positive nuclei in brain indicated that cell death and/or damage in the CNS. Infected susceptible mice also showed gross neurological symptoms like hemiplegia and/or paraplegia of hind limb, circling movement, eye lesions etc. In adult mice, the virus reached the brain through intranasal and intra-cerebral route of inoculation. However the pathogenesis noticed only in intra-cerebrally inoculated mice. No pathogenesis noticed in adult mice through all other routes employed in this study. From this study, we concluded that in young susceptible mice the virus might migrate through the nervous tissues and finally reach the CNS. However in adult mice, the nerve tissues neither pick up nor transport the virus to CNS. The pathogenesis in young mice might be due to the virus replication induced damages in the nervous system. The results are preliminary and a detailed study is necessary to confirm of mechanism of axonal transport in Chandipura virus infection.

Role of TLRs and Cytokines, Two Central Components of Host Immune Response, in the Immunopathogenesis of Viral Hepatitis E

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Hepatitis E virus (HEV) infection is endemic in India. The disease manifestation ranges from self limiting acute viral hepatitis (AVH) to life threatening acute liver failure (ALF). The clinical course of the disease, possibly, thought to be immune-mediated. Toll-like Receptors (TLRs), a class of evolutionary-conserved protein, plays a key role in sensing pathogen associated molecular patterns (PAMPs). Downstream signaling of TLRs modulates the production of cytokines and plays a crucial role in determining the course of immune response and disease pathogenesis. Thus, the present study was aimed at elucidating the role of TLRs and their cytokine modulation in the immunopathogenesis of HEV. Anti-coagulated blood was collected from 50 AVH-HEV, 30 ALF-HEV patients, and 50 apparently healthy-controls (HC). PBMCs were separated using Ficoll-Hypaque. One part was processed for RNA extraction (unstimulated) another cultured in RPMI-1640 and pulsed (Stimulated) with recombinant HEV-ORF2 protein (452–617 a.a). Gene-expression levels of TLR (2, 3, 4, 7 and 8) were checked in unstimulated and stimulated group using semi-quantitative Real-time-PCR. Lymphocyte proliferation-index was estimated using Colorimetric-MTT assay. Cytokine levels were checked in the Serum and culture-supernatant using Cytokine-Bead-Array. TLR3 silencing experiments were performed in the PBMCs of representative HEV patients. Post silencing cytokine levels were estimated. TLR3 gene-expression in AVH was significantly higher than ALF (215 Vs 21 fold-increase; ($p < 0.0001$). Proliferation-Index of AVH was significantly higher than ALF (3.25 ± 0.876 Vs 1.748 ± 0.253; $p < 0.008$). Significantly higher amount of IFN-γ detected in the culture-supernatant of AVH Vs ALF (292.3 ± 88 pg/ml Vs 3.235 ± 0.8 pg/ml; $p < 0.0001$). IFN-γ: IL-4 ratio in the AVH was 89.76 and in ALF was 0.589. Circulating TGF-β levels were significantly elevated in patients as compared to HC (HC: 57.40 ± 27.48 ng/ml, AVH: 322.8 ± 212.2 ng/ml, ALF: 334.5 ± 203.8 ng/ml). After silencing TLR-3 using specific siRNA significant decrease in IFN-γ in the PBMC culture-supernatant observed (without-siRNA 25.57 pg/ml Vs with siRNA 4.30 pg/ml; $p = 0.0349$). A lower level of TLR3 gene-expression followed by minimal levels of INF-γ production leads to a low proliferation index which contributes to a marked Th2-shift in ALF-patients. On the other hand, AVH-patients demonstrated robust Th1-type of immune-response mediated by TLR3 pathway, thus providing a strong pointer towards involvement of TLR3 in the immunopathogenesis of HEV infection and can be a potential therapeutic target for antiviral agents.

Transgenic RNAi-Derived Field Resistance to Cassava Brown Streak Viruses

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Cassava (*Manihot esculenta*) is an important subsistence food crop cultivated in the tropical regions of Africa, Asia and Latin America. Cassava is vulnerable to at least 20 different viral diseases, among which cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) are the most important. Although CMD occurs in almost all cassava-cultivating regions of sub-Saharan Africa, CBSD has emerged as the most important viral diseases of cassava in Africa and is one of the biggest threats to global food security. CBSD is caused by two distinct species of ipomoviruses, Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) belonging to the family Potyviridae. Previously CBSD was reported only from the coastal lowlands of East Africa, but recently it has taken an epidemic form in entire East Africa, further spreading into West and South of Africa. Through a collaborative project called Virus Resistant Cassava for Africa (VIRCA), researchers at the Danforth Plant Science Center (USA) with two partner institutions in Uganda and Kenya have demonstrated the proof of principle for the control of CBSD by RNAi. Here, we discuss the first evidence of effective RNAi-based control of an ipomovirus in both the model host *Nicotiana benthamiana* and the natural host cassava. We further demonstrate effective cross-protection against diverse CBSD-causing virus isolates belonging to two distinct species, both in the green house experiments, as well as the field trials in Uganda.

Sequence Analysis, Infectivity Analysis and Phylogenetic Relationship of a Begomovirus Associated with the Mosaic Disease of Cassava in India

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Cassava (*Manihot esculenta* Crantz, Family. Euphorbiaceae) is the third largest source of dietary carbohydrates in the world. Cassava mosaic disease, very common in the cassava crop, is caused by the geminiviruses Indian cassava mosaic virus (ICMV) and Sri lankan cassava mosaic virus (SLCMV) in India. This investigation describes an SLCMV DNA cloned from CMD-affected cassava, using Rolling Circle Amplification (RCA). Its sequence analysis and infectivity on *Nicotiana benthamiana*, using biolistic inoculation is described. Infected materials were collected from Attur, Tamil Nadu. RCA technique was used to clone geminiviral DNAs. Total DNA was extracted from symptomatic cassava samples and used as a template for the RCA. A 2.7 kb cloned fragment was obtained in pTZ57R vector (Fermentas) and sequenced. Sequences were analysed by using the BLAST programme of the NCBI server. For infectivity analysis of the cloned fragment of viral segment was released from the vector backbone of the recombinant plasmid by digestion with Pst I restriction enzyme [MBI Fermentas], self-ligated, amplified by RCA and was used directly for the analysis of infectivity on 30–35 days old *Nicotiana benthamiana* plants. The sequence resembled DNA-A of begomoviruses and was found to be having the highest sequence identity (99%) to SLCMV. The name SLCMV-Attur is proposed for the isolate. Biolistically inoculated *Nicotiana benthamiana* showed symptoms of leaf rolling and deformation after 30 dpi. The results of this study comparing the sequence variations and phylogenetic analysis with full-length begomoviral sequences reported from plants belonging to various taxonomic families from Indian subcontinent and rest of the world indicated that a strong relationship exists between begomoviruses infecting plants belonging to the same family and their geographic location. Biolistic inoculation results gave conclusive evidence that the cloned DNA is infectious in *Nicotiana benthamiana* plants.

Synergistic Interaction Between Two Bipartite Begomoviruses, Tomato Leaf Curl New Delhi Virus (ToLCNDV) and Tomato Leaf Curl Palampur Virus (ToLCPalV) Infecting Tomato

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In a survey conducted on the diversity of begomoviruses infecting tomato in Northern India, nearly 30% of the tomato leaf samples had

mixed infection of two bipartite begomoviruses, ToLCNDV and ToLCPalV. The plants showed brilliant yellow blotches, yellow mottling and severe leaf curl symptoms. To study the interaction between the two bipartite begomoviruses, the genomic components of ToLCPalV and ToLCNDV were cloned and partial tandem repeat constructs of DNA A and DNA B components were made in the Ti-plasmid derivative pBIN19. The DNA A and DNA B components of ToLCNDV and ToLCPalV were agroinoculated to *Nicotiana benthamiana*, tomato and cucurbitaceous hosts to study the interaction between the components. Agroinoculation of ToLCPalV alone produced downward leaf curling, mild yellowing, leaf curling in *N. benthamiana* and tomato at 10 days post inoculation (DPI). The ToLCPalV constructs were readily infectious on bottle gourd and cucumber produced yellowing, downward leaf curling and yellow blotches. Contrastingly, ToLCNDV components induced vein clearing, yellow mottling and severe leaf curl symptoms in *N. benthamiana* and tomato; the ToLCNDV components were not able to induce any symptoms in cucurbitaceous hosts. When agroinoculations were done by exchanging the components (DNA A of ToLCNDV with DNA B of ToLCPalV) on tomato, characteristically severe symptom expression, downward leaf curling, brilliant yellow blotches in leaf lamina, reduction of leaf lamina were seen in the plants inoculated with ToLCNDV A and ToLCPalV B. No such severe symptoms were seen in plants inoculated with combination of ToLCPal A and ToLCNDV B. Mixed inoculation with all four components led to severe symptoms expression though dominated by ToLCPal B mediated symptoms. Semi-quantitative PCR analysis of viral replicative forms clearly showed that all four components replicate efficiently on par with each other that the viral DNA components attained 500 ng/100 mg of leaf at 15DPI. The vector whitefly picked up the virion particles from the plants inoculated with homologous, heterologous and mixed combinations transmitted it to 85% of test plants. The acquiring and transmission of all the four components efficiently by the vector, indicates how association of two viruses may get established and perpetuated by subsequent transmission to healthy plants. Importance of these results in the context of resistance breaking and epidemics due to emergence of new variants is discussed.

Cucumber Green Mottle Mosaic Virus Based Vector for Gene Expression in Plants

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Cucumber green mottle mosaic virus (CGMMV), a member of genus Tobamovirus, infects cucurbits worldwide. The present study was undertaken to use the CGMMV genome for useful purposes. The complete genome of an isolate of CGMMV from Delhi characterized previously was used to design an a vector for transient expression of protein in plants. The genome of CGMMV was cloned in two parts; the one contain 5′ backbone that included the 5′ untranslated region (UTR), replicase protein and movement protein genes and the other part contained coat protein gene and 3′ UTR. Two modular constructs, 5′ module and 3′ module were developed by adding an intron (IN) sequence and recombination sequences (RS) to the 3′ end of the 5′ backbone and multiple cloning sites, IN and RS to the 5′ end of 3′ backbone. When the mixture of 5′, 3′ and integrase constructs were agroinfiltrated in *Nicotiana benthamiana*, recombination of the 5′ and 3′ genome was evident by RT-PCR and formation of virus particles was observed in electron microscope. To examine the ability of the vector system to express heterologous protein, beta-glucuronidase (GUS) gene was cloned in the MCS of 3′ module. When a mixture of 5′ module, 3′ GUS module and an integrase gene constructs was agroinfiltrated to N. benthamiana and cucumber leaves, GUS expression was detected at 10–12 days post infiltration. As CGMMV produces mild symptoms, the vector based on CGMMV will be potentially useful to produce pharmaceutical proteins in cucurbits.

Molecular Cloning and Prokaryotic Expression of the Genes Encoding Antibody to the Recombinant Coat Protein of Papaya Ringspot Virus

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Papaya ringspot virus (PRSV) [genus Potyvirus, family Potyviridae] is a major limiting factor for papaya and cucurbit cultivation worldwide. The present study was undertaken to clone and express the genes encoding antibody to the recombinant coat protein (rCP) and to assess the engineered monoclonal antibody (mAb) for the detection of PRSV. A 33 kDa rCP gene of PRSV was expressed in E. coli and purified rCP of about 50 μg was used to immunize the mouse through intraparitoneal route and subsequently three booster doses were given using 25 μg of the protein. The serum was drawn at 45 days post immunization showed high reactivity with PRSV infected leaf samples as well as with the purified rCP in ELISA. The spleen was isolated from the rabbit in order to purify mRNA to clone the antibody variable region genes (VH and VL) using universal degenerate primers. The VH and VL genes were 351 and 360 nucleotides long respectively, which contained the framework regions and complimentary determining regions and belong to the family IgG1 and kappa chain, respectively. The VH and VL genes were used to develop the expression constructs in pET28a(+) vector and 14 kDa protein was obtained in E. coli. The amount of purified VH and VL proteins was 3–4 mg/liter of bacterial culture. The crude sonicated pellet as well as purified VH and VL proteins were used as an engineered mAb to detect PRSV in the infected tissues and showed high binding affinity with purified protein in dot immunobinding assay. The present study successfully generated engineered mAb fragments to PRSV in E.coli. This is the first report of engineered mAb to PRSV. The approach may be useful to produce diagnostic mAb against other plant viruses.

Detection of Begomovirus Infection in *Capsicum annum*: Perambalur District of Tamil Nadu

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Chilli (*Capsicum annum*) is an important spice crop cultivated throughout Tamil Nadu. Leaf Curl disease of chilli has emerged as serious problem in the Perambalur district, the major chilli growing area of Tamil Nadu. During 2010–2011, very high disease incidence (up to 100% of plants) was observed in farmers field in Esanai, Anugur, Konnaripalayam, Narnamangalam villages. The characteristics field symptoms were upward curling, puckering and reduced size of leaves. Severely affected plants were stunted and produced small and no fruits. Electron microscope examination of field samples revealed few, typical geminate particles isolated by Honda et al., method. The presence of begomovirus was confirmed by polymerase chain reactions (PCR) using the replication protein primers ChiLCVFP 5′-ATGAAATA TGAACARCCG-3′ and ChiLCVRP 5′-CCATCCRAACATTCAGG

G-3′, which gave a approximately 0.85 kb product for all four samples. Sequencing of the PCR products yielded a 800–840 bp product for all four samples (Acc. No. JN887127, JN887128, JN887125, JN410657). A BLAST search of GenBank revealed close (95%) similarity of the sequence with Chilli leaf curl Multan virus-India [India/Guntur/2009] (HM007100), Pepper leaf curl virus isolate Varanasi, complete genome (EF190217), Chilli leaf curl Multan virus segment A, complete sequence (FM179613), Pepper leaf curl Pakistan virus isolate Khanewal 1 clone PC8 segment A, complete sequence (DQ116878), Chilli leaf curl virus isolate Sh1, complete genome (JN604491). Hence the virus isolated from Chilli is considered to be begomovirus namely Pepper leaf curl virus.

Molecular Identification of Cucumoviruses Infecting Chrysanthemums and Their Possible Management

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Chrysanthemum morifolium Ramat are popular for flowers, exhibiting an incredible range of diversity in size, shapes, colours and long self-vase life. CSIR-NBRI, Lucknow maintains more than 350 cultivars of chrysanthemum as germplasm which are being propagated by cuttings/suckers. Chrysanthemum cultivation has setback of virus infections in nature jeopardizing its quality and production. Several groups of viruses have been reported infecting chrysanthemum including Cucumoviruses. Cucumoviruses have three type members: Cucumber mosaic virus (CMV), Tomato aspermy virus (TAV) and Peanut stunt virus (PSV) which are aphid transmittable and have single-stranded, tripartite, plus sense RNA genome. The natural occurrences of cucumoviruses on chrysanthemums affect their quality production; therefore, attempts were made for characterization and identification of cucumoviruses along with their possible management. Infection of CMV and TAV was detection by biological assays, serological and RT-PCR tests in the chrysanthemum plants exhibiting severe mosaic, marginal yellowing, vein banding, ring-spot on leaves, floret and flower deformation, and poor growth symptoms observed in germplasm centre of CSIR-NBRI. Both the viruses were identified at molecular level by cloning and sequencing of their complete RNA3 genomes: CMV (EF153733) and TAV (EU163411) which revealed movement and coat protein ORFs separated by IR and flanked by 5′ and 3′ UTRs. Based on highest more than 97% sequence identities and closest phylogenetic relationships, the virus isolates EF153733 and EU163411 were identified as CMV of subgroup IB and TAV, respectively. The elimination of detected viruses (CMV and TAV) has been successfully achieved in a commercial cultivar of chrysanthemum cv. Pooja employing shoot apical meristem culture. A total of 78.1% CMV and TAV-free shootlets were obtained from the regenerated shoot meristem as indexed by DAC-ELISA, of which only 65.6% were found truly virus-free when confirmed by RT-PCR. Virus-free shootlets were rooted and acclimatized under glasshouse. These plants showed better growth and quality of blooms as compared to diseased ones. Development of inbuilt resistance in C. morifolium against CMV was also attempted by Agrobacterium-mediated transformation of petiole explants using pRoK2 binary vector harbouring coat protein (CP) gene of CMV under the control of CaMV 35S promoter. A total of 257 explants were transformed and 73 putative transgenic plants from seven independent co-cultivation events were obtained. Molecular analysis of these plants confirmed the successful integration of CP transgene in 63% plants, of which 12.3% plants were able to transcript and translate the transgene. Expression of coat protein did not evoke any abnormal phenotype. Transgenic plants showed delayed resistance when challenged by CMV-chrysanthemum strain which produced good quality blooms as compared to the susceptible ones. The present study provides extended molecular identification of CMV and TAV isolates infecting chrysanthemums from India which provides better understanding of the taxonomy of virus/es. The regeneration and transformation methodologies evaluated for chrysanthemum would be of practical value and help in extending the genetically engineered resistance to plant viruses to other economically important crops.

Molecular Detection and Identification of Begomoviruses Affecting Important Ornamental Plants in India

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A number of plant species are grown in India as ornamentals for their aesthetic values. Several viruses have been reported to cause various diseases on them which affected their quality and production. During the survey in 2011 some ornamental plants viz. *Hibiscuss rosa-sinensis*, crape jasmine (*Tabernaemontana coronaria*), night blooming jasmine (*Cestrum nocturnum*), *Jatropha podagrica*, *Jatropha multifida*, *Jatropha integerrima*, Hollyhock (*Alcea rosea*), Aster (*5555555*) and ornamental ageratum species growing at various gardens in Lucknow U.P., India were found to be exhibiting begomovirus like symptoms. The ~1.2 Kbp amplicons of begomovirus were successful amplified in naturally infected ornamental plant species by PCR using begomovirus specific primers. The 1.2 kb amplicons obtained were sequenced by both the direction and sequence data were deposited in GenBank database under Accession numbers: JN807763 (*H. rosa-sinensis*), JN807764 (*T. coronaria*), JQ012790 (*C. nocturnum*), HQ848382 (*J. podagrica*) HQ848381 (*J. multifida*), JQ043440 (*J. integerrima*), JQ911766 (*A. rosea*), JQ954859 (*A. alpinus*) and JQ911767 (ornamental ageratum). The sequence analyses results were suggested occurrence of diverse begomovirus species on these ornamental plant species viz. Cotton leaf curl Multan virus on *H. rosa-sinensis*, *Pedilathus* leaf curl virus on T. coronaria, Tomato leaf curl Pakistan virus on *C. nocturnum*, Jatropha mosaic India virus on *J. podagrica*, Tomato leaf curl Patna virus on *J. multifida*, Papaya leaf curl virus on *J. integerrima*, Hollyhock yellow vein mosaic virus on Hollyhock, Papaya leaf curl virus on Aster and *Ageratum enation* virus on ornamental ageratum were partially identified. The details of research work on detection and identification of begomoviruses done by us at NBRI, Lucknow will be discussed in the conference.

Quantitative and Qualitative Yield Loss of Cassava Tubers Infected with Sri Lankan Cassava Mosaic Virus

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Cassava in Tamil Nadu is cultivated as a tuberous root crop and its roots are the major source of dietary and industrial starch serving as input for the major starch and sago industries. Tamil Nadu has an area of 95,000 ha (40% of the total area under cassava in India, 2006) and 60% of cassava produced is utilized industrially to produce starch, sago and other value added products. Since, cassava is mainly propagated

vegetatively, it is particularly prone to viral infections, which tends to build up in successive cycles of propagation (Calvert and Thresh, 2002). In Tamil Nadu, the cassava mosaic disease has been reported to be caused by Sri Lankan cassava mosaic virus (Dutt et al., 2005 and Rajinimala et al., 2006). Crop loss data due to infection with Cassava Mosaic Disease have been reported from many countries including India (Fauquet and Fargette, 1990). The average yield loss caused by cassava mosaic disease was estimated to be 50% (Fauquet and Fargette, 1990). From India Malathi et al. (1985) has reported that the disease causes a yield loss of 17–88%. Cursory perusal of the literature revealed that many studies have been attempted to quantify yield losses due to CMD infection but information on qualitative loss is limited. Hence, we attempted to study both the quantitative and qualitative loss due to SLCMV infection in cassava variety CO2. This study was conducted in an area of 50 cents in total (Virus indexed healthy plants in an area of 29 cents and SLCMV infected plants in an area of 21 cents). The yield obtained from infected plants was 969 kg when compared to healthy plants yield 3392 kg. Further the quality of the tubers was also analyzed and it was found that the starch content of tubers collected from healthy tubers was 28% whereas it was 21.5% in infected tubers. The protein content of tubers from healthy plants was 0.44% and it was 0.39% in virus infected tubers. Our studies indicate that infection with Sri Lankan cassava mosaic disease can reduce the starch content of the tubers to the tune of 7%. Since, Tamil Nadu has more than 800 cassava based industries (which depends mainly on the cassava starch), this study has significance to the economy of Tamil Nadu and farmers.

Mycoviruses: An Emerging Potential Field of Virus Research in Indian Scenario

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Mycoviruses are viruses that infect fungi selectively. Mycovirus research is an emerging and potential field of virus research but is still in its infancy, especially in Indian context. In the present study 18 isolates of different fungi were screened for the presence of virus like particles (VLP's). Negative staining and electron microscopy indexed 7 *Chrysosporium* sps., *Candida albicans*, *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Microsporum fulvum*, 7 *Fusarium* sps. Isometric (short, medium and large ranging from 20, 33–40 and 66–70 nm respectively) VLP's were most commonly associated with positively indexed fungal cultures of *Chrysosporium* sps., *Candida albicans*, *T. mentagrophyte*, *A. fumigatus*. Rods were found in Fusarium isolates. *Chrysosporium pseudomerdarium* and *Candida albicans* were selected for further studies. In *C. pseudomerdarium*, VLP's elimination was attempted through hyphal tipping and thermotherapy. Immediate heat treatment was successful in eliminating VLP's from *C. pseudomerdarium*. Similarly, C. albicans was treated with cycloheximide at 10, 20, 50, 100, 200 μg/ml concentration. VLP's were eliminated at 50 μg/ml. Partial purification of VLP's of *C. pseudomerdarium* and *C. albicans* was done. Extraction of nucleic acid directly from mycelium gave band in both the isolates through Agarose Gel Electrophoresis. Nature of nucleic acid was ascertained by RNAse and DNAse treatment. Presence of band in low salt (0.01 SSC) RNAse test of *C. pseudomerdarium* led to conclusion that nucleic acid can be dsRNA. High salt and DNAse treatment did not reveal any band in any of the isolates. Studies of fungal virulence and hypovirulence can increase our understanding of molecular mechanism influencing expression of virulence in plant pathogens and expand potential of fungal virulence as unique mechanism of action for biological control.

Molecular Characterization of Coat Protein Gene of *Garlic common latent virus* Isolates from Distinct Geographical Locations of India: An Evidence for Distinct Phylogeny and Recombination

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The coat protein (CP) gene of five Indian *Garlic common latent virus* (GarCLV) isolates was determined. Length of the CP gene was found to be 960 bp, encoding a protein of 319 amino acids. Comparative nucleic acid sequence analyses revealed a 4.3% divergence among the Indian isolates with an overall 11.9% diversity among all available isolates from around the world. Closest and farthest *Carlavirus* species to GarCLV was found to be *Coleus vein necrosis virus* (CVNV) and *Cole latent virus* (CLV), respectively. Amino acid sequence comparison showed significant variability in the N terminal of CP of GarCLV. Various protein analysis tools identified eleven conserved domains and motifs within CP sequence including highly conserved domains such as Flexi CP and Flexi N CP, specific to carlaviruses and potexviruses. Phylogenetic analysis of GarCLV isolates revealed the clustering of all isolates into two subgroups, one corresponding to isolates from the USA and the China designated as GarCLV Subgroup I, and the other corresponding to isolates from other countries designated as GarCLV Subgroup II. Similarly, phylogenetic analysis using the CP of 37 *Carlavirus* species including GarCLV also grouped them into two sub groups, we proposed them as Carla Subgroup I which included 26 *Carlavirus* species infecting diverse plant species and Carla Subgroup II which included remaining 12 *Carlavirus* species including GarCLV. Intraspecies recombination study revealed that only one of the Indian isolate i.e. GarCLV-Anand (Accession no. JQ818258) was found to be a recombinant of two Indian isolates GarCLV-JN (Accession no JQ818255) and GarCLV-Kolar (Accession no JQ818257). Interspecies recombination study suggested the absence of exchange of genetic material from *Carlavirus* species to GarCLV but GarCLV contributed its genetic fragment for at least two other *Carlavirus* species. This is the first report of molecular variability and recombination in GarCLV isolates.

Detection and Localization of Potato Virus A (PVA) by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) in Infected Potato Plants

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Potato virus A (PVA) belongs to Potyviridae is the largest virus family infecting plants which confined to the members of the Solanaceae. Different combinations of primers targeting coat protein gene, master mix and varying PCR conditions were standardized for RT-PCR detection of PVA. This standardized RT-PCR protocol gave a sharp and specific band of ~340 bp without any multiple bands. The PCR fragment of ~340 bp (PVA-CP) was gel eluted and ligated in a pPrime cloning vector and it was transformed in E. coli strain JM107. It resulted in several recombinant white colonies carrying the target DNA on a selective media. A colony PCR was carried out wherein a specific fragment of 330 bp was observed which confirmed the recombinant clones. A second confirmation was done by restriction digestion which released a fragment of 330 bp. The fragment so obtained was sequenced and the sequence was found to be 337 bp long. The BLAST analysis of the sequence revealed that the query

had a similarity match of 96 and 95% with that of the coat protein gene sequence reported from Finland and China respectively. After confirming the standardized protocol, it was further used to localize the distribution of PVA in different parts like bark, top, middle and base of the sprouts, leaves, center and periphery of tubers and roots wherein, all the parts showed the presence of the virus. Later, different cultivars were screened for the infection of PVA which revealed that few cultivars were infected while some were free from virus. So, this standardized RT-PCR protocol can be employed for quick and reliable detection of PVA in seed certification programmes.

Molecular Characterization and Genetic Diversity Among the Begomoviruses Infecting *Jatropha curcas* in India

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Jatropha curcas L. of Euphorbiaceae family is grown in India for biodiesel production. However, Jatropha production is limited due to jatropha mosaic disease caused by Begomovirus which shows severe mosaic, blisters, leaf distortion and stunting symptoms. Because of high economic importance of *J. curcas* and begomovirus, this study has concerned on genetic diversity exists among the begomoviruses infecting *J. curcas* grown in India. To study the genetic diversity exists among the begomoviruses infecting *J. curcas*, total DNA were isolated from the Jatropha leaf sample collected from various locations in India viz. Lucknow & Bahraich (U.P.), Hyderabad (A.P.), Nagpur (M.S.), Bhavnagar (Gujarat), Jodhpur (Rajasthan) and Raipur (Chhattisgarh). The partial DNA A ($\sim$1.2 kb) of begomovirus isolates of *J. curcas* were amplified by PCR using begomovirus specific primers (Rojas et al., 1993) and amplicons were sequenced and deposited in GenBank database: JN698953, JN698952, JN698951 (from Hyderabad); JN807767, JN807768 (from Bhavnagar); and JQ178365 (from Lucknow). BLASTn analysis of begomovirus isolates of *J. curcas*: JN698953, JN698952, JN698951 (Hyderabad) and JN807767, JN807768 (Bhavnagar) revealed highest identities (99–91%) with Jatropha mosaic India virus. However, JQ178365 (Lucknow) showed 95% identities with Tomato leaf curl Karnataka virus. Further, the complete DNA A ($\sim$2.7 kb) was also amplified by RCA, cloned into pCAMBIA2301 vector, sequenced and sequence data were deposited in GenBank under Acc. Numbers: HM230683 & JN692494 (Lucknow) and JN135236 & JN698954 (Bahraich). Sequence analysis of complete DNA A molecule of Lucknow and Bahraich isolates revealed 89–90% sequence identities with each other and highest (90%) identity with Jatropha mosaic India virus (JMIV) and 88–89% with *Jatropha curcas* mosaic virus (JCMV), Indian cassava mosaic virus (ICMV) and Sri Lankan Cassava mosaic virus (SrLCMV) and less identities with African cassava mosaic virus (ACMV) and Jatropha leaf curl virus (JLCV). During phylogenetic analysis of these four isolates along with JMIV, JCMV, ICMV, SrLCMV, ACMV and JLCV isolates, all the isolates clustered in 5 different groups. The Lucknow isolate (JN692494) shows closest relationship with JCMV Jalgaon and Dharwad isolates. Bahraich isolates (JN135236 and JN698954) clustered together and showed close relationship with JMIV Lucknow isolate (HM230683). These studies indicated existence of some genetic diversity among the begomoviruses infecting *Jatropha curcas* in India, which needs further investigations.

Using Molecular Techniques to Determine the Endosymbiont Diversity and Biotype of Whiteflies, the Vector for All Begomoviruses

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The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a serious pest that damages many agricultural crops and are known vector for more than 100 emergent plant virus species (Begomoviruses). Whiteflies are the plant-sap sucking insects having obligatory association to the prokaryotic endosymbionts. Various endosymbionts associated with whiteflies are categorised as Primary (P) endosymbionts and Secondary (S) endosymbionts. The main function of P endosymbionts is to synthesize essential missing aminoacid for the host. S-endosymbionts form a heterologous group with respect to their location and function. *Bemisia tabaci* consists of several biotypes that differ in several biological traits that affect host range, their capacities to inflict plant disorders and/or viral diseases, and their susceptibility to various insecticides. The two most widespread biotypes of *B. tabaci* in southern Europe and the Middle East are referred to as the B and Q types. This investigation reports the biotype and endosymbiont population of *B. tabaci* prevalent in Delhi. Here in this study, DNA was isolated from the individual as well as from 20 mg of whiteflies collected from the brinjal plants maintained in the greenhouse of University of Delhi South Campus. Polymerase Chain Reaction (PCR) along with the specific primers for the conserved region of MtCOI and 16S rDNA were used to determine the biotype and the endosymbiont population of the whiteflies. MtCOI specific primers resulted in amplification of 800 bp DNA fragment from the genomic DNA of individual whitefly while 16S rDNA specific primers resulted in DNA amplification of 600 bp, 700 bp, 900 bp and 700 bp amplicon for 16S rDNA region of *Arsenophonus*, *Wolbachia*, *Rickettsia*, and *Hamiltonella* endosymbionts respectively. The DNA sequencing of the MtCOI and 16S rDNA genes led to the determination of the biotype as well as confirmed the endosymbiont population diversity in the whiteflies. The results indicated the Q-biotype of the whiteflies harbouring *Arsenophonus*, *Rickettsia*, *Wolbachia* and *Hamiltonella* as S-endosymbionts. This piece of information has led to the better understanding of the host which would in turn be helpful in studying viral transmission aspect as well.

Study on the Host Range of Sri Lankan Cassava Mosaic Virus Using SLCMV Infectious Clone

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Cassava mosaic disease (CMD) is one of the major constraints in the cultivation of cassava, an important tropical root crop of our country (Fauquet and Fargette (1990). Srilankan cassava mosaic disease causes cassava mosaic disease in India. Elsewhere the first epidemiological evidence on CMD was provided by Storey (1938). However, presence of alternative host for SLCMV was provided by Jose et al. (2008). They have documented the alternate host by mechanical inoculation with crude extract of SLCMV on 39 species of plants belonging to Solanaceae (38 species of *Nicotiana* and *Daturastramonium*). But they failed to inoculate on cassava by sap inoculation. In order to pinpoint the host range of SLCMV pure virus has to be used. Earlier, Mittal et al., 2008 demonstrated Agro inoculation of SLCMV clone to cassava, *Nicotiana tabaccum* and *Arabidopsis thaliana* which resulted in production of typical symptom but not much evidence have provided on the host range using the infectious clone. Hence, a study was undertaken during

2012 to inoculate SLCMV clones to various plant species including cassava. In our study, we used clones of SLCMV developed by Indranil Dasgupta to study host range. SLCMV clone was inoculated onto the dicotyledonous hosts belonging to the genus Euphorbiaceae (3 species), Malvaceae (2 species) Solanaceae (8 species) and Cucurbitaceae (5 species) and Cruciferaceae (1 species). On infection with SLCMV clone the following symptoms viz., vein thickening, deformation of leaf and lateral shoots, stunting and sterility on *Nicotiana tabaccum*, *N. glutinosa*, *N. benthamiana*, *N. rustica*, Tomato and Cowpea, whereas, typical mosaic symptom and stunting were observed on cassava. The incubation period after inoculation for symptom development ranged from 35–40 days on all the Nicotiana species, for tomato and cowpea the visible symptom appeared 30 days after inoculation and on cassava the symptom appeared 40–50 days after inoculation. The percentage of infectivity was higher in case of laboratory plants when compared to tomato, cowpea and the natural host, the cassava. Other crop species belonging to Cucurbitaceae, Cruciferaceae, Malvaceae, Solanaceae, Euphorbiaceaea well as mock inoculated plants did not produce symptoms.

Sequence Analysis of Cloned Geminiviral and Satellite Molecules Associated with Okra (*Abelmoschus esculentus*) Affected with Yellow Vein Mosaic Disease

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Okra (*Abelmoschus esculentus*)is an important vegetable crop in India. The Yellow Vein Mosaic disease of okra is caused by a complex consisting of the monopartite begomovirus Bhendi yellow vein mosaic virus (BYVMV, family: Geminiviridae) and a small satellite DNA β component. Yellow vein mosaic disease is a major limitation in the production of Bhendi, the yield loss estimated to a range from 50% to 94%. Despite the importance and widespread nature of Bhendi Yellow Vein Mosaic Disease (BYVMD), not much information is available on the extent of sequence variability of the causative viruses and satellites. Materials and Methods: For this purpose, infected okra leaves were collected from various parts of India and checked for the presence of BYVMV by amplifying the CP gene from ten different samples [Andhra Pradesh (AP), West Bengal (WB) and Orissa (Or), Aurangabad (Au), Coimbatore (Co), Jalna (Jal), Vijayawada (Vij), Jalgaon (Jalg) and Varanasi (Var)]. Thereafter, cloning and sequence analysis of full-length viral DNA and associated betasatellites was done from selected four and six samples respectively. The results indicated that CP sequences fell into two groups each showing more than 95% identity within the group but less than 80% between the groups. One group showed highest identity to BYVMV CP sequences, while other to Mesta Yellow Vein Mosaic Virus CP sequences. The cloned betasatellites shared more than 95% similarity to betasatellites associated with BYVMD. The recombination analysis of the clones was performed by using Recombination Detection Program (RDP) RDP analysis could detect an event of possible recombination between BYVMV and MeYVMV in one of the full-length isolate. This report indicates strongly that BYVMD in India is associated with at least two viruses, BYVMV and MeYVMV. It also indicated that recombination is quite widespread in begomoviruses affecting okra. Sequence analysis of cloned betasatellites from seven locations indicated that there was high sequence identity (90% or <90%) among all the cloned molecules.

Genome Sequence of a Sweet Cherry Isolate (JK10) of Cherry Necrotic Rusty Mottle Virus (CNRMV) from India

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Cherry necrotic rusty mottle virus is a graft transmissible, unassigned member in the family Betaflexiviridae. CNRMV infects sweet cherry (Prunus avium) and has been reported in North America, Europe and Japan. Diseased plants show brown angular necrotic spots, rusty chlorotic areas, shot holes of the leaves, blisters, gum pockets and general necrosis of the bark. In India, sweet cherry is grown mainly in the North-Western states of Jammu and Kashmir (J&K), Himachal Pradesh (H.P.) and in hilly regions of Uttarakhand (U.K.). This perennial crop is usually propagated by grafting and, therefore, is easily infected with viruses when contaminated material is used as a propagation source. Therefore, it is desirable to devise diagnostic protocols for CNRMV in quarantine and certification programs. To understand the health status of Cherry orchards and to determine the incidence of the virus in India, a survey was conducted in the months of May and September in Srinagar, Pulwama, Shopian, Sopore and Ganderbal regions of J&K and Rohru, Narkanda and Bhutti regions of H.P. For the preliminary detection of CNRMV, symptomatic leaf and twig samples were collected and RT-PCR was carried out. The incidence of CNRMV was found to be 7% and 66% from J&K and H.P., respectively. In order to characterize the virus at the molecular level, the CP gene and TGB were amplified by RT-PCR using specific primers. The partial genome sequence of JK10 isolate consists of 2203 nucleotides. Comparison of the sequence with the already submitted complete CNRMV sequences revealed a similar genetic organization with the ORF 2, ORF 2a, ORF 3, ORF 4, ORF 5 and ORF5a in similar positions and 3′UTR region of almost identical size. The sequence of JK10 isolate showed 91% and 84% similarity to the type isolate of CNRMV from Germany (AF237816) at the nucleotide and amino acid level, respectively. Phylogenetic analysis based on JK10 isolate coat protein gene with selected members of the family Betaflexiviridae showed that the isolate is most closely related to CNRMV flowering cherry isolate, FC4 from Japan (EU188438) sharing 94% and 96% identity at nt and aa levels respectively.

Preliminary Studies on Natural Occurrence of *Clerodendrum* Mosaic in Agra Region

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Natural occurrence of severe chlorotic mosaic symptoms on *Clerodendrum aculeatum* hedges around botanical garden was observed. A survey of North-Eastern part of Agra including Dayalbagh, Rambagh, Trans Yamuna colony, Paliwal Park and Agra university; along with South-Eastern region of Agra including various areas of Taj for visual examination of symptoms was conducted and revealed an explosion in chlorotic mosaic symptoms. Approximately 65–70% plants exhibited mosaic symptoms. Ethno-medical importance of various species of *Clerodendrum* genus has been reported in various indigenous systems of medicines. The genus is being used for the treatment of various life threatening diseases such as syphilis, typhoid, cancer, jaundice and hypertension. Few species are ornamental and being cultivated for aesthetic purposes. Genus has potential to be developed as potent remedial agents from natural resources. Major biological activities reported for this genus are

antihypertensive, antitumour, antidiabetic, antihyperlipidemic, larvicidal, antidiarrhoel antimicrobial activities. There are few reports of viral diseases affecting plants of this genus. Owing to the enormous ethno-medicinal importance of the plants, preliminary investigations were undertaken to establish the viral nature of the disease, virus infectivity assay on 25 plants belonging to 6 different families was done, by leaf rubbing method using Celite as an abrasive 0.1 M phosphate buffer, pH 7.0 was used for extraction of infected sap. Local chlorotic spots appeared on Cowpea, *Acalypha*. Systemic infection was seen in *Lageneria* spp., *Cucumis* spp., *Luffa* spp., *Vignaspp*, etc. Systemic chlorotic spots, vein clearing, leaf curling and reduction of leaf lamina was observed in mechanically inoculated test-plants. Histopathological studies of infected and healthy leaves using trypan blue rose bengal and phloxin B stains revealed proliferation in vascular bundles and chloroplast clumping in infected leaves. No inclusion bodies were observed in the epidermal peel of infected plants. Electron microscopy of partially purified virus preparation of *Clerodendrum* of infected plants revealed the presence of isometric virus particles 50–60 nm in size.

New Ornamental and Cucurbitaceous Hosts of Groundnut Bud Necrosis Virus in India

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Natural infection of Groundnut bud necrosis virus (GBNV) on *Chrysanthemum indicum* (Chrysanthemum), *Dahliacoccinea* (Dahlia) and *Cucumismelo* (Muskmelon) hosts was identified on the basis of nucleocapsid (N) protein gene characteristics. The partial N gene of GBNV from muskmelon, chrysanthemum and dahlia of 477 nucleotide long encoding 159 amino acids amplified using species specific forward and degenerate reverse primers. Comparative sequence analyses of GBNV revealed 93–100% and 94–100% identities at nucleotide and amino acid level respectively with the corresponding region of GBNV N gene from different hosts. Similarly, in phylogenetic relationships, the N gene sequences in this study clustered with the GBNV sequences from other hosts used for comparisons, this forms the first evidence of occurrence of GBNV in ornamental and cucurbitaceous crop in India.

Detection and Elimination of Potyvirus from *Gladiolus* spp. for Its Quality Production

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Gladiolus (*Gladiolus psittacinus* L. of family Iridaceae) is a popular garden and potted flower plant grown worldwide including India. Gladiolus cultivars are of various colors, sizes, shapes and flowering time which make it a high choice for commercially cultivation. The commercial production of gladiolus is being hampered by infection of several plant viruses deteriorating the quality of blooms. Approximately 200 gladiolus cultivars are being maintained and grown in CSIR-NBRI, Lucknow for cut-flowers and annual flower show. The leaf mosaic, color breaking and deformation of flowers, and corm distortion symptoms were observed in many gladiolus plants of different cultivars. The natural occurrence of potyvirus was detected byRT-PCR tests in Vink's Glory, Aldebaran and Sylvia cultivars. The elimination of potyvirus was attempted by in vitro culturing in combined with chemotherapy of cormel explants from infected gladiolus of cvs. Vink's Glory, Aldebaran and Sylvia. The infected cormels were surface sterilized and cultured in MS media supplemented with 1.0 mg/L BAP, 0.5 mg/L IAA and 2 mg/L 2,4 D growth hormones and various concentrations of virazole (30, 40 and 50 mg/L). The successful regeneration was achieved in MS media supplemented with 30 mg/Lvirazole. At 40 mg/L concentration of virazole, corms survived but there was no growth, however, 50 mg/L concentration was found lethal. Three randomly chosen regenerated shoot lets of Sylvia cultivar were tested by RT-PCR to confirm the potyvirus and two/three plants were found to be virus-free. The virus-free shootlets are being multiplied by in vitro propagation to obtain more virus-free plants. Elimination of potyvirus infection from gladiolus and development of the virus-free culture using chemotherapy would be useful for the floriculture industry for the mass propagation and quality production of blooms.

Molecular Detection of Canna Yellow Mottle Badnavirus Associated with Mottling Disease of Canna in India

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Canna spp. (of family Cannaceae) are the perennial, rhizomatous ornamental plants growing in tropical and subtropical areas including India. Canna is popular for its attractive bunchy blooms of red, orange and yellow colors. Canna has been reported to be susceptible for the natural infection of many viruses such as Canna mosaic virus, Cucumber mosaic virus, Potyvirus, and Canna yellow mottle virus (CaYMV). Among them, CaYMV infection has been reported to be much frequent. The association of a small non-enveloped bacilliform virus with the canna yellow mottle disease was first ever reported in Japan during 1985. CaYMV (genus Badnavirus; family Caulimoviridae) cause yellow streaks along the veins and flower color breaking in canna. The similar yellow streaks and flower color breaking symptoms were observed in canna plants growing at canna plot of CSIR-NBRI, Lucknow during the survey conducted in 2011–2012. A few of symptomatic as well as asymptomatic leaf samples from the same vicinity were collected for different varieties of Canna spp for the detection of CaYMV. Total genomic DNAs isolated from all samples, employing QIAamp DNA mini kit (QIAGEN, India), were used as template for PCR amplification using CaYMV forward and reverse primer pair spanning the most conserved region of RT/RNaseH of CaYMV. During agarose gel electrophoresis, PCR amplified product showed the expected $\sim$550 bp band in many diseased samples, but no amplification in asymptomatic sample. Two independent PCR amplified products were gel purified and got sequenced. The sequence data of 565 nucleotides obtained were analyzed to resolve any ambiguity and submitted to GenBank under the accessions: JX228965 and JX228966. The virus isolates under study showed 97% nucleotide sequence identities with each and 96% sequence identities with several isolates of Canna yellow mottle virus: (EF189148, HE774735, HE774734, HE774733, EF189147 and EF189149) reported worldwide. During phylogenetic analysis of both the isolates under study clustered together and showed closest relationships with CaYMV-V17 isolate (EF189148) reported from Austria and close relationships with other CaYMV isolates reported worldwide. Based on high sequence (96%) identities and close phylogenetic relationships with several CaYMV isolates, the virus isolates under study have been identified as isolates of CaYM. To the best of our knowledge, this is the first ever record of CaYMV infection in canna from India. The data (sequence information) obtained from the present study may be used to develop the diagnostic probe for the sensitive and reliable detection of CaYMV for the

screening large number of canna plants of different varieties. Besides this, the sensitive and reliable PCR protocols optimized for the amplification of CaYMV genome may be used for complete identification of CaYMV.

Molecular Detection and Elimination of Cucumber Mosaic Virus from *Gerbera jamesonii* for Better Flower Quality Production

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Gerbera (*Gerbera jamesonii*) belongs to the family Asteraceae and is commonly known as African Daisy. It is popularly used as decorative garden and container plants, besides sold mostly as cut-flower sticks. Gerbera has been cultivated almost in all parts of country, yet more commercially in Karnataka, Maharashtra and North-Eastern states including Uttar Pradesh. Gerbera cultivation has a major setback in the commercial production because of the several plant viruses which deteriorate the commercial quality of flowers. One of the commercially important cultivar of gerbera cv. Zingaroo was found to be infected by severe chlorotic mosaic, floret and flower deformation disease. The natural occurrence of Cucumber mosaic virus (CMV) associated with the disease of *G. jamesonii* has been detected by western blot immunoassay using CMV antiserum (PVAS242a) and RT-PCR tests employing coat protein gene specific primers. In present study, the elimination of CMV from infected gerbera has been attempted by in vitro culturing of floral buds and pedicel explants. The basal MS medium supplemented with combination of plant growth hormones (1.0 mg/L BAP and 0.5 mg/LIAA) was optimized for gerbera regeneration. The regenerated shootlets were first tested by RT-PCR using CMV-CP specific primers for presence or absence of CMV. One of the randomly selected shootlet was found to be free from CMV and further multiplied using their proliferation shoots in regeneration medium additionally supplemented with Adenine sulfate (Ads, 0.5 mg/L) growth hormone. A total of 15 regenerated shootlets were rooted in rooting medium (MS medium supplemented with 0.5 mg/LIAA and 35.0 mg/L Ads hormones. The rooted plantlets were re-confirmed by RT-PCR, acclimatized and established in glasshouse. Elimination of CMV infection from gerbera and development of virus-free gerbera plants would be helpful to provide healthy planting material to gerbera growers and for improvement of better quality production of gerbera in the country.

Immunodiagnosis of Peanut Mottle Virus Using Polyclonal Antibodies to Bacterial Expressed Core Recombinant Capsid Protein

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Peanut mottle virus (PeMoV), a serologically distinct Potyvirus (family Potyviridae)is one of the major seed borne virus affecting groundnut and soybean all over the world. The symptom of the virus in peanut is mild and thus its occurrence is often unnoticed in the field. PeMoV occurring in India has not been characterized at molecular level. In the present study, the coat protein (CP) gene of PeMoV isolate from peanut in Andhra Pradesh was cloned, sequenced and used to express in *E.coli*. Polyclonal antiserum (PAb) to the core recombinant CP of PeMoV was generated and used in serological diagnostic tests. The full length sequence of PeMoV CP (JX088125) gene showed 99% similarity with the PeMoV Isolate (DQ868539) from Israel. The core CP gene (540 bp) was cloned into pET28a(+) expression vector and the recombinant CP was expressed as a fusion protein containing N and C terminal hexa-histidine tag. A high titre PAb produced against the recombinant CP efficiently detected PeMoV in the infected peanut leaf samples. The PAb was used in ELISA to test 1641 peanut samples during 2010–2012 from several fields near Hyderabad, which showed 12% incidence of PeMoV. This is the first report of immunodiagnosis of PeMoV using polyclonal antiserum against bacterial expressed viral antigen.

Transgenic Constructs and Transformation of Watermelon cv. Sugar Baby for Developing Transgenic Resistance Against Watermelon Bud Necrosis Virus

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Watermelon bud necrosis virus (WBNV), a member under the genus Tospovirus is a serious limiting factor for watermelon cultivation in India. Transformation frequency in of watermelon is very low and therefore it is difficult to develop virus resistant transgenic watermelon plant. In the present study, we have developed a full length nucleocapsid protein gene and a core NP gene transgenic constructs from an isolate of WBNV characterized from northern India. To achieve optimal conditions for regeneration of watermelon cv. Sugar Baby, six types of explants (immature cotyledon, mature distal cotyledon, mature proximal cotyledon, distal hypocotyls, proximal hypocotyls, basal epicotyls) were evaluated on MS medium supplemented with different concentrations and combinations of hormones. The results showed that proximal immature cotyledons cultured in MS +BAP (2 mg/l) + IAA (0.1 mg/l) achieved the highest rate (76%) of regeneration. *Agrobacterium tumefaciens* strain EHA 105 carrying the construct in a binary vector pBI121, which contained the GUS reporter gene and a kanamycin-resistance gene nptII, was employed for optimizing the transformation efficiency. The optimal conditions for transformation of watermelon were evaluated and 14.2% of GUS transformed plants were obtained. The optimised protocol was then adapted to transform watermelon plants using both the transgene constructs of WBNV. However, a very low rate (0.9%) of transformation using WBNV based transgenes was achieved.

Molecular Detection of Cherry Virus A From Sour Cherry in India

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Cherry virus A (CVA) belongs to the genus Capillovirus within the family Betaflexiviridae. CVA is an important virus disease of cherry having worldwide distribution. It is believed that CVA causes severe symptoms and disease in combination with other stone fruit viruses in cherry and non cherry hosts. To investigate the status of virus and virus like disease on cherry a preliminary survey was conducted in the experimental field of CSIR-Institute of Himalayan Bioresource

Technology, Palampur. Eight sweet cherry (*Prunus avium*) and five sour cherry (*P. cerasus*) leaf samples were collected and tested for five viruses that are known to infect Prunus species by reverse transcription-polymerase reaction (RT-PCR). Viruses that were analyzed included Little cherry virus 1 (LChV-1), Little cherry virus 2 (LChV-2), Cherry necrotic rusty mottle virus (CNRMV), Cherry virus A (CVA) and Prunus necrotic ringspot virus (PNRSV). Only CVA was detected from all sour cherry and five sweet cherry plants by RT-PCR assay with a new set of primers CVA5839U/CVAL specific to a 1539-bp fragment of the CVA replicase gene and 3′ UTR. To confirm RT-PCR results, CVA amplification products were sequenced (accession no. FN669549). Sequence analysis of the 1539 bp by BLAST search in GenBank showed 91% similarity to the type isolate from Germany (Accession no. NC_003689) and 99% similarity to the Indian isolate (Accession no. FN691959) of CVA at nucleotide level. To the best of our knowledge, this is the first report of CVA from sour cherry in India.

Begomovirus on Plant of Family *Asteraceae* in Gorakhpur District of North-Eastern Uttar Pradesh

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During the survey of Asteraceous plants in Gorakhpur district of North-Eastern Uttar Pradesh a severe yellow mosaic and vein clearing symptoms were recorded on Ageratum conyzoide. The symptomatic leaves were analyzed through PCR assays with specific primers (TLCV); detect positive ~800 bp amplicons. The PCR amplified product was directly sequenced and sequence was submitted in GenBank with accession no. GQ412352. Sequence similarity and phylogenetic analysis confirmed the presence of begomovirus Ageratum enation virus (AEV) on *Ageratum conyzoides*. In current scenario, AEV is widely distributed throughout Asia. The frequency with which new strains of AEV are appearing in several agricultural crops and non-crop species which indicates that the virus species has more virulent strains in field and pose a serious threat to cultivated and non cultivated crop species. In last 5 years its number is increasing very fast. So far many strains have been reported from a variety of plants. It indicates that in coming years Ageratum enation virus will pose serious constraint next to Tomato leaf curl New Delhi virus to agricultural crops due to its more virulent strains with broad host range. The widespread occurrence of AEV on various crop and non crops species, suggests us to formulate strict control strategy measure to check further spread of this virus in new locality. Existence of begomovirus has been increased at the alarming level in the India and it has been reported on various crops by different workers from the different regions of India. Therefore an immediate attention is required to check the further spread of this begomovirus in nature.

Identification of a Watermelon Mosaic Virus associated with Mosaic Disease of *Catharanthus roseus* in North-Eastern Uttar Pradesh and its Histopathological Effects

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During survey in North-Eastern of Uttar Pradesh, severe chlorosis, crinkling and curling symptoms were noticed on leaves of *Catharanthus roseus* plant. The virus was partially purified from the symptomatic leaves by differential centrifugation. Electron microscopy of the partially purified preparation revealed the 760 nm × 14 nm filamentous flexuous particles. The causal virus was found transmitted through mechanical transmission to healthy *Catharanthus roseus* plants as well as through *Aphis gossypii*, *A. nerii*, *A. craccivora*, *Brevicoryne brassicae*, *Myzus percicae*, and *Macrosiphoniella sanbormii*. Immuno-diagnostic studies (DAC-ELISA and DIBA) showed positive serological relationships of the causal virus with polyclonal antiserum of Watermelon mosaic virus (WMV). The RT-PCR assays amplified fragment of ~330 bp with coat protein specific primer of WMV. The particle morphology, serology and RT-PCR results confirmed that the virus associated with chlorotic leaves of *Catharanthus roseus* in the present study is Watermelon mosaic virus. The virus infection also found to deform the palisade cells in shape and size and number of chloroplast were reduced in tissue of infected leaves under histopathological observation.

Cowpea Mild Mottle Virus: A Causal Agent of Mosaic Disease Complex in Soybean Under Delhi Condition

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Cowpea mild mottle virus (CPMMV), a member of genus Carlavirus (family Betaflexiviridae) transmitted by whitefly (*Bemisia tabaci*), contains long flexuous particle, positive sense ssRNA of about 8.12 kb. It is an emerging as a threat to cultivation of field crops in tropical and subtropical regions of Africa and Asia. During 2011–2012 soybean crops grown at Indian Agricultural Research Institute (IARI), New Delhi, experimental field was found to be severely affected by mosaic disease with disease incidence of 18.6–71.2%. Infected soybean produced symptoms like mosaic, mottling, leaf deformation and stunting. The virus could infect soybean, French bean, mungbean, urdbean, cowpea, tobacco, fenugreek and asparagus bean through mechanically sap inoculation. Seed transmission of virus was ranged from 0.62 to 14.2%, depending on soybean cultivars. Under electron microscopy a long flexuous particle measuring 620–650 × 12–15 nm in size from symptomatic soybean leaves was observed. Carlavirus specific primer detected the associated virus with mosaic disease of soybean as a member of Carlavirus amplifying 930 nt fragment of the viral genome and this virus was further confirmed as a strain of CPMMV using another specific primer pair amplifying 216 nt fragment. One soybean isolate from Delhi designated as CPMMV-D1 was characterised by sequencing of 1289 nt fragment of 3′ end of the genome covering CP (867 nt), nucleic acid binding protein (NABP) gene (303 nt) and 3′UTR (120 nt). Sequence analysis showed that the present isolate shared 68–73% nt identity with other isolates available in GenBank. In phylogenetic analysis, CPMMV-D1 placed separately in phylogenetic tree. The present isolate differed from previously reported two Indian groundnut isolates CPMMV-S and CPMMV-M, sharing 73% nt identity. Earlier it was presumed that Soybean mosaic virus (SMV) is the causal agent of mosaic disease in soybean under Delhi condition but present study ruled out the SMV-etiology and determined CPMMV-etiology of mosaic disease in soybean under Delhi condition. This is the first report of identification and molecular characterization of CPMMV infecting soybean in India.

Determination of Tridimensional Structure of *Mandarivirus* Coat Protein (in silico)

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Most or all of the flexible filamentous plant viruses share a common protein fold and helical chemistry and are used widely in nanobiotechnology as various engineering platforms. Little is known about the structure of flexible filamentous plant virus coat proteins compared to rigid rod tobamoviruses and icozahedral plant viruses. Mandarivirusis a monotypic filamentous plant virus genus in the family Alphaflexiviridae with Indian citrus ringspot virus (ICRSV) as type species. ICRSV contains a genome of 7560 base positive sense single stranded RNA coding six different proteins. Coat protein (CP), the only structural protein of this virus is a 325 residue peptide of 34 kDa that self assembles into a flexible helical particle of around 650 nm in length. An attempt was made to construct the 3D model structure of ICRSV CP through threading the templates available in protein databank using iterative threading assembly refinement server which is an integrated platform for automated protein structure and function prediction. Secondary structure of the ICRSV CP has 101 helix and 224 coils with a confidence score ranging from 6–9 on an average. The predicted 3D structure of ICRSV CP has a TM score of 0.33 ± 0.11. This TM score is in the range of 0.17 to 0.5, which indicate the predicted 3D model cover a satisfactory level of topology with the native structure. The structural analogue of the 3D model of ICRSV CP was found to be crystal structure of CP of Papaya mosaic virus with a TM score ~ 0.5. Six out of the top 10 alignments of the 3D model of ICRSV CP with a normalized Z score of ≥ 2 reported from the following threading programs MUSTER, PROSPECT2, HHSEARCH, SP3, PPA-I, HHSEARCH I and SPARKS were with the crystal structure of CP of Papaya mosaic virus, a flexible filamentous virus of Potexvirus genus. Potexvirus is the closest relative of Mandarivirus and CP of both viruses show homology at the aminoacid level in sequence analysis. 3D model of the ICRSV CP generated was verified with structure validation programme PROCHECK and Ramachandran plot of the model showed 96% of the aminoacid residues in allowed region with 83.3% in most favoured region. This implicates that the model generated is of moderately good quality. The structure of CP generated may serve as a model for new experimental advancement with Mandarivirus coat protein as a platform for engineering nanoparticles for vaccine production and peptide expression like its closest relative Potexvirus.

In vitro Expression and Purification of Coat Protein Gene of Garlic Common Latent Virus (GarCLV) from Garlic and Its Application in ELISA Based Diagnostics

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Coat protein (CP) gene of *Garlic common latent virus* (GarCLV) was over-expressed in *Escherichia coli* strain BL21 expression system as 40 kDa fusion protein bearing Histidine tag (6His) at its both N and C terminals. The purified protein reacted positively in western blotting with anti GarCLV polyclonal antiserum (Bioreba, Switzerland) and hence, used as immunogen for the production of polyclonal antisera in New Zealand white rabbit. Antisera to GarCLV (titre 1:2000) detected the virus by direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA) in GarCLV positive garlic sample. Further, the specific reactivity of the antisera was confirmed through western blotting and immunosorbent electron microscopy (ISEM). Antiserum developed was successfully utilized for detection of the GarCLV infection in 46 out of 48 different garlic accessions. The immunoreagents developed will be useful for the virus indexing in garlic tissue culture programme and quarantine certification programme as well.

Simultaneous Detection of Apple Chlorotic Leaf Spot Virus and Apple Mosaic Virus in Apple by Duplex RT-PCR

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Apple Chlorotic Leaf Spot Virus (ACLSV; familyBetaflexiviridae genus Trichovirus) and Apple mosaic virus (ApMV, genus Ilarvirus) are economically important viruses of apple (Malus × domestica Borkh.). A duplex Reverse Transcription Polymerase Chain Reaction (RT-PCR) assay was developed for simultaneous detection of these two viruses with an internal control (NADH dehydrogenase subunit 5 gene) to minimize the risk of getting false negative results. Specific primers were designed against coat protein gene of both the viruses and reported sequences of internal control were used in this study. Uniplex RT-PCR assay was standardized for the detection of these viruses (ACLSV &ApMV) individually. Later the standardized RT-PCR conditions were used for duplex RT-PCR analysis with an internal control wherein the amplicons appeared as faint bands. Therefore, RT-PCR conditions were again optimized by modifying the composition of PCR mix i.e., buffer, dNTPs, primer and Taq DNA polymerase and by minor change in the PCR cycling conditions. This optimized PCR mix and PCR conditions showed sharp expected size of amplicons in duplex RT-PCR with respect to two viruses and multiplex RT-PCR with respect to internal control. The results were confirmed by sequencing the amplified product and then by BLAST analysis. Robustness of the technique was further validated wherein; duplex RT-PCR was carried out to detect both the viruses in field infected apple plants. It gave clear bands of desired sizes along with an internal control. The duplex RT-PCR assay has detection sensitivity as that of uniplex RT-PCR assay for respective viruses. So, the duplex RT-PCR provides a simple, rapid, sensitive and convenient way for simultaneous detection of ACLSV &ApMV by reducing the time and cost of the consumables.