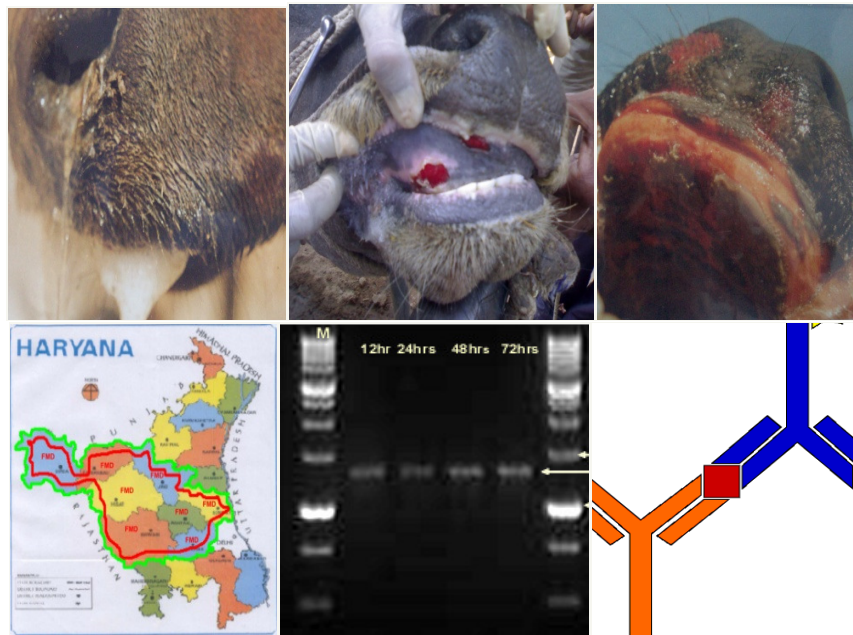# Course Compendium

Control of infectious diseases of animals with particular emphasis on FMD Control Programme

(Sept. 18 – Oct. 8, 2012)

ICAR Centre of Advanced Faculty Training

Department of Veterinary Microbiology

College of Veterinary Sciences

LLR University of Veterinary and Animal Sciences

Hisar 125004, Haryana

Course Compendium

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Course Director
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ACKNOWLEDGEMENTS

FOREWORD

Of recent, agriculture sector has witnessed a static growth and reached its plateau. In order to enhance the contribution of agriculture to national GDP, there is an urgent need to develop livestock sector. This could only be achieved by putting more emphasis on animal health for better livestock production. This has put much greater responsibilities upon the veterinary services under WTO regulations to control the economically important infectious animal diseases. Foot-and-mouth disease (FMD) is one of the economically important and devastating viral diseases affecting cloven-hoofed animals. The disease is endemic in India, occurs throughout the year and causes economic losses to the tune of >2 billion $ annually. In light of this, FMD Control Programme (FMD-CP) was launched in India during 10th Plan in selected regions to create FMD-free zones through mass immunization of cattle and buffaloes. During the 12th Plan, the FMD-CP has been expanded in the country. There is an urgent need for capacity building to undertake extensive surveillance and sero-monitoring for FMD-CP.

The mass vaccination under FMD-CP in Haryana has provided sufficient protection against FMDV at herd level. However, control of infectious diseases requires constant inputs in the form of trained manpower, besides other requirements. Keeping in view the above, the Centre of Advance Faculty Training, Department of Veterinary Microbiology is organizing a three weeks training course on *'Control of infectious diseases of animals, with particular emphasis on FMD Control Programme'* under the aegis of ICAR, New Delhi.

The Course Compendium has been prepared by the Course Coordinators, Drs. R. Sharma and N. K. Kakker and contribution made by the distinguished faculty members who have vast experience in the control of infectious diseases. I congratulate the organizers of the course and faculty of the CAFT for their sincere efforts in bringing out the training manual before the start of the training.

I hope that the lectures delivered and 'hands on practical training' provided by the learned research workers will be useful for the participants towards control of infectious diseases for the betterment of livestock health and production in their respective States. Further, it is envisaged that the success of FMD-CP will pave the way for launching a National FMD Eradication Programme in the country.

Dr. (Ms.) Nita Khanna
Director Research
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Preface

Infectious diseases are the leading cause of morbidity and mortality of domestic animals. Heavy economic losses occur in their management and control annually in India and worldwide. As an example, foot-and-mouth disease alone causes direct and indirect losses of more than two billion US dollars annually. Infectious diseases caused by bacteria, such as mastitis, pasteurellosis, brucellosis, tuberculosis, colibacillosis, salmonellosis, etc. and viruses such as FMD, avian influenza & other poultry viral diseases, swine fever, rabies, sheep-pox, blue-tongue, PPR, etc. are particularly of immense importance in India.

Control of infectious diseases of domestic animals is obviously a top priority for the growth of livestock industry of all nations, including India. Vaccination is one of the most cost-effective and proven control strategies for infectious diseases. Rinderpest, the only animal disease and next to smallpox, has been declared by Food and Agricultural Organization of the United Nations in 2011 as eradicated with the use of efficacious vaccine and disease surveillance & monitoring programmes. Although every infectious disease poses unique difficulties in its control, this singular global achievement has taught some general lessons to veterinary professionals for devising strategies for control of other infectious diseases at regional, national, continental and global levels. Control and elimination strategies for FMD, avian influenza, brucellosis, hemorrhagic septicemia, rabies, poultry viral diseases, etc. are already laid down and being implemented at various levels.

Disease surveillance and sero-monitoring of vaccination programme using validated immunoassays and molecular assays serve as watchdogs to ensure success of the disease control programme. DIVA strategies for different infectious diseases have been and are being developed and employed for various purposes by producing evidence of presence/absence of the infectious agent in the target region. 'Penside' diagnostic tests are being developed and validated for field applications, particularly for early containment of rapidly spreading agents of infectious diseases, and agro-bioterrorism agents.

The theme of the 25th CAFT course is *'control of the infectious diseases, with particular emphasis on FMD control programme'*. The Manual presents a compilation of lecture write-ups contributed by resource faculty of several years' scientific experience in their respective fields. The protocols of various diagnostic and laboratory tests that have been included in the manual have been developed or are being used routinely by the respective demonstrators for several years. The participants will be provided 'hands-on-training' on these tests. It is hoped that these techniques would be of immense importance towards capacity building in the field of diagnostics leading to effective animal disease control programmes. Timely submission of the write-ups by the entire resource faculty, and untiring efforts of Dr. Ravindra Sharma and Dr. Naresh Kakker as Course Co-ordinators have made it possible to bring out the 25th CAFT Course Manual in time. The Manual should prove to be a good companion to all the trainees during the course and later as they return home with their updated knowledge bank.

Dr. Ajit Singh
Course Director

ICAR Centre of Advanced Faculty Training, Department of Veterinary Microbiology, LLRUVAS, Hisar-12504

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25th CAFT Training Course
'Control of infectious diseases of animals, with particular emphasis on FMD control programme' (*Sept., 18, 2012 to Oct., 8, 2012*)

List of Participants

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List of speakers and practical demonstrators

Theory Lectures

S.N.	Title	Speaker*
1.	Foot-and-Mouth Disease: General Perspectives	N. K. Kakker, *R. Sharma and Maloshrie Bora
2.	Control of Foot-and-Mouth Disease By 2020: A National Target	*R. Sharma and N. K. Kakker
3.	FMD-Control Program in Haryana and Delhi	*R. Sharma and N. K. Kakker
4.	Strategies for Monitoring of FMD-Control Programme	*N. K. Kakker and R. Sharma
5.	Surveillance of Foot-and-Mouth Disease Outbreaks before and after launch of FMD Control Programme in Haryana, India.	N. K. Kakker and *R. Sharma
6.	Vaccination Strategies against Equine Influenza	*B. R. Gulati, B.K. Singh, N. Virmani and R. K. Singh
7.	Approaches to the Prevention and Control of Avian Influenza Viruses in Poultry	*S. Kapoor
8.	Marker Vaccines and DIVA Strategies for the Control and Eradication of Infectious Diseases	*R. Sharma, N. K. Kakker and A. Lather
9.	Development of Humoral Immune Response against Infectious Diseases	*S. K. Kadian and S. M. Ray
10.	OIE Recommended Tests for Diagnosis of Animal Diseases	*N. K. Kakker and R. Sharma
11.	Rapid 'Penside' or 'Point- of- Care' Diagnostic Tests for Viral Diseases of Domestic Animals	*Ajit Singh
12.	Management of Herpes Virus Infection in Horses: Diagnosis and Vaccination Strategies	*B.K.Singh, N.Virmani and B.R.Gulati
13.	Bluetongue in India: Molecular Techniques for Epidemiological Studies	*Minakshi, K. Ranjan, P. Kumar, Basanti, Supriya, M. Shafiq, and G. Prasad
14.	PPR in Small Ruminants: Diagnosis and Control	*S. K. Batra and S. Charan
15.	Control of Hemorrhagic Septicaemia in Cattle and Buffaloes	*Arvind Kumar
16.	Prerequisites for control of infectious diseases with special reference to hemorrhagic septicemia in bovines	*Shiv Charan and S.K. Batra
17.	Strategies for control of brucellosis in animals in India	*Puran Chand
18.	Diagnosis and control strategies for bluetongue in India	Guest Speaker: Dr. A.B. Pandey, IVRI, Mukteswar

Practicals

1.	Double Antibody Sandwich ELISA for Serotyping of FMD Virus	N. K. Kakker, R. Sharma and Maloshrie Bora
2.	Liquid Phase Blocking ELISA for detection of antibodies against FMD Virus Serotypes	N. K. Kakker, R. Sharma and A. Lather
3.	Monoclonal Antibody based ELISA for the detection of Foot and Mouth Disease Carrier Animals	R. Sharma, N. K. Kakker and A. Lather
4.	Recombinant 3AB3 NSP ELISA for Differentiation of Foot-and-Mouth Disease Infected and Vaccinated Animals	R. Sharma, N.K. Kakker and Maloshrie Bora
5.	Serum Neutralization Test for detection of antibodies against Foot-and-Mouth Disease Virus	R. Sharma and N. K. Kakker
6.	Virus Neutralization Test for Equine Herpes Virus-1 Diagnosis	B. K. Singh
7.	Serological Diagnosis of Equine Influenza by Hemagglutination	N. Virmani and B. R. Gulati
8.	Estimation of Antibody titres to Haemorrhagic Septicaemia in serum samples of cattle and buffalo – Use of HS ELISA Kit	Arvind Kumar
9.	Mouse model of Hemorrhagic Septicemia: *In Vivo* titration of *Pasteurella multocida* (B:2) in the different organs of mice	Shiv Charan
10.	Diagnosis of Brucellosis in animals through Conventional and Fluorescence Polarization Assays	Puran Chand
11.	Analysis of the BoCD4$^+$ T and BoCD8$^+$ cells of Bovine Milk by Flow Cytometery	R. Chhabra and Anshu Sharma

FOOT-AND-MOUTH DISEASE: GENERAL PERSPECTIVES

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Foot-and-Mouth disease (FMD) is one of the most important viral infections of the cloven footed animals causing heavy economic losses to livestock industry on account of direct loses in the form of productivity (mainly due to loss in milk production and reduction in the working capability of animals) and indirect losses in the form of restriction on international trade of livestock and their products including germ-plasm. In era of globalization, FMD attains an important priority as chances of disease threat have been increased many folds due to open trade between countries within a particular continent.

The disease is caused by the FMD virus (FMDV), an *Aphthovirus* of the family *Picornaviridae* that includes small (20-30 nm in diameter), non-enveloped, positive stranded RNA viruses consisting of many important pathogens of human and animals. The FMDV exists in seven immunologically distinct serotypes viz. O, A, C, Asia 1 and South African Territories (SAT) -1, -2 and -3. Within these seven serotypes, more than 140 subtypes have been identified. Some of these subtypes are strongly divergent and the vaccines made against one subtype may protect against the others only after repeated vaccinations. The FMD is highly contagious in cloven-footed animals: most prevalent in cattle and buffaloes followed by sheep and goats, whereas pigs mostly act as reservoirs. In addition, many species of cloven-hoofed wildlife, such as deer, antelope and wild pigs may become infected. The disease is more severe in exotic and cross-bred animals. In addition, many species of cloven hoofed wildlife such as deer, antelopes and wild pigs may become infected.

International Status of FMD

The FMD occurs in most of the cattle raising regions of the world. The disease was eradicated in Australia, New Zealand and North America. Australia had its last outbreak in 1972. The first and last outbreak in Canada occurred in 1952. The United States of America has been declared FMD-free since 1929. In Europe, the United Kingdom remained practically free from FMD since the last major outbreak of 1967-68 with the exception of isolated outbreaks in Channel Island in 1974, 1981 and recently in 2001 and 2007 that has affected the 'FMD-free status' of UK, France and Germany. In other European countries, the incidence of the disease has declined with the exception of Italy, Greece and the countries of

South-eastern Europe. The western European countries eradicated the disease in the last decade but outbreaks have been recorded in 2001. FMD is endemic in many parts of South Africa, South America, Middle East and Asia and some parts of Europe.

FMD: Indian Perspective

In India, FMD is endemic and occurs in all parts of the country, throughout the year. Of the seven FMDV serotypes prevalent worldwide, only four serotypes i.e. O, A, C and Asia 1 were earlier reported to be circulating in India. Of these, FMDV serotype O has been the most prevalent, followed by Asia 1 and A in the northwest region of the country, while FMDV serotype C has not been detected for the last about 16 years. Presence of FMDV in India presents a major blockade in a move towards global liberalized trade in animals and the efforts to achieve the idea of global food security and global market access for animals and animal products.

The incidence of FMD outbreaks and virus type distribution during 2010-2011 in different parts of India has been depicted in Table 1.

Table 1: FMD outbreaks and distribution of virus types in India (2010-2011)[1]

S. No.	State	No. of Outbreaks	Distribution of virus types		
			O	*A*	*Asia 1*
Northern Region					
1.	Haryana	2	2	-	-
2.	Himachal Pradesh	1	1	-	-
3.	Jammu & Kashmir	5	1	-	-
4.	Punjab	7	3	-	-
5.	Uttar Pradesh and Uttrakhand	3	2	-	-
Western Region					
6.	Gujarat	9	7	-	1
7.	Maharashtra	8	6	-	2
8.	Rajasthan	13	3	-	1
Southern Region					
9.	Andhra Pradesh	3	2	1	-
10.	Karnataka	37	27	-	-
11.	Kerala	42	8	-	-
12.	Tamil Nadu	13	13	-	-
Eastern Region					
13.	Assam	16	10	6	-
14.	Arunachal	9	6	-	1
15.	Bihar	11	10	-	-

16.	Manipur	2	2	-	-
17.	Mizoram	1	1	-	-
18.	Meghalaya	6	3	-	3
19.	Nagaland	3	3	-	-
19.	Orissa	1	1	-	-
20.	Tripura	5	-	1	4
21.	West Bengal	27	16	-	4

Central Region

22.	Madhya Pradesh	29	27	2	-
	Total	253	154	10	16

[1]Source: Annual Report 2010-11, Project Directorate on FMD, Mukteshwar, Nainital, Uttrakhand
* Of the 253 outbreaks, clinical samples from 73 outbreaks were untypable.

Clinical Signs and Symptoms:

The incubation period of FMD virus ranges from 2 to 14 days depending on the infecting dose, strain of virus involved and susceptibility of the host. In pigs, the incubation period may be as short as 18 hours after contracting the virus. The disease is characterized by high fever (up to 106°F) that declines after two or three days and lesions in the mouth (tongue, dental pad, gums, lips) leading to excessive secretion of foamy or stringy saliva. The painful lesions cause profuse drooling, foot stamping and lip smacking. The blisters are formed on the feet, coronary band and interdigital cleft that may rupture and cause lameness. Sheep and goats may develop only a few vesicles on the coronary band and in the mouth. In adult milch animals, milk production can decline significantly and vesicles may also appear on the teats and udder, particularly of lactating cows and sows. Due to mild signs in sheep, diagnosis may be delayed and virus may spread to other animals. In newborn calves, the disease can lead to myocarditis (inflammation of the heart muscle) and death. Some infected animals remain asymptomatic and become FMD carriers.

Vesicles usually heal within 7-10 days, whereas recovery of the tongue lesions may take some longer time. If proper care is not taken, lesions on the feet and mammary gland frequently develop secondary infections, resulting in mastitis, under running of the sole and chronic lameness. In pigs, the complete horn of the toe may be lost, whereas in cattle and deer one or both horns of the foot may be lost.

Transmission

FMDV can replicate and be excreted from respiratory tract of animals. Airborne excretion of virus occurs during the acute phase of infection. The virus spreads mainly by direct contact (inhalation and ingestion), although indirect spread through fomites is

important. The virus in dried blood, milk, meat and carcasses can be stable and may serve as source of infection. All secretions and excretions may be infective before the animal is clinically ill. The greatest concentration of the virus is found in vesicular fluid. Infected or carrier animal (e.g. in livestock fairs) and migratory herds are contributory factors for spread of FMDV infection. The environmental factors, namely, temperature (maximum and minimum), relative humidity (morning) and wind speed have a significant correlation on the incidence of FMD outbreaks.

Diagnosis

FMD is characterized by fever, lameness, and vesicular lesions on the tongue, feet, snout, and teats. However, in young animals mortality can be high, where the virus can affect the myocardium of heart. The accurate diagnosis of FMDV infection is of great importance for both control and eradication campaigns in FMD endemic areas. The control and eradication of FMD depends upon quick reporting, an accurate and swift diagnosis, followed by rapid and effective implementation of control measures. The recognition of FMD signs in cattle, buffaloes and pigs provide straightforward clinical diagnosis. However, it can often be difficult in sheep and goats as the clinical signs are mild and transient. Laboratory diagnosis is, therefore, necessary for disease confirmation. Laboratory diagnosis procedures are based on either identification of virus serotype involved or antibody response following an active phase of the disease.

Diagnosis through identification of virus serotype and laboratory tests

Identification of virus serotype is done by virus isolation or by the demonstration of FMD viral antigen/ nucleic acid in samples of tissue or vesicular fluid. Detection of specific antibody in serum (serotyping) can also be used for diagnosis. Serodiagnosis is enhanced by newly developed non-structural proteins (NSPs) assays that enable detection of past or current infection irrespective of vaccination status. The preferred procedure for the detection of FMDV antigen and identification of viral serotypes is sandwich ELISA. The results can be obtained within 3-4 hours after sample is received in the laboratory; a negative sample is confirmed by inoculation of sample into cultures (BHK-21), followed by confirmation of the virus serotype by ELISA.

FMDV serotype can also be diagnosed by the detection of specific antibody response. Several tests/techniques have been developed to detect the presence of FMDV serotype specific antibodies. Of these, ELISA is more versatile, reproducible and simple to perform

and ELISA reagents have longer shelf life. The polymerase chain reaction (PCR) offers potential advantages over the other conventional tests. A Real-time PCR capable of detecting all the seven serotypes of FMDV has been developed that has a high sensitivity and specificity for detection of FMDV genome in all seven serotypes. The only disadvantage of Real-time PCR is that it is very expensive and not suited for field purpose.

Differentiation of infected animals from vaccinated animals (DIVA strategy)

The conventional diagnostic tests are unable to differentiate FMD infected and vaccinated animals. The detection of antibodies to non-structural proteins (NSPs) of FMDV has been used to detect the past or present infection of any of the seven FMD virus serotypes. Further, detection of antibodies against NSP antigens could also be used for differentiation of antibodies due to vaccination/infection. When animals are vaccinated, they mount antibody response only against coat (structural) proteins of the virus. On the other hand, in infected animals antibodies also develop against NSPs (viral polymerases and proteases) of the virus because virus actually replicates inside the host cells.

Prevention and Control of FMD

Since FMD is endemic in India, the outbreaks can be controlled by regular vaccination of the susceptible animals. Conventional vaccines are typically inactivated virus mixed with an adjuvant and having immunity of 4-6 months. The protection is conferred for a minimum of 3-4 days after vaccination in cattle. Inactivated whole virus FMD vaccines are formulated as mono- or polyvalent vaccines with suitable adjuvant to enhance their potency. Such vaccines have been used successfully worldwide. Due to short lived protective immunity following FMD vaccination, there is need to vaccinate animals annually or bi-annually. Mass prophylactic vaccination against FMD, usually practiced in the susceptible population, is an important step towards control of FMD in endemic areas which is helpful in reducing the number of outbreaks.

Role of Veterinarian

i. Disease Reporting: The veterinarian can play a very important role in prevention and control of FMD, which depends upon quick reporting, an accurate and swift diagnosis, followed by rapid and effective implementation of control measures. The recognition of FMD signs in cattle, buffaloes and pigs by vets in field conditions can provide straightforward clinical diagnosis. Further laboratory diagnosis is necessary for disease confirmation which

are based on either identification of virus type involved or antibody response following an active phase of the disease.

ii. Collection and Dispatch of Clinical Sample: The collection of appropriate sample at proper time by the field veterinarian will help in the quick laboratory diagnosis of serotype involved in FMD outbreak(s). The tissue of choice is epithelium from an unruptured or recently ruptured oral (tongue, gum, dental pad, upper palate, etc.) vesicle. Epithelial tissue samples should be collected in transport medium containing equal amounts of glycerol and 0.04 M phosphate buffer pH 7.2-7.6, preferably with added antibiotics. Where epithelial tissue is not available, for example, in advanced or convalescent stages, samples of oesopharyngeal fluid can be collected by means of a probang cup for isolation of virus. Alternatively, serum samples from affected (convalescent) and in contact animals may also be collected for retrospective diagnosis.

Precautions should be taken while sending samples suspected for FMD to typing laboratory. These include prevention of leakage and consequent contamination so as to ensure safe arrival of samples in the laboratory. The samples should be transported on ice in leak-proof containers.

iii. Prevention and Control: The role of Veterinary Surgeon in prevention and control of FMD is very important as an extension worker. He can make aware the animal owners/ villagers about the economic importance of the disease and advantages of proper vaccination. Further, the veterinarian can help in prevention and control of FMD by conducting regular prophylactic vaccination of all the susceptible animals in the area.

In case of outbreaks, spread of the disease can be effectively controlled by isolation of the affected animal and disinfection of the surroundings of the infected premises. The excretions and secretions of the animals should also be properly disinfected prior to disposal. Ring vaccination should be followed in the surrounding 20-25 km area using disposable syringe & needle.

CONTROL OF FOOT-AND-MOUTH DISEASE BY 2020: A NATIONAL TARGET

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Foot-and-Mouth disease (FMD) is one of the most important viral diseases of the livestock causing heavy economical losses (to the tune of more than 2 billion US dollars per year) to livestock industry on account of direct loses in the form of productivity and indirect losses in the form of restriction on international trade of livestock and their products including germ-plasm. The disease is highly contagious in cloven-footed animals: most prevalent in cattle and buffaloes followed by sheep and goats, whereas pigs mostly act as amplifier. In addition, many species of cloven-hoofed wildlife, such as deer, antelope and wild pigs may become infected. FMD is endemic in many countries of the world including India, but several nations have attained FMD-free status through stamping out policy and/ or systematic vaccination programme. These countries enjoy economic benefits from international trade in animals and livestock products. Further, geographic isolation and restricted marketing avenues in livestock sector under WTO regime can favour FMD eradication. In era of globalization, FMD control attains an important priority as chances of disease threat have been increased many folds due to open trade between countries within a particular continent. The continuous occurrence of FMD in developing countries may be mainly attributed to the poor socio-economic conditions, movement of live infected animals, followed by trade in animal products constitutes the greatest risk for spread of FMDV. Further, continuous evolution of FMDV gives rise to new strains that cause periodic upsurges in the number of cases and thus increase the risk of spread into new areas.

Of the seven FMDV serotypes prevalent worldwide, serotypes O, A and Asia-1 are currently circulating in India, whereas serotype 'C' has not been detected since about 15 years. Of these, FMDV serotype O is the most common followed by A and Asia-1. Presence of FMDV in India presents a major blockade in a move towards global liberalized trade in animals and their products, and the efforts to achieve the idea of global food security and global market access for animals and animal products.

CONTROL OF FMD

The strategy evolved for the control of FMD varies from country to country: geographical location, economic development and status of FMD incidence. In developed

countries the most effective way remains the slaughter of affected and in-contact susceptible animals and restriction of animal movements. In India, FMD control cannot be achieved either by slaughter or stamping out policy as adopted by western countries. However, the control of FMDV in India is a challenging task due to several factors such as i) large geographical area with different agro-climatic and socio-economic zones, ii) large array of susceptible livestock population, iii) antigenic plurality of the virus and high transmissibility iv) limited availability and huge cost of FMDV vaccine, v) short duration of immunity, vi) unrestricted animal movement and vii) lack of legislation for compulsory reporting and vaccination.

Recently, efforts have been made to develop alternative FMD control strategy, which is directed towards creation of "disease free zone" (DFZ) in endemic Countries. The DFZ may be carved out in three ecosystems based on disease incidence in epidemic zone, endemic zone and sporadic zone. The scheme consists of i) control of livestock movement in to and out of the DFZ, ii) systemic mass vaccination of all the susceptible animals in the area, iii) creation of buffer zones, iv) sero-monitoring of vaccination programme, v) emergency measures to be taken in the event of outbreak and vi) epidemiological surveillance in the DFZ for residual foci and surrounding area.

In order to promote the long term coordinated FMD control approach, FAO developed the Progressive Control Pathway for FMD (PCP-FMD), a set of control program activity stages leading to FMD freedom, and Regional Roadmaps (RR), which describe the anticipated progress along the PCP at national and regional level to 2020.

In 2009, FAO proposed that the PCP and RR approach be adopted as the international framework for coordinated action under GFTADS, in each of the currently endemic regions at the OIE/FAO International Conference on FMD held in Paraguay. At global level, seven FMD epidemiologic regions have been recognized containing specific viral variants, specific targeted vaccines and regional early warning systems ("7 virus pools"), and Regional Roadmaps are suggested as an approach for 5 regions (West Eurasia, South Asia, East Africa, West/central, and Southern Africa) that do not currently have Continental (The Americas) or sub Regional (South-east Asia) FMD Roadmaps.

The PCP approach describes a set of intermediate control stages that should culminate in the preconditions for gaining and maintaining FMD freedom. The initial stage (Stage 1) is applicable in almost all the countries, and aims at gaining the information required developing

a national strategy; disease incidence, country capacity, and socio-economic drivers. Stage 1, therefore, generates significant valuable data at national, regional and global level (to free countries at risk). The next two stages focus on progressive application of control measures, leading eventually to a situation where all outbreaks are exogenous (from outside the country) and from which the jump to official recognized disease freedom (Stage 4) could be made.

The PCP approach has been developed and applied by FAO-OIE, at regional meetings to develop Regional Long Term Roadmaps in West Eurasia (14 countries), in Africa (52 countries, 2009) and the Middle-East (10 countries, 2009) and south Asia (7 countries, 2010). The PCP approach involves four sequential stages (Stage 0 to 3) of control within non-free countries, leading to official recognized freedom with or without vaccination (Stages 4 and 5, respectively). The FAO-OIE reviewed the status of each SAARC country in January 2011 and placed India at Stage 2 level. The PCP stage assessment will be repeated every year on set criterion to monitor the progress. A potential SAARC regional road map developed by FAO envisages *SAARC countries free of clinical FMD by 2020.*

In lines with the above strategies, Govt. of India had launched FMD Control Program (FMD-CP) during 10th Five Year Plan in three different geographical regions of the country comprising of 54 districts. Epidemiological data being generated by Project Directorate on FMD, IVRI Campus, Mukteshwar and its 8 regional centres & 15 network units all over India helped in launching the Control Programme. The region I comprised 8 districts: Kanyakumari (Tamil Nadu); Quilon, Pathanamthitha, Trivandrum (Kerala) and four Union Territories of Andaman & Nicobar Islands, Dadra & Nagar Haveli, Daman & Diu and Lakshadweep. The region II was the largest comprising of 33 districts of the states of Punjab (8), Haryana (8), Uttar Pradesh (16) and Delhi as one district. The region III included 13 districts, also in the states of Maharashtra (5), Gujarat (4) and Andhra Pradesh (4).

The program envisages total funding by Govt. of India for vaccine including other supporting assistance (manpower, infrastructure and logistic support), framing of legislation to restrict animal movement and compulsory vaccination of all animals in the defined regions. Besides, pre- and post-vaccination monitoring & surveillance of vaccinated animals is being done by AICRP on FMD to assess the extent of exposure of the population to FMDV. It is envisaged that the success of FMD-CP will pave the way for launching a National FMD Eradication Program in the country.

FMD-CONTROL PROGRAM IN HARYANA AND DELHI

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Foot-and-mouth disease (FMD) is a highly contagious and economically important disease affecting all the ruminants and pigs including many wild life species (Sutmoller *et al*., 2003). The disease has serious impact on international trade of animals and their products including germ-plasm. Besides, there are indirect losses in terms of severe trade restrictions on account of WTO/GATT agreement. In India, where world's largest livestock population exists, FMD is endemic, occurs in all parts of the country throughout the year and a leading cause of loss of livestock economy.

In view of importance of the disease, the Govt. of India launched a FMD-Control Program (FMD-CP) in January 2004 including Haryana with an idea of developing 'FMD-free zones'. Sero-monitoring data, epidemiology of the disease and DIVA reactivity from different phases of vaccination under the FMD-CP in the state of Haryana were utilized to assess the implementation and impact of FMD-CP in Haryana and Delhi.

FMD-CP in Haryana and Delhi: A Success Story

The FMD Control Program (FMD-CP) was launched in eight districts of Haryana (Bhiwani, Fatehabad, Hisar, Jhajjar, Jind, Rohtak, Sirsa, and Sonepat; Fig.1) and Delhi in January 2004. The animals in the remaining 13 districts of Haryana were also vaccinated under Assistance to States for Control Animal Diseases Plan (ASCAD).

Fig. 1: Map of Haryana showing eight districts covered under FMD – Control Program.

The Regional Research Centre on FMD, Hisar participated actively in the implementation of FMD-CP by providing logistic support in the form of surveillance and sero-monitoring work in all the 21 districts of Haryana and Delhi. This Centre actively engaged in FMD surveillance, epidemiology and sero-monitoring of FMD-CP in Haryana. The surveillance for FMD was undertaken through interaction with livestock farmers during frequent visits to village fields, Kisan Melas and Farm Darshans by project officials as well as reporting by field vets and a network of Diagnostic labs in different districts. The strategy followed in case of FMD outbreak(s) comprised of immediate visit to the affected area, collection of suitable samples from different species affected, collection of epidemiological data and advice for undertaking necessary preventive measures. Each outbreak was promptly attended and meticulously followed. The suitable material was collected and processed for serotyping by sandwich ELISA.

A total of 229 FMD outbreaks/Cases were recorded in Haryana over a period of ten years between January 2000 and December 2010. Of these, 204 FMD outbreaks were recorded before (2000 to 2003) and only 25 after (2004 to 2010) the launch of FMD-CP. Maximum outbreaks (94) were recorded in 2003-04 which reduced to three in 2004-05, only one each in 2005-06, 2006-07 and 2008-09, two each in 2007-08, 2009-10 and 2010-11 (Fig.2, 3). In maximum outbreaks, cattle and buffalo were simultaneously affected. Among the prevalent virus serotypes, FMD virus serotype O was the predominant serotype during 2000-2003 whereas during 2004-2010, FMD virus serotypes O and A were in almost equal proportions followed by Asia-1. However, FMD virus serotype C has not shown its presence during all these years. The significant reduction in FMD outbreaks can be attributed to the implementation of FMD-CP in Haryana since January 2004.
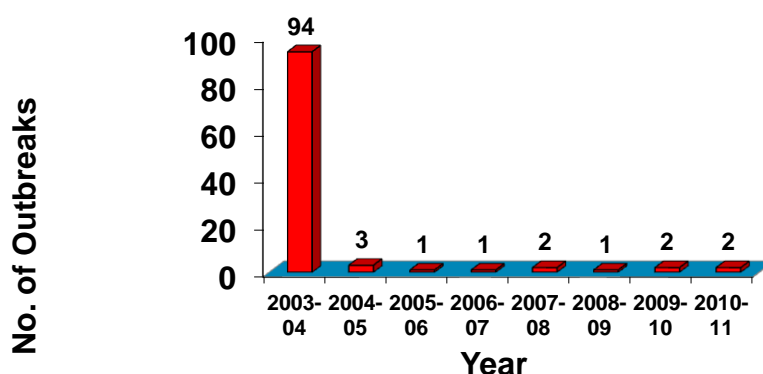

Fig. 2: Year-wise distribution of FMD outbreaks before and after mass vaccination under FMD-Control Program in Haryana
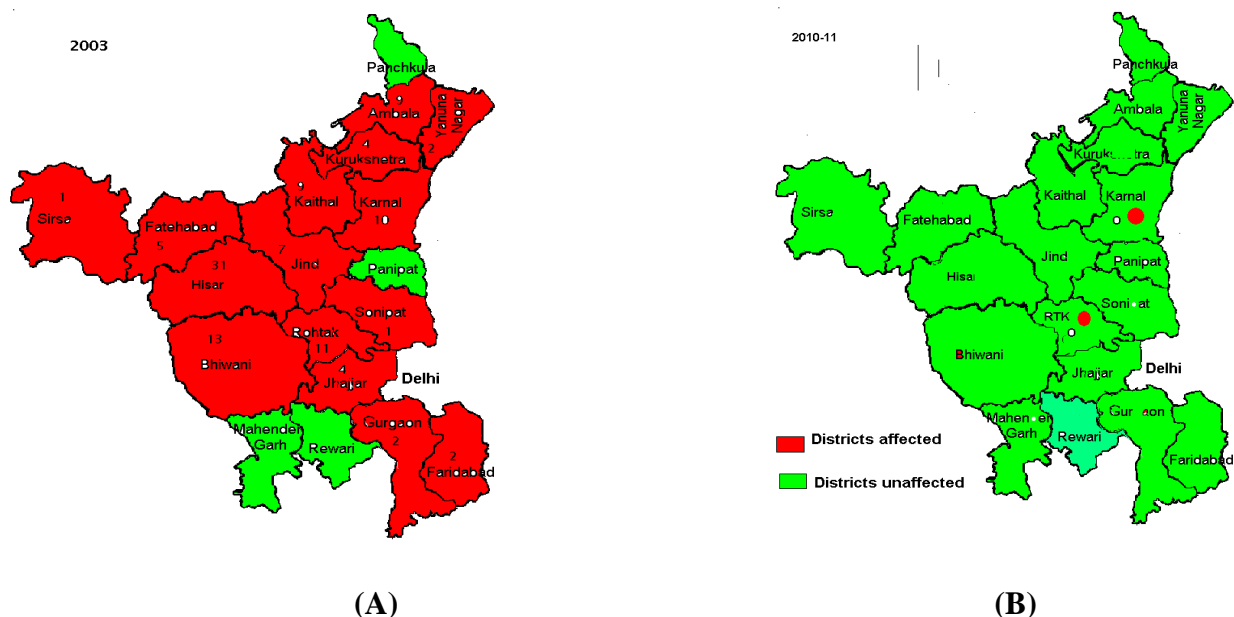
Fig. 3: Distribution of FMD outbreaks before launch of FMD-Control Programme (A) and after 10th phase of mass vaccination (B).

The sero-monitoring of the FMD-CP was done by analyzing the serum samples collected from FMD vaccinated animals comprising pre- (samples at the time of vaccination) and post- (approximately 4 weeks after vaccination) vaccination. The serum samples were collected from 10 cattle and 10 buffaloes from each of the randomly selected 10 villages in each district as per the guidelines under FMD-CP. These samples were processed against three FMD serotypes by Liquid Phase Blocking ELISA (LPB-ELISA). A total of 27936 (12464, pre- and 15472, post-vaccination) serum samples from ten phases were analyzed from all the eight districts of Haryana under FMD-CP. In addition, a total of 1237 (766, pre- and 471, post-vaccination) serum samples from ten phases were analyzed from Delhi under FMD-CP. The results were analyzed by calculating the per cent of animals showing protective antibody titres ($> \log_{10} 1.8$).

The overall per cent sero-conversion during different phases of vaccination against FMD virus serotypes O, A and Asia-1 in cattle and buffaloes together from eight districts under FMD-CP revealed that animals in Haryana and Delhi state developed 70- 80% herd immunity against prevalent FMD virus serotypes after tenth phase of vaccination which were maintained up to 10th phase of vaccination in both the states. This data provided logistic support that in the state of Haryana and Delhi, mass FMD vaccinations after ten rounds of vaccination in FMD – CP districts as well as eight vaccinations in ASCAD districts provided sufficient protection against FMD at herd level despite the fact that the disease was present in neighbouring states.

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(Against FMDV type O) (Against FMDV type A) (Against FMDV type Asia1)
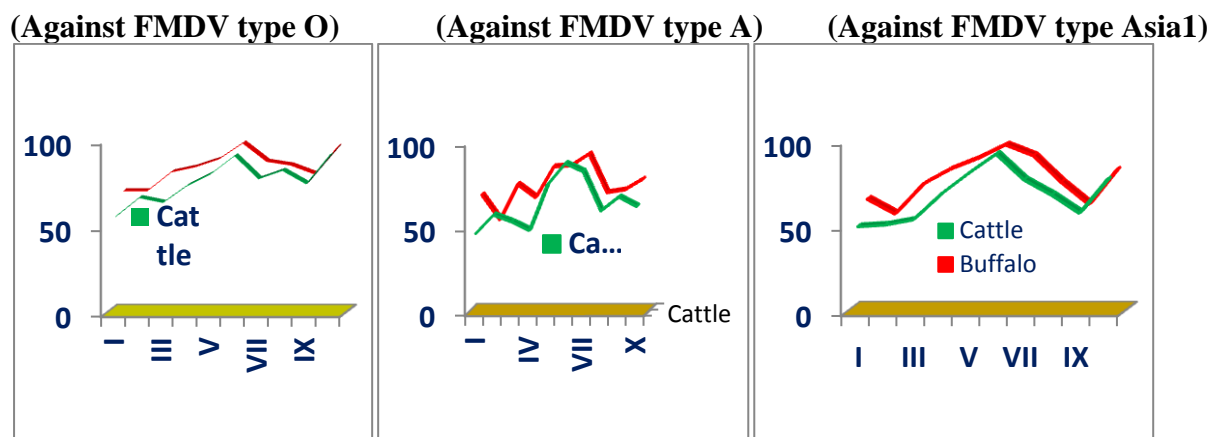
X axis: Different phases of FMD-CP; Y axis: Per cent of animals showing protective antibody levels

Fig. 4: Per cent of animals showing protective antibody levels against FMDV serotype O, A and Asia-1 after different phases of mass vaccination under FMD-CP.

Further, the serum samples have also been analyzed periodically for anti-NSP (3A/ 3AB3) antibodies for monitoring the FMD carrier status in vaccinated subjects under National FMD sero-surveillance. The percentage of animals which were found positive for anti-NSP antibodies during pre- 1st vaccination (before start of FMD CP) and pre- 4th vaccination (after two years of FMD-CP) were 31.94 and 18.40%, respectively which further lowered to 12.12% during pre-8th vaccination (after four years of FMD-CP). A significant (P<0.01) reduction in anti-NSP antibody profile in Haryana was reported six years after launch of FMD-CP. The district wise anti-NSP antibody profile in eight districts of Haryana under FMD-CP is shown in Fig. 5. This further supports the fact that incidence of FMD virus circulation in the state has significantly reduced due to vaccination.
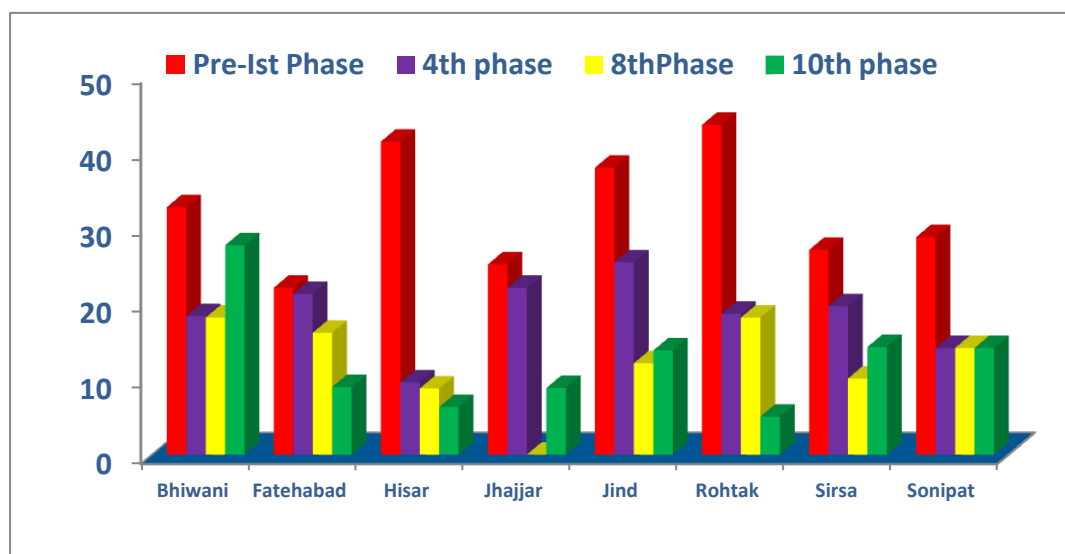
X axis: Districts under FMD-CP; Y axis: Per cent of carrier animals

Fig. 5: Carrier status of animals before launch of FMD-Control Programme and after different phases of mass vaccination.

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In view of the above data on the epidemiology, FMD-CP and National sero-surveillance, Haryana state can serve as a model **"FMD Free Haryana".** The Regional Research Centre on FMD, Hisar is also supporting other network units in north-west zone i.e., Punjab, J&K, Himachal Pradesh, Delhi and Rajasthan. The reduced incidence of the disease in the states of Himachal Pradesh, Punjab, Haryana and Delhi support the candidacy of these northern states for "Zonal freedom from FMD". It is envisaged that the success of FMD-CP will pave the way for launching a National FMD Eradication Programme in the country.

ACKNOWLEDGEMENTS

STRATEGIES FOR MONITORING OF FMD- CONTROL PROGRAMME

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The control and eradication of FMD depends upon quick reporting, an accurate and swift diagnosis, followed by rapid and effective implementation of control measures. The Regional Research Centre on FMD, Hisar is actively engaged in the monitoring (FMD surveillance, epidemiology and sero-monitoring) of FMD-CP in Haryana and Delhi. The surveillance for FMD is undertaken through frequent visits to Government Veterinary Hospitals, Government Veterinary Dispensaries, Sub-Divisional Officers and Deputy Directors (Animal Husbandry) as well as network of Diagnostic labs in different districts regularly. Likewise, information on FMD is also collected through interaction with livestock farmers during village visits, Kisan Melas and Farm Darshans by project officials. In case of FMD outbreak(s) the affected area is immediately visited, suitable samples (tongue/ gum/ muzzle/ palate/ foot epithelium in case of clinical cases; blood/ serum in case of old outbreaks for retrospective studies) & epidemiological data collected from different species affected, and Govt. Animal Husbandry officials & farmers advised for undertaking necessary preventive measures. Each outbreak is promptly attended and meticulously followed.

The accurate diagnosis of FMDV infection is of great importance for both control and eradication campaigns in FMD endemic areas. The recognition of FMD signs in cattle, buffaloes and pigs provide straightforward clinical diagnosis. However, it can often be difficult in sheep and goats as the clinical signs are mild and transient. Laboratory diagnosis is, therefore, necessary for disease confirmation. Laboratory diagnosis procedures are based on either identification of virus type involved or antibody response following an active phase of the disease.

A. Diagnosis through identification of virus type:

i) Isolation of virus: Identification of virus type is done by virus isolation or by the demonstration of FMD viral antigen/ nucleic acid in samples of tissue or vesicular fluid. In laboratory, detection of FMD viral antigen is done in suspensions of epithelium using a combination of ELISA and virus isolation in cell culture. The ELISA typically detects 70–80% of positive FMDV samples (epithelial suspensions) and the results can be reported

within hours of sample receipt. The remaining samples are confirmed as positive only after visualisation of a recognisable cytopathic effect (CPE) in cell cultures.

Primary cell culture of bovine and porcine origin have exhibited susceptibility to FMDV from infected tissues, however, the most sensitive culture system for virus isolation is primary bovine thyroid cells. Bovine kidney cell culture and BHK-21 is also susceptible to FMDV. Suckling mice assay (via intraperitoneal route) is also an alternative to cell cultures. An alternative to the virus isolation is cell suspension plaque test which also quantify the virus in sample. A special clone of BHK21 cell lines BHK-21CT (clone Tubingen, Germany) is used for this test. This test is routinely used at National Reference Laboratory of FMD, Insel Riems, Germany.

However, the cell culture system is laborious, time consuming and relatively low sensitive. It also requires careful handling of specimens and a bio-safety laboratory and loses sensitivity due to presence of inhibitors such as interferons.

ii) Double Antibody Sandwich ELISA for Serotyping of FMD Virus: The detection of FMDV antigen and identification of viral serotypes is done through double antibody sandwich ELISA which has been standardized by the Central Laboratory of Project Directorate on FMD. The reagents and other biologicals necessary for the test are produced centrally and distributed to all the Regional Centres (8) and Network Units (15) of FMD throughout the country. The results are obtained within 3-4 hours after sample is received by the laboratory; a negative sample is confirmed by inoculation of sample into sensitive cultures followed by confirmation of the virus serotype by ELISA. Such kind of assay can take up to 4 days, a time frame compatible with the need to rapidly detect disease and initiate and appropriate disease control strategy. At the FAO/World Reference Laboratory for FMD, the preferred procedure for detection of FMDV antigen and identification of viral serotypes is through sandwich ELISA.

Although complement fixation test (CFT) has also been used to detect the FMDV antigen but ELISA is preferred over CFT because it is more sensitive and is not affected by anti-complementary factors. Recently, a monoclonal antibody based rapid chromatographic strip test has also been developed for the pen-side detection of FMDV.

iii) Retrospective Diagnosis of FMD Virus: For routine diagnosis and serotyping of FMD virus from clinical epithelial tissues, double antibody sandwich ELISA is the test of choice. Sometimes, it becomes difficult to collect suitable clinical material due to either late receipt of information about FMD outbreak or mild/ sub-clinical form of the disease in animals. In

such cases retrospective diagnosis of FMD outbreaks can be provided by analysing sera sample collected from recovered/ convalescent animals by LPB-ELISA [discussed under D(ii) below].

B. Diagnosis through nucleic acid based tests:

A portion of the clinical sample collected/ virus isolated is sent by the Regional Centres and Network Units to the Central Lab of PD on FMD, IVRI Campus Mukteshwar. The Central Lab of PD on FMD, IVRI Campus Mukteshwar is well equipped to undertake the molecular diagnosis and epidemiology of FMD through various indigenously standardized assays.

i) Conventional PCR: The PCR offers potential advantages over other conventional tests. The risk of false negative associated with poor sample handling is limited. Further, cell culture loses the sensitivity due to presence of inhibitors like interferons and presence of some enzymatic inhibitors.

A particularly high sensitivity has been reported by RT-PCR ELISA for the serotyping of FMDV which could possibly be used in the field. The Central Lab of PD on FMD has developed one tube RT-PCR that reduces assay complexity and potential operator errors.

ii) Real-Time PCR: Due to poor precision, low resolution, absence of automation, only size based discrimination, poor quantitative performance (Ethidium bromide for staining is not very quantitative) and post PCR processing, the conventional PCR is being replaced by real-time PCR.

Recently, *TaqMan* technology has combined the 5′nuclease activity of the *Taq* DNA polymerase and fluorescent resonance to detect and quantify amplification product in a closed tube format. Using this technology real-time PCR has been developed to detect the nucleic acid which is a most sensitive and rapid method for detection of nucleic acid. Real Time chemistry allows for the detection of PCR amplification during the early phases of the reaction that provides a distinct advantage over traditional PCR. In the Real time PCR, the problem of carry-over is significantly reduced because of the real time measuring principle, which is based on closed tube system.

The assay has also been applied for FMDV and it is capable of detecting all seven serotypes. It has a high sensitivity (97%) and specificity for detection of FMDV genome in all seven serotypes of reference FMD virus. Viral RNA can be detected in oral and nasal samples from experimentally infected animals 24-96 hours before the onset of clinical signs.

The only disadvantage of Real-time PCR is that it is very expensive and not suited for field purpose. In future, use of robotic extraction of RNA will increase the throughput of samples whilst reducing the timescale for the issue of laboratory diagnostic results.

C. New approaches in FMD Virus Diagnosis:

i) Pen side test: A confirmatory diagnosis at the site of suspected FMD outbreak would circumvent problems associated with the transportation of samples to the laboratory and would be especially useful in endemic situations for a faster diagnosis, to increase disease awareness and to improve epidemiological information. A Solid-phase chromatographic strip test has been developed for FMD diagnosis. The test uses a monoclonal antibody with broad but specific reactivity for FMD viruses and is based on the detection of FMD viral proteins. The test is very simple and quick, requires no sophisticated equipments and can literally be done pen side in about 15 minutes. The main disadvantage is that it is less sensitive than tests that can be carried out in laboratories and can only be used on cases that show vesicles.

ii) Integrin ($\alpha v \beta 6$) ELISA: Recently, IAH, Pirbright has developed a sandwich ELISA by utilising recombinant $\alpha v \beta 6$ (major integrin to which VP1 of FMDV binds) as a capture reagent (ligand). The assay is able to detect all seven serotypes simultaneously, is more specific, sensitive and less time consuming than routine antigen capture ELISA.

iii) Micro array-based detection and typing of FMDV: To enhance the rapidity of the diagnosis, a microarray-based test has been developed that uses a FMDV DNA chip, designed from the VP3-VP1-2A region of the genome. A total of 23 different FMDV strains representing all seven serotypes were detected and typed by using FMD DNA chip.

D. Diagnosis of virus type specific antibodies through serological tests:

FMD virus type can also be diagnosed by the detection of specific antibody response. Several tests/ techniques have been developed to detect the presence of FMDV type specific antibodies e.g. passive haemagglutination, counter immunoelectrophoresis, complement fixation (CFT), complement fixation inhibition, solid phase radio-immunoassay, indirect immunofluorescence, serum neutralization (SNT), ELISA, etc. Based on the elevated antibody titres against any one serotype of FMD, the disease can be diagnosed retrospectively also.

The CFT was a test of choice over SNT before today's ELISA due to its high specificity (but low sensitivity) but later it was found that ELISA is more versatile, reproducible and simple to perform than CFT. ELISA reagents have longer shelf life

compared to CFT reagents and can be readily standardised. The international standard test for FMD antibody detection is the SNT and ELISA.

i) Serum Neutralization Test: The SNT has been used extensively since long back by several workers for estimation of FMDV specific antibodies and potency of vaccine in animals. Though the SNT has been most frequently used but due to requirement of cell culture, variability in results, cumbersome operation and requirement of containment facilities, the test is not widely accepted.

ii) Liquid Phase Blocking ELISA: Several authors have described different types of ELISA for detection of FMDV specific antibody with different sensitivity, specificity and reproducibility. The liquid phase blocking ELISA (LPB ELISA), described by Hamblin *et al*. (1986) has become a widely accepted standard test and adapted by a large number of laboratories world-wide. This has replaced the SNT for routine screening because it is quicker, more reproducible, correlates well with the SNT and does not suffer from the biological variability. As in sandwich ELISA, the LPB ELISA has been standardized by the Central Laboratory of Project Directorate on FMD and biological reagents necessary for the test are produced centrally and distributed throughout the country.

iii) Solid Phase Competitive ELISA: The IAH, Pirbright has developed a solid phase competitive ELISA (SPCE) for the detection of FMDV antibodies using the same reagents as used in LPBE. The SPCE has the advantage over the current OIE prescribed tests of robustness and the ability to be readily adapted for mass testing. The test provides estimates of the analytical sensitivity, precision, repeatability and reproducibility and establishment of the working cut off point. Recently, this test has been accepted by OIE as a prescribed test for international trade.

E. Sero-monitoring of FMD-Control Programme:

The sero-monitoring of the FMD-CP is done by analyzing the serum samples collected from FMD vaccinated animals comprising pre- (samples at the time of vaccination) and post- (approximately 4 weeks after vaccination) vaccination. The serum samples are collected from 10 cattle and 10 buffaloes from each of the randomly selected 10 villages in each district as per the guidelines under FMD-CP. These samples are processed in four serial dilutions (1:32, 1:64, 1:128 and 1:256) against three FMD serotypes by Liquid Phase Blocking ELISA (LPB-ELISA) as described at D(ii) above. The results are analyzed by calculating the per cent of animals showing protective antibody titres ($> \log_{10} 1.8$).

F. Differentiation of Infected and Vaccinated Animals (DIVA strategy):

The conventional diagnostic system (e.g. ELISA) is unable to differentiate FMD vaccinated and infected animals (DIVA strategy). Detection of antibodies against non structural proteins (NSPs) has been used as a tool for DIVA strategy. Various diagnostic tests have been used for DIVA strategy: agar-gel immuno-diffusion test using virus infection-associated antigen (VIAA), 3D-ELISA, liquid phase ELISA for the detection of VIAA, the enzyme-linked immuno-electrotransfer blot (EITB) using purified rDNA derived NSP antigens. The Central Laboratory of Project Directorate on FMD has developed an indigenous 3AB3 NSP ELISA which is being used all over the Regional Centres and Network Units of FMD throughout the country for monitoring the antibody levels against FMDV NSPs indicating thereby the residual virus activity, persistent infection and the career status of animals in the field after every round of mass vaccination under FMD-CP. The reagents and other biologicals necessary for the test are produced centrally and distributed to all over the country to have a uniform pattern of testing. The serum samples have been analyzed periodically for anti-NSP antibodies for monitoring the FMD carrier status in vaccinated subjects under National FMD sero-surveillance. The percentage of animals which were found positive for anti-NSP antibodies during pre- 1st vaccination (before start of FMD CP) and pre- 4th vaccination (after two years of FMD-CP) were 31.94 and 18.40%, respectively which further lowered to 12.12% during pre- 8th vaccination (after four years of FMD-CP). A significant ($P<0.01$) reduction in anti-NSP antibody profile (<10%) in Haryana was demonstrated six years after launch of FMD-CP. This data supports the fact that incidence of FMD virus circulation in the state has significantly reduced due to vaccination.

The results are encouraging and it has been demonstrated that with every subsequent vaccination, there is a significant decrease in the percent of animals demonstrating anti-NSP antibodies.

The protocols of all the tests being used in epidemiological surveillance and sero-monitoring of FMD-CP in Haryana and Delhi and as standardized by the Central Laboratory of Project Directorate on FMD, IVRI Campus Mukteshwar have been described under the Practicals later in this manual.

SURVEILLANCE OF FOOT-AND-MOUTH DISEASE OUTBREAKS BEFORE AND AFTER LAUNCH OF FMD CONTROL PROGRAMME IN HARYANA, INDIA.

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Foot and mouth disease (FMD) is a highly contagious major viral infection and a major constraint to international trade in livestock and animal products including their germ plasm. Considering the endemicity of the disease, Govt. of India had launched FMD-Control Programme (FMD – CP) in January 2004 across the country including Haryana with the idea of developing 'FMD-free zones'. This Centre is actively engaged in FMD surveillance, epidemiology and sero-monitoring of FMD-CP in Haryana. The present communication compares the occurrence of FMD outbreaks and distribution of virus serotypes involved before (2000 to 2003) and after (2004 to 2010) launch of FMD – CP in Haryana, India.

A total of 229 FMD outbreaks have been recorded in Haryana over a period of ten years between January 2000 and December 2010. Of these, 204 FMD outbreaks were recorded before (2000 to 2003) and only 25 after (2004 to 2010) the launch of FMD-CP. Maximum outbreaks (111) were recorded in 2003 which reduced to 15 in 2004, three in 2005, only one each in 2006 and 2007, two each in 2008 and 2009 and one in 2010. Between 2000-2003, just before the launch of FMD-CP, the disease was recorded round the year with maximum outbreaks during January - March months and highest outbreaks recorded in Hisar (53) district. The number of outbreaks reduced to eight in Hisar during 2004-2007 and further reduced to only one in 2007. In maximum outbreaks, cattle and buffalo were simultaneously affected. Among the prevalent virus serotypes, FMD virus serotype O was the predominant serotype during 2000-2003 whereas during 2004-2010, FMD virus serotypes O and A were in almost equal proportions followed by Asia-1. However, FMD virus serotype C has not shown its presence during all these years. The significant reduction in FMD outbreaks can be attributed to the implementation of FMD-Control Programme in Haryana since January 2004.

In view of the data on the epidemiology, Haryana state can serve as a model towards **"FMD Free Haryana"**. Further, the reduced incidence of the disease in the north-west states i.e., Punjab, J&K, Himachal Pradesh, Delhi and Rajasthan states support the candidacy of these states for "Zonal freedom from FMD". It is envisaged that the success of FMD-CP will pave the way for launching a National FMD Eradication Programme in the country.

VACCINATION STRATEGIES AGAINST EQUINE INFLUENZA

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Historically, equine influenza vaccines were mainly composed of whole inactivated viruses, which provide protection against influenza through induction of a short-lived humoral immunity. This is in contrast to immunity stimulated by natural infection, which is more robust and longer lived due to the stimulation of both humoral and cellular immune responses. The development of new strategies of vaccination – that mimic more closely the stimulation of the immune system induced by EIV infection – has been the focus of EIV vaccine development in the last two decades. Thus, modern vaccines composed of either live attenuated influenza virus, DNA plasmids or poxvirus-vectors coding for influenza virus proteins have been developed and some have been commercialized. A new approach to EIV vaccination using live-attenuated influenza virus engineered by reverse genetics, is also under development. Different vaccination approaches for equine influenza have been reviewed.

Equine influenza A viruses (EIV; H7N7 and H3N8 subtypes) are a leading cause of respiratory disease in the equines. Although the H7N7 EIV subtype has not been detected recently, the H3N8 subtype still remains a serious concern with significant economic consequences for the horse industry. Current vaccination strategies for EIV can be divided into the administration of either 'inactivated' or 'live' vaccines. 'Inactivated' vaccines include killed whole virus, subunit proteins and DNA vaccination while 'live' vaccines include attenuated virus or living virus- vectored vaccines.

A. INACTIVATED VACCINES

a. Inactivated whole influenza virus vaccines: Protection from influenza disease conferred by conventional inactivated vaccines is strongly associated with the levels of circulating antibodies against HA, provided that the vaccine strain and the challenge infection strain are genetically and antigenically similar. Whole inactivated virus vaccines adjuvanted with aluminium phosphate or aluminium hydroxide failed to induce influenza virus specific CTL activity (Hannant *et al*., 1994). Amorphous aluminium hydroxyphosphate gel (alhydrogel or adjus-phos) are common adjuvants, which, in mice, stimulate IL-4 synthesis and activate Th_2 cells, with enhanced IgG1 and IgE production (Vogel *et al*., 1998). Other adjuvants include polymers of acrylic acid cross-linked with polyallylsucrose, or a lipidic, non-aluminum, dual phase adjuvant (MetaStim). Carbomer, an emulsifying agent – a polyacrylic acid with an extremely high molecular weight – is also used as an adjuvant. The intranasal inoculation of

the inactivated influenza vaccine with cholera toxin B (CTB) as adjuvant has been shown to induce a local immune response, composed of virus-specific VN and IgA antibodies, which protects ponies from infection with EIV (Hannant *et al*., 1999).

b. Subunit vaccines: Current subunit vaccines contain purified HA and NA proteins. These membrane proteins (antigens) are generally adjuvanted with *Quillai*a saponin (Quil A) or integrated into immuno-stimulating complexes (ISCOM) to improve their antigenicity. In the late 1980s, an ISCOM vaccine containing HA antigen from A/eq/Solvalla/79 (H3N8) was shown to induce IgGab antibody lasting about 25 weeks in trachea and nasopharynx of horses (Hannant *et al*., 1987). In the mid 1990s, an ISCOM vaccine (Equip[TM]; Pitman-Moore) containing HA protein from A/eq/ Newmarket/77 (H7N7) and A/eq/Brentwood/79 (H3N8) was evaluated in ponies. With an equivalent amount of HA antigen, an ISCOM vaccine was shown to induce SRH antibodies more efficiently than an inactivated whole virus vaccine (Mumford *et al*., 1994). More recently, an ISCOM-based vaccine containing HA antigen from Newmarket/77 (H7N7), Borlange/91 (H3N8) and Kentucky/98 (H3N8) (EQUIP F, Schering-Plough Animal Health) provided strong protective immunity against EIV challenge. Vaccinated ponies were completely protected against clinical signs of the disease but 43% showed virus shedding after challenge infection (Crouch *et al*., 2005).

c. DNA vaccines: Administration of DNA plasmids containing a gene for an immunogenic protein offers a different form of vaccination and efficient protection against influenza infection and has been demonstrated in several species viz. mice, ferrets, chicken (Fynan *et al*., 1993, Webster *et al*., 1994). DNA vaccination results in the *in vivo* expression of antigenic proteins, leading to the stimulation of both humoral and cellular immune responses. The impact of skin and mucosal immunization with a plasmid DNA vaccine coding for the HA protein of A/eq/Kentucky/1/81 influenza virus was therefore studied in the horse (Soboll *et al*., 2003; Lunn *et al*., 1999). Vaccinated horses were partially to completely protected against clinical signs of the disease and virus shedding induced by challenge infection with a homologous strain of influenza virus, 30 days after the last vaccination. DNA vaccination induced serum IgGa and IgGb antibody responses (i.e. an isotype response similar to EIV infection) but no mucosal IgA responses were detected. Protection resulting from DNA vaccination was associated with the presence of both serum IgGa/IgGb antibodies and mucosal IgG, supporting the idea that mucosal IgA is not essential for protection (Lunn *et al*., 1999; Soboll *et al*., 2003).

B. LIVE VIRUS/ VECTOR VACCINES

The demonstration that the immune response induced by experimental infection with equine influenza virus protects horses against re-infection for up to 1 year (Hannant *et al* 1988) suggests that an EIV vaccine mimicking natural infection should be more efficient in affording protection than conventional inactivated vaccines. Thus, the use of a modified live attenuated EIV or a live virus-vector coding for EIV proteins constitute the main approaches investigated in the last 20 years to produce a live equine influenza vaccine.

a. Live attenuated influenza virus vaccine: Immunization with a live attenuated influenza virus closely mimics natural infection. Antigens are presented to the immune system via both exogenous and endogenous pathways, and vaccines are therefore expected to stimulate an immune response similar to those induced by infection.

b. Reassortant influenza virus: An influenza virus with a limited growth in mammals (e.g. avian influenza virus) can be reassorted with a mammalian influenza virus to obtain a live attenuated virus able to induce a protective immune response. A reassortant expressing internal proteins of the avian influenza virus A/Duck/ New York/6750/78 (H2N2) and surface glycoproteins of the EIV A/eq/Georgia/1/81 (H3N8) was evaluated for efficacy and safety in hamsters and ponies. The reassortant induced an antibody response and was shed after infection. Five-and-a half months after the exposure to the reassortant, ponies were partially protected from a challenge infection with the parental EIV, the duration of virus shedding was also slightly reduced (Mumford *et al*., 1998). Vaccines based on avian/equine reassortants certainly would be too risky to use. Reassortants could be safe in horses but induced disease in birds (Banbura *et al*., 1991). The recent infections of humans with avian H5N1 influenza virus in Asia would raise a lot of safety concerns for the use of such reassortants for horse vaccination.

c. Temperature-sensitive (Ts) or cold-adapted influenza virus: In the late 1980s, the *Ts* clone 8B1 (bearing a *Ts* lesion on the PA gene) was derived from the re-assortment of a human influenza A *Ts* donor virus (bearing *Ts* lesions on the polymerase and NP genes) with a wild-type A/eq/Cornell/16/74 (H7N7) EIV. In the horse, intranasal instillation of clone 8B1 did not cause detectable clinical signs or febrile responses. Vaccine virus that was shed retained the *Ts* phenotype. When challenge infected (28 days after the last vaccine exposure) with the parental equine H7N7 influenza virus, ponies were partially to fully protected from clinical signs of the disease and virus shedding (21 of 40 vaccinated ponies did not shed virus after challenge infection) (Holmes *et al*., 1988). In the mid 1990s, clone 8B1 virus was used as the donor virus with the wild-type A/eq/Kentucky/1/81 (H3N8) influenza virus to produce

a H3N8 *Ts* reassortant virus (Clone 255). Ponies vaccinated by a nebulized suspension containing the reassortant virus showed no clinical signs after immunization with Clone 255 but did seroconvert for haemagglutination inhibiting (HI) antibody after exposure to the reassortant, indicating antigenicity of the reassortant. When they were challenge infected with the A/eq/Kentucky/1/81 parental influenza virus 1 or 2 months after immunization, vaccinated ponies were fully protected against clinical signs of disease but virus shedding was observed in 20–33% of vaccinated ponies. Ponies remained protected against clinical signs of disease when they were challenged 7 and 10 months after immunization, but the percentage of ponies shedding virus increased to 55% (Holmes *et al* 1991).

Cold adapted, *Ts* live EIVs were also produced by serial passage in embryonated hens' eggs at temperatures gradually reduced to 26°C (Youngner *et al*., 2001). More recently, a cold-adapted, Ts, modified-live EIV vaccine (FluAvert IN Vaccine; Intervet) − derived from the wild-type A/eq/Kentucky/1/91 (H3N8) influenza virus − has been evaluated and is available commercially in North America. Vaccinates showed significant clinical protection when re-challenged 6 months post-vaccination and lower rectal temperatures when challenged 12 months after vaccination. This vaccine has also been shown to protect ponies against infection with a heterologous influenza virus (Chambers *et al*., 2001).

d. Virus vectored vaccines: Live recombinant virus-vector vaccines are constructed by inserting selected genes from the pathogen of interest into live, infectious, but non-disease-causing viruses. Recombinant poxviruses have been widely used for vaccination (Paoletti *et al*., 1996; Moss *et al*., 1996). Poxviruses are genetically stable and allow the insertion of a large segment of foreign DNA. Recombinant poxviruses derived from vaccinia or avipoxvirus (canarypox virus) are commercially available and several recombinant canarypox-based vaccines have been developed for the horse (Minke *et al*., 2006).

In 2003, a modified live canarypox virus-vectored influenza vaccine (ProteqFlu[®4]; Merial Ltd., UK) was licensed in the European Union for use in horses. This live, vectored vaccine is safe, as canarypox virus undergoes an abortive infection in mammalian cells (Plotkin *et al*., 1995). This vaccine contains two canarypox recombinant viruses coding for the HA proteins from Newmarket/2/93 and Kentucky/94 EIV strains (European and American representative strains, respectively) adjuvanted with Carbormer 974P. The efficacy of this new commercialized vaccine has recently been published (Edlund *et al*., 2005; Paillot *et al*., 2006). After the first immunization, all vaccinated ponies developed detectable levels

of SRH antibodies and a few ponies mounted an anamnestic response after the second immunization. Singly vaccinated ponies did not shed virus and were protected or developed only mild signs of the disease after challenge infection with EIV and similar results were obtained with ponies vaccinated twice.

One concern with poxvirus-based vaccines is the presence of pre-existing immunity specific to poxvirus and its impact on subsequent vaccination with the same recombinant. Suppression of the antibody response to recombinant antigen encoded by a VACV-based vector has been reported (Kanesa-thasan *et al*., 2000). However, vaccination with canarypox virus-based vaccines had no discernable effect on the efficiency of subsequent immunization using the same vector coding for homologous or heterologous antigens.

e. Live attenuated influenza virus designed by reverse genetics: Reverse genetics allows the generation of entirely artificial recombinant influenza viruses from cloned DNA plasmids (Schickli *et al*., 2001). In the mid 1990s, application of the reverse genetics method to equine influenza virus was discussed after the generation of an attenuated influenza virus by the insertion of a mutation in the cytoplasmic tail of NA protein (Bilsel *et al*., 1994). Ten years elapsed before the generation by reverse genetics of attenuated EIVs encoding a carboxyl-terminus truncated NS1 protein (Quinlivan *et al*., 2005). An infectious recombinant virus was recovered after the transfection of a dog kidney cell line (MDCK) with plasmids coding for each of the eight influenza virus RNAs (PB1, PB2, PA, HA, NP, NA, M and NS) simultaneously with protein expression plasmids (PB1, PB2, PA, NP and NS1). Derived from the A/eq/Kentucky/5/02 (H3N8) influenza virus, the 3 recombinant viruses obtained showed a low replication capacity both *in vitro* in MDCK cells and also *in vivo* in embryonated hens' eggs (9 to 11-day old) or in mice after an intranasal infection. The ability of the recombinant viruses to inhibit the production of IFNα/β by infected cells (previously associated with the NS1 protein in wild-type virus was also decreased (Kittel *et al*., 2004). An EIV with an altered NS1 protein could be a good master strain for live attenuated viral vaccines, reverse genetics allowing the insertion of HA and NA genes of epidemic EIV strains. The same approach has been suggested in the context of human vaccination (Schickli *et al*., 2001). Reverse genetics is an efficient method to generate recombinant live attenuated influenza virus. However, such recombinants for EIV remain to be tested *in vivo* in horses to assess their antigenicity and efficacy in protection against challenge infection.

CONCLUSION

Today, the main types of equine influenza vaccines in use contain whole inactivated virus or subunits. Protection afforded by this first generation of vaccines is based on high levels of protective antibodies. Second generation vaccines (i.e. live attenuated and poxvirus-based vaccines) are now available. These stimulate both humoral and cellular immune responses and so mimic more closely the protective immunity induced by natural infection with influenza virus. These vaccines are not yet widely used and it is necessary to evaluate their performance in the field. The delivery routes of DNA vaccines remain a major issue, despite their ability to protect horses against influenza. It is anticipated that live attenuated influenza virus engineered by reverse genetics will almost certainly be the next generation of vaccines against equine influenza. Several laboratory animal models (e.g. rodents, ferrets and monkeys) are used to evaluate vaccine efficacy against influenza A virus infection. Such laboratory models need to be developed for assessing potency of EIV vaccines in experimental animals before finally testing in natural host – the horse. However, the horse model allows field studies of a range of vaccine techniques in the natural host population, and integration of natural external factors (e.g. population dynamics, genetic diversity) that can influence the outcome of vaccination. Therefore, the horse is an interesting model of vaccination in the battle against the evolutionary strategy of influenza viruses.

REFERENCES

Banbura MW, Kawaoka Y, Thomas TL and Webster RG. 1991. *Virology* **184**:469–71.

Bilsel P and Kawaoka Y. 1994. In: Nakajima H, Plowright W, editors. Equine infectious diseases. VII. Proceedings of Seventh International Conference on Equine Infectious Diseases. Newmarket, UK: R&W Publications Ltd.; p. 165–8.

Chambers TM, Holland RE, Tudor LR, Townsend HG, Cook A and Bogdan J. 2001. A new modified live equine influenza virus vaccine: phenotypic stability, restricted spread and efficacy against heterologous virus challenge. *Equine Vet J* 33: 630–6.

Crouch CF, Daly J, Henley W, Hannant D, Wilkins J and Francis MJ. 2005. *Vet Immunol Immunopathol*. **15**: 345-55.

Edlund Toulemonde C, Daly J, Sindle T, Guigal PM, Audonnet JC and Minke JM. 2005. *Vet Rec* **156**: 367–71.

Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC and Robinson HL. 1993. *Proc Natl Acad Sci USA* **90**: 11478–82.

Hannant D, Easeman R and Mumford JA. 1999. In: Wernery U, Wade JF, Mumford JA, Kaaden OR, editors. Equine infectious diseases. VIII. Proceedings of the Eighth International Conference on Equine Infectious Diseases. Newmarket, UK: R&W Publications (Newmarket); p. 118–9.

Hannant D, Jessett DM, O'Neill T, Livesay GJ and Mumford JA. 1994. In: Nakajima H, Plowright W, editors. Equine infectious diseases. VII: Proceedings of Seventh

International Conference on Equine Infectious Diseases. Newmarket, UK: R&W Publications Ltd.; p. 169–74.

Hannant D, Jessett DM, O'Neill T, Sundquist B, Mumford JA. In: Powell DG, editor. Equine infectious diseases. V. Proceedings of the Fifth International Conference. Lexington, KY: University Press of Kentucky; 1987. p. 66–73.

Hannant D, Mumford JA, Jessett DM. 1988. Duration of circulating antibody and immunity following infection with equine influenza virus. *Vet Rec* **122**: 125–8.

Holmes DF, Lamb LM, Anguish LM, Coggins L, Murphy BR and Gillespie JH. 1988. In: Powell DG, editor. Equine infectious diseases. V. Proceedings of the Fifth International Conference. Lexington, KY: University Press of Kentucky; p. 88–93.

Holmes DF, Lamb LM, Coggins L, Murphy BR and Gillespie JH. 1991. In: Plowright W, Rossdale PD, Wade JF, editors. Equine infectious diseases. VI. Proceedings of the Sixth International Conference. Newmarket: R&W Publications Ltd.; p. 253–8.

Kanesa-thasan N, Smucny JJ, Hoke CH, Marks DH, Konishi E and Kurane I. 2000. Safety and immunogenicity of NYVACJEV and ALVAC-JEV attenuated recombinant Japanese encephalitis virus-poxvirus vaccines in vaccinia-nonimmune and vaccinia-immune humans. *Vaccine* **19**: 483–91.

Kittel C, Sereinig S, Ferko B, Stasakova and Wolkerstorfer A. 2004. *Virology* **324**: 67–73.

Lunn DP, Soboll G, Schram BR, Quass J and Drape RJ. 1999. Vaccine **17**:2245–58.

Minke JM, Fischer L, Baudu P, Guigal PM, Sindle T and Mumford JA. 2006. *Vet Immunol Immunopathol* **111**: 47-57.

Moss B. 1996. *Proc Natl Acad Sci USA*;**93**: 11341–8.

Mumford JA, Jessett DM, Dunleavy U, Wood JLN, Hannant D and Sundquist B. 1994. *Vaccine* **12**: 857–63.

Paillot R, Kydd J, Sindle T, Hannant D, Edlund-Toulemonde C, Audonnet JC, Minke JM and Daly JM. 2006. *Vet Immunol Immunopathol.* **112**: 225-33.

Paoletti E. 1996. *Proc Natl Acad Sci USA* **93**: 11349–53.

Plotkin SA, Cadoz M, Meric C, Leroy O and Excler JL. 1995. *Dev Biol Stand* **84**: 165–70.

Schickli JH, Flandorfer A, Nakaya T, Martinez-Sobrido L, Garcia-Sastre A and Palese P. 2001. *Philos Trans R Soc Lond B Biol Sci* **356**: 1965–73.

Soboll G, Nelson KM, Leuthner ES, Drape R and Macklin MD. 2003. *Vaccine* **21**: 3081–92.

Vogel FR, Powell MF, and Alving CR. 1998. A compendium of vaccine adjuvants and excipients. 2nd ed. Bethesda, MD: National Institute of Allergy & Infectious Diseases.

Wattrang E, Jessett DM, Yates P, Hannant D. 2003. *Viral Immunol* **16**: 57–67.

Youngner JS, Whitaker-Dowling P, Chambers TM, Rushlow KE and Sebring R. 2001. *Am J Vet Res* **62**: 1290-4.

APPROACHES TO THE PREVENTION AND CONTROL OF AVIAN INFLUENZA VIRUSES IN POULTRY

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Avian influenza (AI) was relatively uncommon until 1997. The number of outbreaks of AI in poultry has increased sharply during the past ten years. The number of birds involved in AI outbreaks has increased 100-fold, from 23 million from 1959 through 1998 to >200 million from 1999 through 2005. Since the late 1990s, AI infections have assumed a completely different profile in the veterinary and medical scientific communities and pose a great public health problem. Some of the recent outbreaks have led to devastating consequences for the poultry industry, negative repercussions on public opinion, and, in some instances, created major human health issues, including the risk of generating a new pandemic virus for humans through an avian-human link.

An avian influenza outbreak has serious implications on the trade of fresh poultry meat. It calls for export ban with infection of H5 or H7 regardless of virulence of isolate. According to OIE criteria, a country may trade freely if it has been shown that highly pathogenic avian influenza (HPAI) has not been present in the country for 3 years, or six months after the slaughter of the last affected animal for countries in which a stamping out policy is practiced with or without vaccination. A country where vaccination is used, can claim avian influenza (AI)-free status when i) there are no new AI outbreaks, ii) sufficient proof that repopulated flocks remain seronegative, and iii) flocks vaccinated against AI test seropositive. Vaccinated flocks need to be monitored for avian influenza infection. However, in the avian influenza outbreak of 2000 in Italy, trade bans were lifted for the first time after a heterologous vaccine was used (Decision 2001/847/EC)

EPIDEMIOLOGY AND PATHOGENICITY

Of the five genera under family *Orthomyxoviridae* the three influenza viruses are classified as separate genera, *Influenzavirus A, Influenzavirus B,* and *Influenzavirus C*, based on the two major internal proteins of influenza viruses, viz. nucleoprotein and matrix protein. Influenza A viruses are the only type reported to cause natural infections of birds and are further divided into subtypes based on their hemagglutinin (H) and neuraminidase (N) proteins. Each virus has one H and one N antigen. Currently, 16 H and 9 N subtypes have been recognized and all possible combinations have been isolated from birds.

The genome of influenza viruses is segmented single-stranded RNA of negative polarity. There are eight RNA segments in influenza A and B viruses while influenza C virus has seven RNA segments. The mRNAs are transcribed from the virion RNA by a virion-associated-RNA-dependent-RNA-polymerase. The eight genomic segments of influenza A virus code for nine structural viz. PB1, PB1-F2, PB2, PA, HA, NA, NP, M1 and M2, and two non-structural proteins viz. NS1 and NS2.

Influenza A viruses that infect poultry can be divided into 2 distinct groups according to the severity of disease they cause: highly pathogenic (HP), and low pathogenic (LP), based on mortality rates of *in vivo* tests and the results of *in vitro* tests. The LP AI viruses are associated with mild form of disease; whereas HP strains cause high morbidity and mortality. The H5 and H7 AI viruses come under HP category. The LP strains cause a mortality of 5-15% where as 76-100% mortality has been observed with HP strains. HPAI is a lethal infection in certain domestic birds (e.g., chickens and turkeys) and has a variable clinical effect (may or may not cause clinical signs and death) in domestic waterfowl and wild birds. Viruses that belong to subtypes H1–H16 that lack the multibasic cleavage site are perpetuated in nature in wild bird populations. Feral birds, particularly waterfowl, are the natural hosts for these viruses and are therefore considered an ever-present source of viruses. Since their introduction into domestic bird populations, these viruses have caused low-pathogenicity avian influenza (LPAI), a localized infection that result in mild disease, primarily respiratory disease, depression, and egg-production problems. It has been postulated that the HPAI viruses emerge from H5 and H7 LPAI progenitors by mutation or recombination. However, the mutation to virulence is unpredictable and may occur soon after the virus is introduced to poultry or after the LPAI virus has circulated in domestic birds for several months.

Although avian influenza is primarily a disease of domesticated poultry such as chickens, ducks, turkeys, pheasants, sub clinical infections do occur in a wide range of feral migratory birds. Antigenically and genetically closely related AI type A viruses have also been isolated from humans, pigs, seals and whales. The infected birds excrete the virus from respiratory tract, conjunctiva and faeces. The infection spreads both through direct contact and indirect contact including aerosol or exposure to virus contaminated fomites. Interspecies transmission between chickens and turkeys, and wild birds particularly ducks may play an important role to introduce avian influenza viruses in susceptible flocks. Clinically normal waterfowl and sea birds may introduce the virus into flocks. Broken contaminated eggs may infect chicks in the incubator.

PREVENTION AND CONTROL

Biosecurity: Stringent biosecurity measures such as removal and slaughter of infected birds, prompt incineration of carcases, disinfection of the premises after removal of infected litter, prevention of the movements of the birds and people from infected to clean areas, and giving an interval between slaughter and repopulation help in containment of an outbreak. The virus can be inactivated by formalin and iodine compounds. Steps should be taken in high-risk areas to prevent access of wild birds to poultry farms. The success of any control or eradication strategy is dependent upon surveillance and diagnostic procedures that ascertain the AI status. Less than optimal virus isolation or serologic procedure or incomplete surveillance strategies may provide negative data that can give false sense of security.

Disease Containment: All infected flocks are humanely destroyed, and carcasses are disposed off in an environmentally acceptable method. Infected premises are thoroughly cleaned and disinfected before introduction of new birds. In the situation of detection of highly pathogenic virus, flocks in the vicinity of infected premises and those from poultry operations that may have had contact with infected premises are also humanely destroyed and disposed of as a pre-emptive measure. Quarantines restricting the movement of poultry and poultry products are placed on infected premises, poultry operations located in the vicinity of infected premises and other poultry operations that may have had contact with infected premises. Birds from quarantined premises are tested and monitored for evidence of AI infection. A segregation protocol is also followed with the aim to minimize, if not eliminate, potential contact between wild birds and domestic or captive birds in the area after a case of HPAI has been confirmed. During highly pathogenic avian influenza outbreaks a Control Area is established and two different zones: (protection zone and surveillance zone) around the infected area are created.

Protection Zone: It is established with a radius of 3 km from the infected farm and will have a minimum duration of 21 days from the date the virus was identified. Birds within the zone will be sampled and tested. Strict biosecurity measures will be in force for all poultry farms in the zone, with access to the zone controlled and vehicles and other materials that leave the premises are cleaned and disinfected. All domestic birds should be confined within a facility erected and laid out in a manner that prevents any direct or indirect contact with wild birds. The public will be notified within the area to increase disease awareness. Live poultry shows, displays or markets are discouraged. The litter or manure is not transported out of the protection zone. The bird hunting activities within the zone are also stopped.

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Surveillance Zone: It is established with a radius of 10 km from the infected farm and will last for a minimum of 21 days from the date the virus is identified. All domestic or captive bird flocks will be identified within the zone. The flock owners in the zone must implement appropriate farm biosecurity measures. The movement of poultry and hatching eggs will be controlled. Transportation of poultry and captive birds out of the surveillance zone will be prohibited for a period of time following establishment of the zone. Assembly of poultry and other birds at fairs, shows or markets will be cancelled.

VACCINATION AS PART OF A CONTROL STRATEGY

Vaccination cannot replace other control measures. It should only be used as an additional tool in a control strategy. Used strategically vaccination compliments a stamping out strategy by slowing/stopping the spread of the virus. The goals of vaccination are i) to create of a buffer zone between infected and non-infected areas ii) protection of AI free areas considered at high risk of infection, iii) vaccination of poultry flocks intended for initial restocking in previously infected areas.

Different vaccination strategies and schedules are required for different situations. In particular, vaccination programmes should be modulated in diverse situations according to the virus strain involved, the characteristics of the poultry producing sector, the capacity of the veterinary infrastructure, and the availability of adequate resources. Based on the eco-epidemiological situation in the affected region/area and the assessment of the risk of AI introduction, different vaccination strategies could be implemented to control AI: (i) routine vaccination performed in endemic areas; (ii) emergency vaccination in the face of an epidemic; and (iii) preventative vaccination carried out whenever a high risk of virus incursion is identified.

The utilization of an AI vaccine was previously not recommended by the OIE. The main reason against vaccination was that vaccination does not prevent infection, or viral shedding, but will often mitigate clinical signs. Therefore, vaccination could allow a highly pathogenic form of the virus to infect and to replicate within a flock without being detected, and allow it to potentially spread to other susceptible birds. Until recently it had not been possible to differentiate between infected and uninfected vaccinated animals, so vaccinated animals were still subject to trade bans. But vaccination could help bring the outbreak under control by reducing the viral load in environment by increasing the amount of virus needed to infect a bird, reducing severity of clinical signs and reducing the shedding of the virus.

There are currently two major forms of AI vaccine available. There is a recombinant form using Infectious Laryngotracheitis or Fowl Pox vaccines as a carrier. The other form is a whole killed virus vaccine. Vaccination of poultry with inactivated influenza vaccine can be an effective tool in the control of avian influenza (AI). One major concern of using inactivated vaccine is vaccine-induced antibody interference with serologic surveillance and epidemiology. DIVA (differentiating infected from vaccinated) strategy can be employed that easily differentiates the birds that were naturally infected with influenza from those that were vaccinated. The whole killed vaccine has been used in a DIVA strategy to vaccinate birds in and around the movement control zone of an infected area. The AI vaccine is still not permitted in Canada. However, in light of the current Asian crisis, where there has been little success to control the spread of AI, a method in addition to stamping-out had to be considered. Based on the reported successful implementation of vaccine by other countries, such as Italy and the U.S.A., one of the recommendations brought forward included vaccinating at risk birds against AI in the face of an outbreak. Mexico has been vaccinating, as a prophylactic measure, against a low pathogenic H5N2 subtype for 10 years. Vaccination in this case has not eradicated the disease. The main reason provided for the ineffectiveness of this vaccine was improper administration or poor quality product.

Role of wild and/ or migratory Birds: Long distances travel is carried out by many bird species leading to immunosuppression. Avian influenza (AI) viruses have been isolated from 105 wild bird species belonging to 26 families. Birds of wetlands and aquatic environments such as ducks, geese, swans, gulls, terns, and shorebirds are thought to constitute the major natural reservoir for avian influenza virus. The virus is usually introduced into one country through the wild fauna and subsequently spread to the domestic flocks either by a direct contact between the wild birds and the domestic ones or by an indirect contact through contaminated fomites such as boots, wheels, straw and by contaminated water. Until recently, the HPAI viruses were considered not to have a wild bird reservoir, but to emerge in from low pathogenic viruses perpetuated in wild water birds. The rapid spread of H5N1 HPAI virus in 2005-2006, with concurrent outbreaks reported in both domestic and wild birds over Asia, Europe, and Africa, has raised concerns about the potential role of migratory birds in the epidemiology of the HPAI infection. The migratory birds can transport HPAI H5N1 over long distances.

'Bridge' Species: Several species of familiar songbirds or perching birds such as crows (*Corvidae* family), sparrows (*Passeridae* family), mynas (*Sturnidae* family) and the

ubiquitous feral pigeon *(Columba livia)* of the *Columbiformes* order, have broad and diverse habitat preferences. Their close association with humans often results in close contact with domestic poultry, especially at open poultry farms where food is readily available. The H5N1 AIV has been reported to fatally infect them. Thus, these species may serve as links between wild birds in natural habitats and domestic poultry, acting as a 'bridge' in the transmission of AI viruses from poultry to wildlife or vice versa. Specific surveillance and monitoring efforts in these potential 'bridge' species should be undertaken at HPAI poultry outbreaks and wildlife mortality events.

International Scientific Task Force on Avian Influenza and Wild Birds

The results of various studies about the role of migratory birds as potential vectors of HPAI virus subtype H5N1, led to the establishment of a Scientific Task Force on Avian Influenza and Wild Birds comprising the following 13 members and observers, including UN bodies, wildlife treaties and specialist intergovernmental and nongovernmental organizations. The objectives of this Task Force are i) to bring together the best scientific advice on the conservation impact of the spread of avian influenza, ii) assess the role of migratory birds as vectors of the virus, iii) issue advice on the root causes of the epidemic as well as technically sound measures to combat it and iv) develop early warning systems.

ANTIVIRALS

The prevention and treatment of influenza virus infection can also be done by antiviral drugs such as: M2 ion channel blockers (amantadine and its derivative rimantadine) and neuraminidase inhibitors (zanamivir and oseltamivir). Amantadine and rimantadine block the ion channel activity of the M2 protein of most influenza A viruses, and viral replication is inhibited by the blockade of hydrogen ion flow, when virus enters the host's cells. The neuraminidase inhibitors interrupt an established infection in its late stages by inhibiting the release of virions from infected cells, which results in the aggregation of virions at the cell surface and the consequent inhibition of viral penetration of mucous secretions and spread to other cells.

Influence of economic indicators, poultry density and the performance of Veterinary Services on the control of high-pathogenicity avian influenza in poultry: HPAI and LPNAI in poultry are notifiable diseases that must be reported to the OIE. The responses of various countries to avian influenza (AI) outbreak situations are quite variable based on their economic status, diagnostic capacity and other factors. Work has been carried out to

determine the association between HPAI control data and a country's poultry density, the performance of its Veterinary Services, and its economic indicators (gross domestic product, agricultural gross domestic product, gross national income, human development index, etc. It has been found that as poultry density increases for least developed countries there is an increase in the number and duration of HPAI outbreaks and in the time taken for eradication of the disease. Member OECD Countries, i.e. those with high-income economies, transparency and good governance, had shorter and significantly fewer HPAI outbreaks, quicker eradication times, lower mortality rates and higher culling rates. Furthermore, countries that had effective and efficient Veterinary Services (assessed using the OIE Tool for the Evaluation of Performance of Veterinary Services) had better HPAI control measures.

INDIA'S SCENARIO

In India, the farmers are expected to report any unusual mortality in poultry to the local veterinary authorities, who thereby send the samples to the nearby Regional Disease Diagnostic Laboratory (RDDL) or to High Security Animal Disease Laboratory (HSADL), Bhopal and National Institute of Virology, Pune for testing. Upon tested positive for HPAI, Department of Animal Husbandry, Dairying & Fisheries, Ministry of Agriculture, Government of India notifies the outbreak of HPAI in that state to the global community through Office International des Epizootis (OIE) (World Organization of Animal Health). It also instructs the affected state to start the necessary actions contained in the Action Plan.

The control and containment operations are required to be undertaken around the infected premise. These include culling of birds; disposal of birds and infected materials; quarantine and restrictions of movements in the operational area, clean-up, disinfection and sanitation followed by post-operation surveillance in and around the infected zone. Poultry is culled in a radius of 3-5 km and surveillance is carried out in a further radius of 5-10 km for 90 days as per the protocol laid down by OIE to regain freedom from Avian Influenza.

CONCLUSIONS

The OIE recommends eradication of HPAI to decrease the virus load in susceptible avian species and environment and thereby decrease the risk of human infection, to secure the production sector and trade, as well as to safeguard food security and the livelihoods of farmers in developing countries. The recent unprecedented and almost worldwide spread of HPAI infections, and the related serious animal and human health implications have increased the need to develop control strategies complementary to a stamping out policy.

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Early detection of disease outbreaks followed by a rapid response is the first line of defence. The reporting and notification of AI by bird owners in infected and at risk countries will be affected positively by a liberal compensation mechanism. Control strategies based on a combination of stamping out, movement restrictions and emergency vaccination could maximize eradication efforts in certain situations.

Controlled elimination of infected poultry, movement restrictions, improved hygiene and biosecurity, and appropriate surveillance should result in a significant decrease of viral contamination of the environment. Vaccination is an additional measure aimed primarily at a reduction of viral replication and viral shedding due to induction of a protective immunity in the target population. A good vaccination program would raise the levels of protective flock immunity and increase the resistance to infection. In combination with the implementation of effective biosecurity measures, vaccination could prevent the introduction of the AI virus, or alternatively in reducing its spread, minimizing the negative impact on poultry production and decreasing potential economic losses.

Further Reading

Action Plan of Animal Husbandry for preparedness, control and containment of avian influenza. 2006. Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, GOI.

D. E. Swayne, G. Pavade, K. Hamilton, B. Vallat and K. Miyagishima. 2011. Assessment of national strategies for control of high-pathogenicity avian influenza and low pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. *Rev. sci. tech. Off. int. Epiz.* **30:** 839-870.

G. Pavade, L. Awada, K. Hamilton, and D.E. Swayne. 2011. The influence of economic indicators, poultry density and the performance of Veterinary Services on the control of high-pathogenicity avian influenza in poultry. Rev. sci. tech. *Off. int. Epiz.* **30:** 661-671.

Pawar S. D, Mullick J., Raut C. G., and Mishra A. C. 2011. Avian influenza surveillance in wild, resident, migratory birds and poultry in India. National Institute of Virology, Annual Report 2010–11, NIV, Pune, India, 103 p, http://www.niv.co.in.

Pawar S. D., Kale S. D., Rawankar A.S., Koratkar, S. S, Raut, C. G., Pande, S. A., Mullick, J., and Mishra A. C. 2012. Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry during 2009-2011 in the West Bengal State, India. *Virol J.* **9**(1):151. [Epub ahead of print].

MARKER VACCINES AND DIVA STRATEGIES FOR THE CONTROL AND ERADICATION OF INFECTIOUS DISEASES

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A vaccine which allows for the differentiation between infected and vaccinated individuals is termed as Marker vaccine. Marker vaccines, being a form of the virus without a particular gene coding for a non protective and non essential antigen so, do not produce a key antibody, while still protecting the individual against the disease. The absence of this key antibody can be detected by suitable diagnostic test, confirming whether the individual has been vaccinated or is genuinely infected. Marker vaccine can be genetically engineered gene deleted, subunit or vectored vaccine. The use of marker vaccine which can differentiate vaccinated from infected (DIVA) animals is important for protecting national livestock allow sero-monitoring of herds and critical as trading tool. Gene-deleted vaccines, DIVA strategies and similar methods have been successfully applied in the control and eradication of classical swine fever, Aujeszky's disease, infectious bovine rhinotracheitis, FMD and avian influenza.

Classical swine fever (CSF)

Classical swine fever (CSF) is an economically important highly contagious disease of swine worldwide. Although Classical swine fever virus (CSFV) field strains vary in the virulence, they all result in serious losses in pig industry. Highly virulent field strains generally cause acute disease and high mortality; moderately virulent field Strains cause sub acute or chronic infections; postnatal infection by low virulent field strains produces subclinical infection and mortality in the new- born piglets. The main strategies to control the CSF epidemics are systematic prophylactic vaccination with live attenuated vaccines (such as C-strain) and non-vaccination stamping out policy. Marker vaccine and companion serological diagnostic test is thought to be a promising strategy for future control and eradication of CSF. Initially, subunit marker vaccines based on baculovirus-expressed E2 glycoprotein of CSF virus have been developed and are available commercially. However, the immune response against these vaccines is delayed, and less protective compared to conventional live attenuated vaccines. The most promising candidates appear to be the vaccines based on viral vectors or chimeric pestiviruses. They have the potential of inducing a similarly strong immunity as conventional live attenuated vaccines and additionally open the possibility to discriminate vaccinated from infected animals.

The vaccine, which is produced commercially by replicating the genetically altered vector in cell culture, contains only the E2 protein. The E2 protein elicits CSFV neutralizing antibodies (and protective immunity) in vaccinated pigs without inducing antibodies to other CSFV proteins. The complementary diagnostic test used in this study was an ELISA using as antigen the Erns protein of CSFV, which is not present in the marker vaccine. With this Erns ELISA it was possible to distinguish pigs with different histories in regard to vaccination and exposure to field virus. The E2 ELISA was used to monitor the antibodies induced by the marker vaccine.

Herpes viruses

Marker vaccines against pseudorabies virus (PRV) and against bovine herpesvirus 1 (BHV1) infections have been developed, along conventional routes and by recombinant DNA technology. These vaccines have been shown to be efficacious in reducing (a) clinical signs after infection, (b) wild-type virus replication after infection, and (c) transmission of wild-type virus in the laboratory and in the field. The best example of a successful marker vaccine-companion kit combination is the set of gE-deleted pseudorabies (PRV), also known as Aujeszky's Disease, products that led to the eradication of PRV in U.S. commercial swine herds. At present, PRV vaccines that lack the gene for the glycoprotein gE are used worldwide in novel eradication programmes.

Glycoprotein gC, gE, gI, gG and gM of bovine herpes virus are non-essential and thus may be deleted with little or no effect on virus production *in vitro* or *in vivo*. These proteins were candidate for the development of marker vaccine against BHV-1. Experimental studies resulted in the selection of the gE mutant as a candidate gene-deleted DIVA vaccine. In addition to this, the majority of the wild-type strains have the gE gene and the antigenic variability of gE is low, which confirms the suitability of gE as a marker protein. Several gE-based companion diagnostic tests have been developed. These tests are based on competitive or blocking ELISAs using gE-specific monoclonal antibodies. For DIVA testing, any immunogenic protein that induces a long-lasting antibody response, including gE, may be used as a marker protein to differentiate gD vaccinated from infected animals.

Nipah Virus

Live canary pox recombinant viruses expressing the F and G proteins of the Nipah virus has been used as a vaccine to control Nipah virus infection in man and animals.

Avian influenza

Tetanus toxoid (TT) is used as an exogenous marker for avian influenza (AI) vaccines in chickens and showed that there is no interference with TT or AI H-specific antibody responses in TT and AI-co-vaccinated chickens. The TT marker was selected on the basis that chickens are highly resistant to tetanus, are not routinely vaccinated with TT, and naturally existing antibodies to TT are absent in chickens from a variety of sources. Furthermore, the antigen is of relatively low cost to manufacture, has minimal regulatory and market acceptance issues and development of an accurate and relatively inexpensive antibody test to the antigen is possible. In this study, evaluation of the levels of naturally acquired antibodies to TT in wild and domestic ducks from Australia, the immunogenicity of TT in Muscovy ducks, and interference by TT on H6- or TT-seroconversion in ducks given separate TT and H6N2 AI vaccines or combined TT/H6N2 vaccines was done. The results of the study supported the suitability of the TT marker for AI sero-surveillance in ducks.

Influenza virus encoded non structural protein NS1 protein is considered a virulence factor due to its ability to block cellular Interferon pathway. Several studies have shown the potential use of NS1 mutant viruses as vaccines for DIVA strategy, and the lack of antibodies against NS1 has been proposed as a DIVA marker. The NS1 is considered as virulence factor due to its ability to block the cellular interferon pathway. The amino-terminal end of NS1 has a double-stranded RNA binding domain, which inhibits the synthesis of IFN-/ by preventing the activation of dsRNA mediated activation of protein kinase R. The carboxyl-terminal end of NS1 contributes to the IFN-antagonistic properties possibly by enhancing NS1 stability and by binding cellular proteins involved in mRNA synthesis. Scientists have characterized an NS1 mutant virus (H5N3/NS1/144) having truncated carboxyl termini of NS1 protein, evaluated its potential use as a live vaccine candidate and its ability to revert to virulence. Within five back passages in chickens, H5N3/NS1/144 reverted to wild-type phenotype, making H5N3/NS1/144 an unsafe live vaccine candidate. Alternatively, the killed form of H5N3/NS1/144 induced similar levels of protection as that of the wild-type H5N3 virus and could be used as DIVA vaccine.

Foot and Mouth Disease virus

Although vaccination offers many advantages in control of FMD, international trade regulations put a heavy penalty on the use of FMD vaccines in the form of import/export restriction of animals and their products. However, after 2001 FMD outbreak in UK, members of European Union have also made provision for emergency vaccination in case of

risk for a future outbreak for those areas where livestock density is very high. Therefore, DIVA strategy is equally important both for countries which are endemic in FMD, where large number of carriers exist and where vaccination is practiced, and for those countries which are supposed to use vaccination in face of an outbreak. There are three basic tools for DIVA strategy for FMDV: virus isolation, NSP serology and detection of viral nucleic acid by PCR.

Due to the low virus titer, intermittent nature of virus recovery and the possible presence of neutralizing antibodies in OPF most of the times, the first two methods do not seem to be suitable for DIVA strategy. The current FMD vaccine used worldwide is an inactivated whole virus particle mixed with an adjuvant. When animals are immunized with such a vaccine, they mount antibody response only against structural proteins of the virus. When an animal becomes infected, antibodies also develop against NSPs (viral polymerases and proteases) of the virus because virus replicates inside host. The conventional diagnostic system detects antibodies only against structural proteins and, therefore, not suitable for DIVA strategy. Detection of antibodies against NSPs might be a tool for DIVA strategy. Immunized animals that are infected and subsequently become carriers will also develop antibodies against NSPs, allowing carriers to be identified in vaccinated stock.

i) Non Structural Protein Serology in detection of FMD Carriers: The agar-gel immuno-diffusion (AGID) test using virus infection-associated antigen (VIAA), isolated from virus cultures, was the first test to be developed. However, in later studies, investigators found that sera from multiple vaccinated animals and from some animals which have even received a single dose of vaccine had antibodies to VIAA. Such kind of immune response elicited by vaccination usually disappear 60-90 days post vaccination as detected by AGID or 90-180 days post-vaccination when 3D-ELISA is used. The reason behind antibodies against NSPs in vaccinated animals is that the FMD vaccines are not purified enough and depending upon the manufacturer, contain various amounts of contaminating NSPs.

Antibody response to NSPs is variable; the response to 3A, 3B, 3D and 3ABC could be detected in cattle as early as 7-10 days post-infection and up to 560-742 days post infection. Although 3D protein is not the best choice to differentiate vaccinated from infected animals, it was shown to be the most antigenic NSP. Further, the 3D ELISA is more sensitive than others but less specific. MacKay *et al*. (1998) discovered that 3ABC is most reliable single indicator of infection (examining with bovine and ovine sera), immune response to 3ABC appeared early after infection and antibodies to 3ABC could be detected for longer

than antibodies to any other NSP. The 3A NSP generally induces a similar response; some animals fail to react against 3B, whilst 3C alone is very weak immunogen. Detection of antibodies to one or more of the NSPs 2C, 3A and 3AB in addition to those against 3ABC, provides further confirmation to infection. The 2C is usually absent in sera from multiple vaccinated animals which is explained by the association of this viral protein with cellular debris that can be separated from the virus harvest prior to inactivation of the supernatant for vaccine. Antibodies to 2C could be detected in cattle up to 365 days after infection. ELISA based assays with various NSPs produced by recombinant baculovirus, in *E. coli* , insect larva or synthetically produced peptides to NSPs have been developed.

There are also three commercial tests to detect antibodies against NSP (3ABC); United Biomedical, Inc., New York, Cedi-Diagnostics B.V., The Netherlands and SYANOVA Biotech AB, Sweden. These tests are also not completely validated. Furthermore, all these tests are very expensive to be afforded by developing countries.

DIVA tests rely on polyclonal or monoclonal hybridoma derived antibody reagents, which can be difficult to prepare and maintain in a quality-assured manner and the quantities required for post-outbreak surveillance. To overcome this problem, recently Foord *et al*., (2007) generated phage display libraries of recombinant antibody single chain variable fragments against FMDV. Recombinant antibodies can be produced in large quantities at low cost in bacteria to guarantee the supply of a consistent and well-characterized reagent. The production of recombinant antibodies does not rely on animal immunization and does not require the maintenance of viable hybridoma cell lines.

ii) Real Time PCR: Due to poor precision, low sensitivity, short dynamic range ($<2 \log_{10}$), low resolution, absence of automation, only size based discrimination, absence of expression of results in numbers, poor quantitative performance (Ethidium bromide for staining in conventional PCR is not very quantitative) and post PCR processing, the conventional PCR is not suitable for accurate diagnosis and Real Time PCR eliminate all these problems. The greatest problem facing the diagnostic application of PCR is the production of false positive results. They are attributable to contamination by nucleic acids, particularly from the previously amplified material (carry over). Any contaminant, even the smallest airborne remnant carried over from the previous PCR procedure or from a strongly positive sample (contamination), may be multiplied and produce a false positive results. In Real time PCR, the problem of carry-over is significantly reduced because of the real time measuring principle, which is based on closed tube system.

The Real Time PCR has also been applied for FMDV and it is capable of detecting all seven serotypes. It has a high sensitivity (97%) and specificity for detection of FMDV genome in all seven serotypes. Viral RNA can be detected in oral and nasal samples from experimentally infected animals 24-96 hours before the onset of clinical signs. In future, use of robotic extraction of RNA will increase the throughput of samples whilst reducing the timescale for the issue of laboratory diagnostic results. Due to its very high sensitivity as compared to virus isolation and conventional PCR it is used as an adjunct to NSP serology. However, it is too expensive particularly for mass screening and needs specialized laboratory.

iii. IgA ELISA as a complementary to DIVA strategy: A promising complement to NSP serology for DIVA strategy is the assessment of IgA mucosal antibody response to FMDV. The FMDV vaccinated cattle do not mount a strong IgA response in saliva as compared to those which are following infection. Since IgA ELISA on saliva samples is faster and allows a higher throughput than virus isolation and PCR test on probang samples, it may efficiently complement the NSP based tests in vaccinated population. But this test is not reproducible and the titer of IgA in saliva varies from time to time during sampling. The titer may also be influenced by the current water or food intake by the animal at the time of sampling.

iv. Simultaneous Detection and Quantification of antibodies to multiple NSPs: In order to reduce the number of false positives samples, EITB assay has been used as a confirmatory test (Bergmann *et al*., 2000). This test uses several NSPs (2C, 3A, 3B, 3ABC and 3D) which are subjected to Western blotting. The membranes carrying the proteins are cut in strips, each strip is then incubated with bovine serum samples and the immune reaction detected by enzymatic action. However, the EITB assay is highly subjective and prone to human errors in its interpretation and also it is time consuming, thereby, limiting its use as a reliable confirmatory test.

Using the micro array or protein suspension array based system, recently Clavijo *et al*. (2006) developed a multiplex bead immunoassay to test simultaneously with a single sample, the immune response to FMDV NSPs 3ABC, 3A, 3B and 3D from experimentally infected and vaccinated cattle. The purified proteins were coupled to microspheres labeled with anti-His monoclonal antibody with different proportions of red and orange fluorescent dyes and reacted against serum specimens. Antibodies reacting against different NSPs, and thus, the different colored beads were detected by use of the Luminex system. This multiplex bead immunoassay can detect the immune response to NSPs in cattle as early as 7 days post-infection.

Marker vaccines and diagnostic tests have proved invaluable in disease eradication and control programs worldwide. Further research is required to have additional marker vaccines and companion test kits for animal pathogens, especially exotic diseases for developing effective strategies for control programmes and disease eradication.

References:

Brahmakshatriya, V.R., Lupiani , B. and Reddy, S.M. 2010. Characterization and evaluation of avian influenza NS1 mutant virus as a potential live and killed DIVA(differentiating between infected and vaccinated animals) vaccine for chickens. *Vaccine*.

Dong, X.N. and Chen, Y. H. 2007. Marker vaccine strategies and candidate CSFV marker vaccines. *Vaccine* **25**: 205–230.

Foord, A. J., Muller, J. D., Yu, M., Wang, L. F., Heine, H. G. 2007. Production and application of recombinant antibodies to foot-and-mouth disease virus non-structural protein 3ABC. *J Immunol Methods* **321**: 142-151.

James, C.M., Foong, Y.Y., Mansfield, J.P., Vind, A.R., Fenwick, S.G and Ellis, T.M. 2008. Evaluation of a positive marker of avian influenza vaccination in ducks for use in H5N1 surveillance. *Vaccine* **26**:5345-5351.

Kitching R.P., Hutber A.M. and Thrusfield M.V. 2005. A review of foot-and mouth disease with special consideration for the clinical and epidemiological factors relevant to predictive modelling of the disease. *Vet. J.* **169:** 197–209.

Kumar, N., Sharma, R. and Kakker, N. K. 2007. Non-structural Protein 3A for differentiation of foot-and-mouth disease infected and vaccinated animals in Haryana (India). *Zoonoses and Public Health* **54:** 376-82.

Maas, A. , Meens, J., Baltes, N., Hennig-Pauka, I. and Gerlach, G.F. 2006. Development of a DIVA subunit vaccine against *Actinobacillus pleuropneumoniae* infection. *Vaccine* **24**:7226-7237.

MacKay D.K.J., Forsyth M.A., Davies, P.R. and Salt, J.S. 1998. Antibody to the non-structural proteins of foot-and-mouth disease virus in vaccinated animals exposed to infection. *Vet. Q.* **20** (Suppl.2): 1-9.

Van Drunen Littel-van den Hurk, S. 2006. Rationale and perspectives on the success of Vaccination against bovine herpesvirus-1. *Vet. Microbiol*. **113:** 275-282.

Yang M., Clavijo A., Li M., Hole K., Holland H., Wang H., Deng M. Y. 2007. Identification of a major antibody binding epitope in the non-structural protein 3D of foot-and-mouth disease virus in cattle and the development of a monoclonal antibody with diagnostic applications. *J. Immunol Methods* **321**:174-81.

DEVELOPMENT OF HUMORAL IMMUNE RESPONSE AGAINST INFECTIOUS DISEASES

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The two major components of the acquired immune system recognize two distinctly different forms of foreign antigens. Some antigens (exogenous) grow in extra cellular fluids and are destroyed by antibodies which are the products of humoral immune response. Other invaders grow inside cells and are destroyed by the products of T cell-mediated immune response. Antibodies are produced by B lymphocytes. This article discusses structure of B-cell receptor (BCR), response of B cells with the antigen, peptide presentation by B cell to T helper cell, activation of B cells and antibody secretion by plasma cells.

B-cell receptor (BCR) and Accessory Molecules

BCR can be regarded as membrane-bound antibodies. BCR confer antigenic specificity on B cells; proliferation of antigen-specific B-cell clones is elicited by the interaction of membrane antibody with antigen. B-cell receptor (BCR) is a transmembrane protein complex composed of membrane immunoglobulin (mIg) and disulfide-linked heterodimers called Igα/Igβ. Molecules of this heterodimer associate with a mIg molecule to form a BCR complex. mIg are usually membrane IgM and IgD, the antigen receptors of naive B cells, which have very short cytoplasmic tails. The cytoplasmic tail is too short to transduce signals. The Igα/Igβ molecules are required for signal transduction and surface
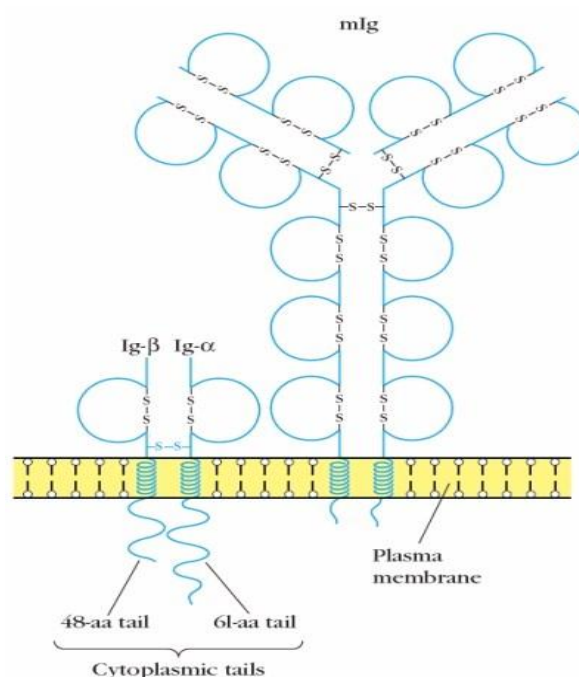
expression of membrane Ig molecules. Antigen-induced clustering of receptors delivers signals to the B cells that initiate the process of B cell activation. The receptor binds to the antigen and internalizes it into endosomal vesicles, and if the antigen is a protein, it is processed into peptides that are presented on the B cells by MHC II molecules for recognition of helper T cells.

Nature of antigens:

- ❖ **Protein antigens (T-dependent or TD Ags)** require Ag specificCD4+ T cells to activate B cells in order to produce antibodies.
- ❖ **Polysaccharides, glycolipids and nucleic acids(T-I antigens)** do not require Ag-specific T helper cell for activation of B cell in order to produce antibodies.

B cell response to TD antigens (T cell dependent antigen)

MHC class II antigen presentation: Once an antigen is internalized, it is degraded into peptides within compartments of the endocytic processing pathway. Internalized antigens take 1–3 h to transverse the endocytic pathway and appear at the cell surface in the form of peptide–class II MHC complexes. The endocytic pathway appears to involve three increasingly acidic compartments: early endosomes (pH 6.0–6.5); late endosomes, or endolysosomes (pH 5.0–6.0); and lysosomes (pH 4.5–5.0). Internalized antigen moves from early to late endosomes and finally to lysosomes, encountering hydrolytic enzymes and a lower pH in each compartment. Lysosomes, for example, contain a unique collection of more than 40 acid-dependent hydrolases, including proteases, nucleases, glycosidases, lipases, phospholipases, and phosphatases. The antigen is degraded into oligopeptides (13– 18 residues) which bind to class II MHC molecules. Because the hydrolytic enzymes are optimally active under acidic conditions (low pH), antigen processing can be inhibited by chemical agents that increase the pH of the compartments (e.g., chloroquine) as well as by protease inhibitors (e.g., leupeptin).

When class II MHC molecule are synthesized within the RER, three pairs of class II αβ chains associate with a preassembled trimer of a protein called **invariant chain (Ii, CD74).** This trimeric protein interacts with the peptide-binding cleft of the class II molecules, preventing any endogenously derived peptides from binding to the cleft while the class II molecule is within the RER. The invariant chain also appears to be involved in the folding of the class II α and β chains, their exit from the RER, and the subsequent routing of class II molecules to the endocytic processing pathway from the trans-Golgi network.

The class II MHC–invariant chain complexes are transported from the RER, where they are formed, through the Golgi complex and trans-Golgi network, and then through the endocytic pathway to lysosomes. As the proteolytic activity increases in each successive compartment, the invariant chain is gradually degraded. However, a short fragment of the invariant chain termed CLIP (for *class II–associated invariant chain peptide*) remains bound to the class II molecule after the invariant chain has been cleaved within the endosomal compartment. CLIP physically occupies the peptide-binding groove of the class II MHC molecule, presumably preventing any premature binding of antigenic peptide. Once a peptide has bound, the peptide–class II complex is transported to the plasma membrane, where the neutral pH appears to enable the complex to assume a compact, stable form.
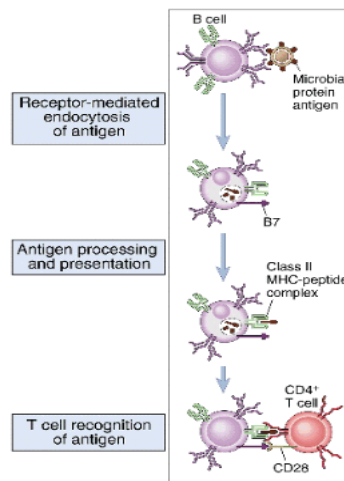
Presentation of protein antigens by B cell to helper T cells

T$_H$ Cells Play Essential Roles in Most B-Cell Responses: Binding of TD antigen to B-cell mIg does not itself induce an effective competence signal without additional interaction with membrane molecules on the TH cell. In addition, a cytokine-mediated progression is required for B-cell proliferation. This process is considerably more complex than activation induced by thymus independent (TI) antigens.

Formation of T-B conjugate: After binding of antigen by mIg on B cells, the antigen is internalized by receptor-mediated endocytosis and processed within the endocytic pathway into peptides. Antigen binding also initiates signalling through the BCR that induces the B cell to up-regulate a number of cell-membrane molecules, including class II MHC molecules and the co-stimulatory ligand B7. Increased expression of both of these membrane proteins enhances the ability of the B cell to function as an antigen-presenting cell in TH-cell

activation. B-cells could be regarded as helping their helpers because the antigenic peptides produced within the endocytic processing pathway associate with class II MHC molecules and are presented on the B-cell membrane to the TH cell, inducing its activation. It generally takes 30–60 min after internalization of antigen for processed antigenic peptides to be displayed on the B-cell membrane in association with class II MHC molecules. Once a TH cell recognizes a processed antigenic peptide displayed by a class II MHC molecule on the membrane of a B cell, the two cells interact to form a T-B conjugate

Contact dependent help mediated by CD40/CD40L interaction: Formation of a T-B conjugate not only leads to the directional release of T_H-cell cytokines, but also to the up-regulation of CD40L (CD154), a TH-cell membrane protein that then interacts with CD40 on B cells to provide an essential signal for T-cell–dependent B-cell activation. Interaction of CD40L with CD40 on the B cell delivers a signal (signal 2) to the B cell that, in concert with the signal generated by mIg cross linkage (signal 1), drives the B cell into G1.
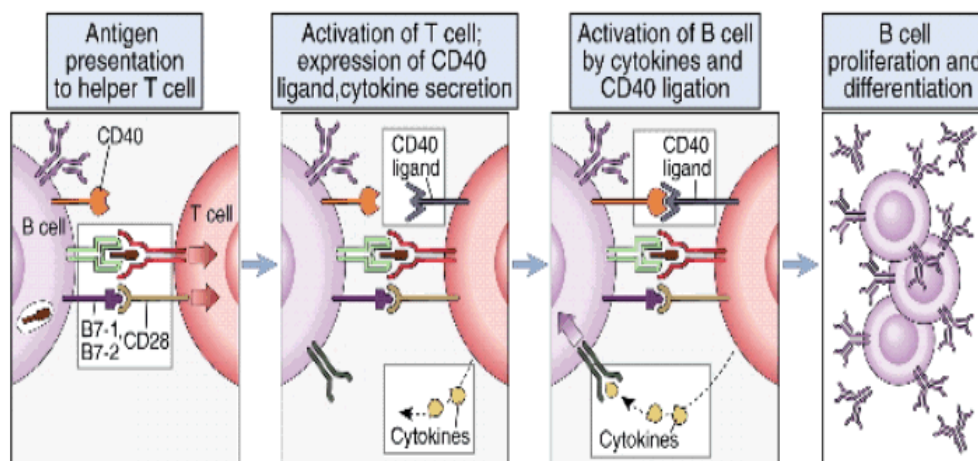
Signals provided by T_H cell cytokines: Although B cells stimulated with membrane proteins from activated TH cells are able to proliferate, they fail to differentiate unless cytokines are also present; suggesting that both a membrane-contact signal and cytokine signals are necessary to induce B-cell proliferation and differentiation. Once activated, the B cell begins to express membrane receptors for various cytokines, such as IL-2, IL-4, IL-5, and others. These receptors then bind the cytokines produced by the interacting TH cell. The signals produced by these cytokine-receptor interactions support B-cell proliferation and can induce differentiation into plasma cells and memory B cells, class switching, and affinity maturation.

Transduction of Activating Signals Involves Igα/Igβ Heterodimers

All isotypes of mIg have very short cytoplasmic tails. Both mIgM and mIgD on B cells extend into the cytoplasm by only three amino acids; the mIgA tail consists of 14 aa; and the mIgG and mIgE tails contains 28 aa. The discovery that membrane Ig is associated with the disulfide- linked heterodimer Igα/Igβ, forming the BCR, solved this puzzle. The BCR is functionally divided into the ligand-binding Ig molecule and the signal-transducing Igα/Igβ heterodimer. The Igα chain has a long cytoplasmic tail containing 61 amino acids; the tail of the Igβ chain contains 48 amino acids. The cytoplasmic tails of both Igα and Igβ contain the 18-residue motif termed the **immunoreceptor tyrosine-based activation motif** (**ITAM**). Interactions with the cytoplasmic tails of Igα/Igβ transduce the stimulus produced by cross linking of mIg molecules into an effective intracellular signal. The cross linking of BCRs results in the induction of many signal-transduction pathways. These include:

Compartmentalization of function within receptor subunits: Both the B-cell and T-cell pathways begin with antigen receptors that are composed of an antigen binding and a signalling unit. The antigen-binding unit confers specificity, but has cytoplasmic tails too short to transduce signals to the cytoplasm of the cell. The signalling unit has long cytoplasmic tails that are the signal transducers of the receptor complex.

Activation by membrane-associated Src protein tyrosine kinases: The receptor-associated PTKs (Lck in T cells; Lyn, Blk, & Fyn in B cells) catalyze phosphorylations (early stages of signal transduction0, essential to the formation of a functional receptor signalling complex.

Assembly of a large signalling complex with PTK activity: The phosphorylated tyrosine in the ITAMs of the BCR and TCR provide docking sites for the molecules that endow these receptors with PTK activity; ZAP-70 in T cells and Syk in B cells.

Recruitment of other signal-transduction pathways: Signals from the BCR and TCR result in the production of the second messengers IP3 and DAG. IP3 causes the release of Ca2+ from intracellular stores, and DAG activates PKC. A third important set of signalling pathways are those governed by the small G proteins Ras and Rac that are also activated by signals received through the TCR or BCR.

Changes in gene expression: One of the important outcomes of signal-transduction processes set in motion with engagement of the BCR or the TCR is the generation or translocation to the nucleus of active transcription factors that stimulate or inhibit the transcription of specific genes.

The B-Cell Co-receptor Complex Can Enhance B-Cell Responses

Stimulation through antigen receptors can be modified significantly by signals through co-receptors. In B cells a component of the B-cell membrane, called the **B-cell co-receptor,** provides stimulatory modifying signals. The B-cell co-receptor is a complex of three proteins: CD19, CR2 (CD21), and TAPA-1 (CD81).

CD19, a member of the immunoglobulin superfamily, has a long cytoplasmic tail and three extracellular domains. The CR2 component is a receptor of C3d, a breakdown product of the complement system, which is an important effector mechanism for destroying invaders. It also functions as a receptor for a membrane molecule and the transmembrane protein TAPA-1. In addition to the stimulatory co-receptor, another molecule, CD22, which is constitutively associated with the B-cell receptor in resting B cells, delivers a negative signal that makes B-cells more difficult to activate. The CR2 component of the co-receptor complex binds to complement-coated antigen that has been captured by the mIg on the B cell. This cross links the co-receptor to the BCR and allows the CD19 component of the co-receptor to interact with the Igα/Igβ component of the BCR. CD19 contains six tyrosine residues in its long cytoplasmic tail and is a major substrate of the PTK activity that is mediated by crosslinkage of the BCR. Phosphorylation of CD19 permits it to bind a number of signalling molecules, including the protein tyrosine kinase Lyn. The delivery of these signalling molecules to the BCR complex contributes to the activation process, and the co-receptor complex serves to amplify the activating signal transmitted through BCR.

Plasma cells: plasma cells (PCs) develop from antigen-stimulated B cells. These cells are found in greatest numbers in the spleen, the medulla of lymphnodes and in the bone marrow. PCs are ovoid cells and have a round, eccentrically placed nucleus with unevenly distributed chromatin. The nucleus may resemble a clock face or cartwheel. PCs have an extensive cytoplasm that is rich in rough endoplasmic reticulum and also have a large, pale-staining Golgi apparatus. PCs can make and secrete up to 10,000 molecules of antibodies per second. The antibody secreted by PCs is identical to its original BCR on its parent B cell. Most PCs are terminally differentiated and do not divide. Their life spans vary from a few days to many months, although most die within few days.

References:

Kuby, J: Immunology, WH freeman and Co., New York.
Tizard, I.R. (2004): Veterinary Immunology: An Introduction. 7th Edition, Elsevier (USA).

OIE RECOMMENDED TESTS FOR DIAGNOSIS OF ANIMAL DISEASES

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With the advancement of research in veterinary microbiology and increasing application of modern techniques of medical microbiology in the diagnosis of animal diseases, most of the economic diseases of veterinary importance can be diagnosed. These techniques mainly target the detection of antigen, antibody or quite recently genomic nucleic acids (RNA/DNA).

Antigen detection remains one of the most confirmatory tests if the sample has been collected from an animal in the active (peracute/acute) phase of the clinical disease. In contrast, antibody detection tests are used to diagnose those infectious veterinary diseases where measurable (in terms of quantity) antibodies are produced in response to any microbe. These tests usually give positive reaction only after a minimum of one week after infection when antibodies are at a level where they can be detected. The monoclonal antibody (MoAb) based enzyme linked immunoassays (ELISAs) have tremendous potential for use in the diagnosis of infectious diseases of animals. In addition, molecular biology based diagnostic methods are increasingly being applied in the diagnosis of infectious animal diseases. Most of these methods are easy to perform in the lab, safe, sensitive, reproducible and automated to facilitate the processing of large number of samples.

Immunoassays Recommended by OIE for the Diagnosis of Animal Diseases:

The Office International des Epizooties (OIE), Paris, France, an international body for monitoring of animal diseases particularly, international movement of animals, their germ plasm and products, have listed the diseases of veterinary importance. The description of these diseases also includes the diagnostic tests for the diagnosis of these diseases in two categories: 'prescribed' and 'alternative'. The prescribed tests are those that are required by the International Animal Health Code for the testing of animals before they are moved internationally. The alternative tests are those that are suitable for the diagnosis of the diseases with in a local setting, and can also be used in the import/export of animals after bilateral agreement. The serological tests recommended by the OIE and listed in its terrestrial Manual (OIE, 2008) for various diseases of veterinary importance are given in the Table 1.

Table 1: Prescribed Immunoassays for some important Veterinary Diseases for International Trade of Animals.

S.No.	Name of the microbe/Disease	Prescribed test(s)	Alternative test(s)
1.	Aujeszky's disease	ELISA, VN	-
2.	Bluetongue	AGID, ELISA, PCR	VN
3.	Foot and mouth disease	ELISA, VN	CF
4.	Paratuberculosis (Johne's disease)	-	DTH, ELISA
5.	Q fever	-	CF
6.	Rabies	ELISA, VN	-
7.	Rift Valley fever	VN	ELISA, HI
8.	Rinderpest	ELISA	VN
9.	Trichinellosis	-	ELISA
10.	Vesicular stomatitis	CF, ELISA, VN	-
11.	Avian infectious bronchitis	-	ELISA, HI, VN
12.	Avian infectious laryngotracheitis	-	AGID, ELISA, VN
13.	Avian influenza	-	AGID, HI
14.	Avian mycoplasmosis	-	Agg, HI
15.	Fowl typhoid and Pullorum disease	-	Agg
16.	Infectious bursal disease	-	AGID, ELISA
17.	Marek's disease	-	AGID
18.	Newcastle disease	-	HI
19.	Bovine anaplasmosis	-	CAT, CF
20.	Bovine babesiosis	-	CF, ELISA, IFA
21.	Bovine brucellosis	BBAT, CF, ELISA, FPA	-
22.	Contagious bovine pleuropneumonia	CF, ELISA	-
23.	Enzootic bovine leukosis	AGID, ELISA	-
24.	Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis	ELISA, VN	-
25.	Malignant catarrhal fever	-	IFA, VN
26.	Theileriosis	IFA	-
27.	Trypanosomosis (Tsetes-transmitted)	-	IFA
28.	African horse sickness	CF, ELISA	VN
29.	Dourine	CF	ELISA, IFA
30.	Equine encephalomyetitis (Eastern and Western)	-	CF, HI, PRN
31.	Equine infectious anaemia	AGID	ELISA
32.	Equine influenza	-	HI
33.	Equine piroplasmosis	ELISA, IFA	CF
34.	Equine Rhinopneumonitis	-	VN
35.	Equine viral arteritis	VN	-

36.	Glanders	CF	-
37.	Venezuelan equine encephalomyetitis	-	CF, HI, PRN
38.	Myxomatosis	-	AGID, CF, IFA
39.	Rabbit haemorrhagic disease	-	HI
40.	Caprine and ovine brucellosis	BBAT, CF	FPA
41.	Caprine arthritis/ encephalitis and Maedi-visna	AGID, ELISA	-
42.	Contagious caprine pleuropneumonia	CF	-
43.	Enzootic abortion of ewes	-	CF
44.	Ovine epididymitis	CF	ELISA
45.	Peste des petites ruminants	VN	ELISA
46.	Sheep pox and goat pox	-	VN
47.	African swine fever	ELISA	IFA
48.	Classical swine fever	ELISA, FAVN, NPLA	-
49.	Porcine brucellosis	ELISA	BBAT, FPA
50.	Swine vesicular disease	VN	ELISA
51.	Transmissible gastroenteritis	-	VN, ELISA

Agg.	Agglutination test	HI	Haemagglutination inhibition
AGID	Agar gel immunodiffusion	IFA	Indirect fluorescent antibody
BBAT	Buffered *Brucella* antigen test	IPMA	Immunoperoxidase monolayer assay
CAT	Card agglutination test	MAT	Microscopic agglutination test
CFT	Complement fixation test	NPLA	Neutralising peroxidase-linked assay
ELISA	Enzyme linked immunosorbent assay	PRN	Plaque reduction neutralisation
FAVN	Fluorescent antibody virus neutralization	VN	Virus neutralisation
FPA	Fluorescence polarisation assay	-	No test designated yet

RAPID 'PENSIDE' OR 'POINT- OF- CARE' DIAGNOSTIC TESTS FOR VIRAL DISEASES OF DOMESTIC ANIMALS

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Definitive diagnosis of animal viral disease involves a combination of methods, including isolation & identification of the viral agent, detection of viral antigen(s) or nucleic acid and host's virus-specific immune response(s) or biomarkers of infection and/or disease. In more than its century-old history, viral diagnostics has evolved from classical serological tests (precipitation, complement fixation test, hemagglutination test, fluorescent antibody technique, etc) to isolation of the agent in embryonated eggs and cell culture to modern immunoassays (radioimmunoassay, enzyme immunoassays) and immune-blotting to modern molecular tests (blot hybridization, polymerase chain reaction (PCR), real-time PCR, etc.) and biosensors.

Progress in viral diagnostics through the decades has been made with the aims either to develop various tests with improved sensitivity, specificity and predictive values or to make rapid, simple, cheap and field- adaptable 'penside' or 'point-of-care' tests. These developments have led to availability of several tests for diagnosis of clinical cases, epidemiological surveys, implementation of disease control programme and vaccination monitoring. Tests differentiating infected from vaccinated animals (DIVA) have been specially developed for use where disease control by vaccination is implemented and for export of animals from endemic region. Modern immunoassays and molecular diagnostic tests in a variety of formats are the mainstay of viral disease diagnostics at present. But, virus isolation in cell culture has still not lost its essential role. Biosensors, nanotechnology-based and biomarkers-based diagnostics exploiting modern biotechnological innovations are still the tests of future (Schmitt and Henderson 2005).

Availability of rapid, sensitive and specific 'on-the-spot' or 'point-of-care' or 'penside' diagnostic tests can be of immense importance for effective and early containment of emergency problems arising from outbreaks of fast-spreading viral diseases in unvaccinated populations, trans-boundary diseases and natural disasters. In addition, the 'penside' tests, which are economical and simple to perform and read, can be useful as routine diagnostic tests in endemic areas. Interestingly, both immunodiagnostic such as latex agglutination test (LAT) and lateral flow immunoassay devices (LFDs), and molecular diagnostic tests such as portable real-time PCR and isothermal loop-mediated amplification

of DNA (LAMP) have been developed as 'on-the-spot' tests, at least at proof-of-concept level. Nano-technological innovations have great promise for providing novel devices and platforms used for development of 'penside tests'. However, only few 'penside' tests have been validated and many more are required to be validated for field applications. The present article reviews the field of 'penside' diagnostic tests for viral diseases of domestic animals.

Rapid 'penside' or 'point-of-care' diagnostic tests for viral diseases

To qualify as 'penside' or 'point-of-care' or 'on-the-spot', the diagnostic test should ideally have the following features: i). Rapid to provide results within minutes, rather than hours, ii). Simple to perform and read result, iii). Cost-effective, iv). Minimal processing of test samples, v). Durable reagents, particularly for applications in tropical countries, vi). Disposable and environmental risk-free, vii).Sensitive, viii).Specific, and ix). Validated for field applications. The 'penside' test can be classified as an immunodiagnostic, molecular diagnostic, biosensor or nanodiagnostic, depending on use of immunological reagents or molecular reagents, biosensor chip or nanomaterials in the test, respectively. 'Penside' tests using biosensors, nanochips and nanoarrays as platforms make the class of future diagnostics. Major diagnostic tests that have been and can be developed as 'penside' are described below:

I.   'Penside' immunodiagnostic tests:

1.   Latex agglutination test: Latex agglutination test (LAT) was first described during 1950s and since then has been developed for most of the infectious diseases. It can detect antigen (Ag) or antibody (Ab) depending on coating of specific Ab or Ag on the latex beads for agglutination reaction. The test is conveniently performed on cavity glass slide or plastic card by mixing the coated latex bead suspension with the test sample containing Ag or Ab. However, LAT is used as a rapid test for Ag detection in clinical sample, more often for infectious agents. The test is rapid i.e., result is declared within 15 min. It is simple to perform i.e., mixing of LAT reagent with clinical sample (serum, plasma, body fluids) after minimal processing and observing agglutination to occur within 5-10 min or so. It can be performed by semi-skilled personnel. The sensitivity and specificity of the test are generally of acceptable levels. Specificity can be further improved by using of monoclonal antibodies in the latex reagent. The cost of the test per sample is less than that of ELISA. The shelf life of the test is several months and it can be presented in a kit. LAT kits for detection of several viral agents have been made commercially available. List of LAT for selected viral agents reported in recent times is given in table 1.

Table 1. Latex agglutination test for detection of selected animal viruses

Virus name	Features & Application	Validation status	Reference
Pseudorabies (Aujeszky's disease in pigs)	gE coated latex suspension, suitable as DIVA test for gE deleted vaccine	Yes, test kit commercially available	Yong *et al.* 2005, Schoenbaum*et al.* 1990
Rotavirus	Rotavirus detection in stool/ fecal samples	*1. SlidexRota-Kit 2®* (BioMérieux) *2. Rotavirus Latex®* kit (Richmond Immunosystems Diagnostics)	i. Ferreira *et al.* 2006. ii. Paesi*et al.* 2012
Hydropericardium syndrome(adenovirus serotype 4)	Recombinant FAV4 protein coated latex suspension	For antibody detection	Kalaiselvi*et al.* 2010
FMDV	Virus infection associated Ag (VIA) or 56 kDa RNA-dependent RNA polymerase-coated latex suspension for FMDV Ab detection	For surveillance & quarantine of imported animals	Sugimura *et al.* 2010
Avian influenza virus	H5N1 subtype of avian influenza virus; Anti-HA MAbs covalently attached latex beads	For virus detection	Chen *et al.* 2007

2. **Lateral flow immunoassay device:** Lateral flow device (LFD) is one of the latest developments in immunoassay devices. The present LFDs work on the principle of lateral diffusion of Ag and gold-labelled Ab (Ab-Au) to make specific Ag-Ab-Au complex which further moves towards two zones: first of coated monoclonal Ab (mAb) to trap the Ag-Ab-Au complex and appear as brown band, and second zone of coated anti-AbmAb to trap the Ab-Au complex to give a brown band. Thus, brown band at both the zones indicate the presence of Ag in the sample and only one band at the second zone (negative control) indicates absence of Ag in the sample (Fig. 1). LFD is becoming popular because it is rapid, simple to perform and simple to read, sensitive and specific, minimal sample processing, good shelf-life, cost-effective and disposable. LFD has been developed for detection of several viral diseases of domestic animals and some of which validated as 'penside' test for field applications. List of LFDs for selected viral agents developed in recent past is presented in table 2.
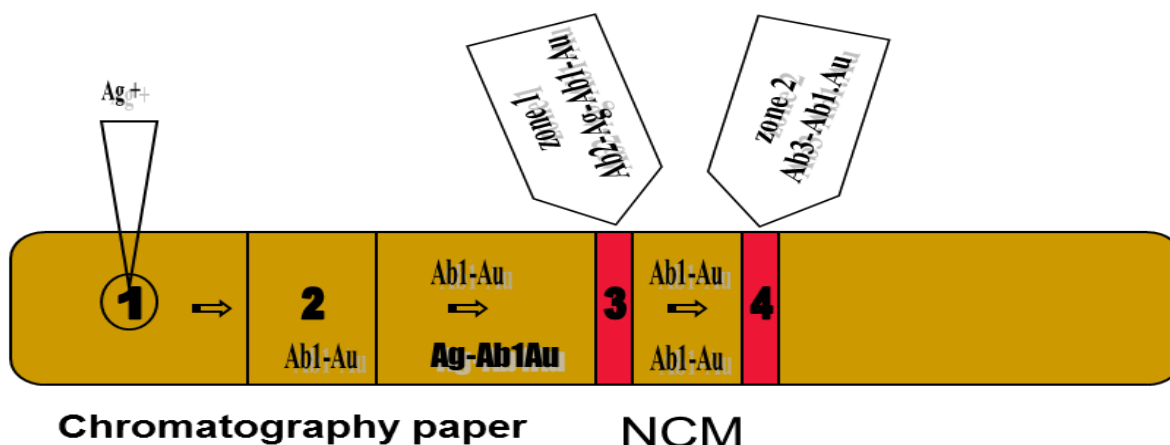
Fig. 1. Lateral flow immunoassay

Table 2. Lateral flow immunoassay device for detection of selected animal viruses			
Virus name	*Features & Application*	*Validation status*	*Reference*
FMDV serotypes O, A, C & Asia 1	Uses monoclonal Ab, For epithelial suspensions, Diagnostic sensitivity 87.%, diagnostic specificity 98.7%, suitable for field application	Proof-of-concept	Oem*et al.* 2009
Avian influenza virus	For virus and anti-viral antibody detection; mAb to nuclear protein epitope	Proof-of-concept	Nielsen *et al.* 2007

II.   'Penside' molecular diagnostic tests:

1.   Portable PCR: Polymerase chain reaction (PCR) is a molecular technique for specific amplification of DNA *in vitro* in several cycles of thermal denaturation of DNA strands (at ≥94°C), annealing with strand-specific oligonucleotide primers (at ≥45°C) and primer extension at 72°C with a thermo-stable DNA polymerase. Just a few copies (theoretically one) of DNA template originally present in any biological (or synthetic) material are exponentially increased to amounts detectable by fluorescence staining in agarose gel or other methods. The technique has revolutionized clinical microbiology laboratory practice, as its applications are far more than other techniques at present. Technical innovations have provided faster protocols for detection of the viral agents, particularly causing difficult-to-contain highly contagious and fast spreading diseases. Real-time PCR is a technique in which increase in DNA amplification level can be monitored in every next cycle for a number of cycles using fluorescent labelled probes (TaqMan[®], Molecular beacons[®] or FRET probes) or DNA intercalating fluorescent dye (SYBR Green).

Portable PCR and real-time PCR are briefcase-packaged battery-operated technologies with lyophilized reagents for detecting viruses in samples at the place of field outbreak. This has been developed particularly for early detection of viruses such as FMD virus, avian influenza virus and others of high potential risk of spread with aim to contain and effectively control the disease at the earliest. In combination with early warning system based on GPS, these technologies can prove extremely useful in countries, where particular disease control programmes are being implemented. These technologies have been tested under field conditions for detection of FMD virus and highly pathogenic avian influenza virus.

2.   **LAMP:** LAMP is a recently developed alternative to PCR for DNA amplification at isothermal conditions in a water bath, obviating the requirement of a thermal cycler machine. Secondly, it is faster and more sensitive than standard PCR. In addition, the results can be read more easily and instantly by colour change in the reaction tube or within 10-15 min in combination with LFD. Because of these features, LAMPs have been developed as portable platforms for detection of several different viruses (Table 3) and the list is fast growing.

Table 3. LAMP for detection of selected animal viruses			
Virus name	*Primers & target gene*	*Other features*	*Reference*
Swine influenza virus Subtypes H1, H3, H5 & H9	6 degenerate primers targeting the conserved gene of SIV polymerase A	Results within 45 min. at 63°C.	Liu *et al.* 2012
Infectious bursal disease virus	RT-LAMP of VP5 gene using six primers	Sensitivity equal to RT-PCR, visual detection, *TfiI* site for discrimination of very virulent (vv)/ non-vv strains, useful for field studies	Wang *et al.* 2011
Porcine circovirus 2	ORF2 gene	55 min 59°C, 100x sensitive than PCR	Zhao *et al.* 2011
Tembusu related flavivirus	RT-LAMP of E gene using 6 primers	50 min 63°C, 100x sensitive than PCR, visual result	Zhao *et al.* 2011
Orf virus	B2L gene using 6 primers	100x sensitive than PCR, products digested with BsrGI	Tsai *et al.* 2009
Classical swine fever virus	RT-LAMP using 6 primers for genomic RNA	60 min 63°C/65°C, 100x sensitive than RT-PCR, visual result	Chen *et al.* 2010,Wongsawat*et al.* 2011.
Foot-and-Mouth disease virus serotype Asia 1 & others	RT-LAMP serotype Asia 1 & for others	45 min at 61°C, field application, Portable LAMP platform evaluated	Chen *et al.* 2011, Ebert *et al.* 2011, Dukes *et al.* 2006,
Equidherpesvirus 1 and 4	LAMP for gC and gE genes of EHV-1 and gC gene of EHV-4	60 min 63°C, 0.1 pfu/tube sensitivity	Nemoto*et al.* 2010
Porcine teschovirus (Teschen disease)	RT-LAMP for RNA-dependent RNA polymerase gene	60 min 65°C, direct visualization, 10x higher sensitivity than PCR	Wang *et al.* 2011.
African Swine Fever Virus	Six primers in RT-PCR	Portable LAMP platform evaluated	James *et al.* 2010, Ebert *et al.* 2011

III. Biosensors:

According to IUPAC, an electrochemical biosensor is defined as 'a self-contained integrated device that allows for specific analytical detection by using a biological recognition element (a biochemical receptor) in direct spatial contact with a transduction element'. It differs from a bioanalytical system because of the direct conversion of biological events to electronic signals and detected directly, rapidly & conveniently. Reflectometry, fluorimetry, resonance, fibre-optic biosensors, etc. are being developed for multianalyte detection in an automated format. Potential applications of biosensors include detection of bioterrorism agents and exotic viral agents (Schmitt and Henderson 2005).

IV. Nanodiagnostics:

Nanotechnology is defined as the systems or devices based on properties of materials of nanometre scale. Over the past two decades, nanomaterials, such as quantum dots, nanoparticles, nanobarcodes, nanowires, nanotubes, nanogaps, and nanoscale films have been found to possess suitable properties for designing novel nanoscale biosensors for nucleic acids, proteins, viruses, and cells (Schmitt and Henderson 2005). Silicon nanowire field-effect transistors (SiNW-FETs) have recently proven useful tool because of their very high sensitivity, selectivity, and label-free and real-time detection capabilities. SiNW-FETs are employed in the detections of proteins, DNA sequences, small molecules, cancer biomarkers, and viruses (Chen *et al.* 2011). Nanotechnology is infantile in the field of diagnostics at present, but may find its ways for detection of bioterrorism agents and subsequently endemic viral diseases in future.

Conclusions and future directions:

Development and availability of validated 'penside'diagnostic tests is highly desired for early management of clinical cases and outbreaks and effective containment of major viral diseases of animals such as foot-and-mouth disease, avian and swine influenza virus, classical swine fever, African swine fever, peste des petit ruminants (PPRV),bioterrorism agents, etc. While, LFDs and LATs are simple, rapid and cheap tests, molecular tests such as real-time PCR and LAMP have been developed as portable platforms for highly sensitive and specific detection of viral nucleic acids of some important diseases including FMD and avian influenza. Proof-of-concept has been shown for development of 'penside' tests based on biosensor, nanochip and nanoarray platforms and some of the 'penside' tests might be validated for field applications in not-so-distant future.

Bibliography:

Chen, H. *et al*. 2011. *Virol. J.* **8**:489-491.

Chen J. *et al.* 2007. *J. Vet. Diagn. Invest.* **19**:155–160.

Chen K.I.*et al.* 2011. *Nano Today* **6**: 131-154.

Chen L. *et al.* 2010. *Virologica Sinica* **25**: 59-64.

Dukes J.P. *et al.* 2006. *Arch. Virol.* **151**: 1093.

Ebert K. *et al.* 2011. Optigene poster downloaded from internet.

Ferreira T.L. *et al.* 2006. *Brazilian J. Microbiol.* **37**:587-589.

James H. J. *et al.* 2010. *J. Virol. Methods.* **164**: 68.

Kalaiselvi G *et al.* 2010. *Veterinarski Arhiv* **80**: 743-752.

Liu Y. *et al.* 2012. *J. Food Agri. & Environ.* **10**: 196-200.

Mori Y. andNotomi T. 2009. *J. Infect. Chemother* **15**:62–69.

Nemoto M. *et al.* 2010. *J. Vet. Diagn. Invest.* **22**:30–36.

Nielsen K. *et al.* 2007. *J. Immunoassay Immunochemistry.* **28**: 307—318.

Oem J.K. *et al.* 2009. *Clin.Vacc.Immunol.* **16**:1660–1664.

OIE 2012.Aujeszky's Disease. Chapter 2.1.2. *OIE Terrestrial Manual* 2012, pp.1-15.

Paesi S. 2012. *Acta Scientiae Veterinariae.* **40**: 1014.

Schmitt B. and Henderson L. 2005. *Rev. sci. tech. Off. int. Epiz.* **24**: 243-250.

Schoenbaum M.A. *et al.* (1990). *J. Vet. Diagn. Invest.* **2**: 29–34.

Tsai SM. *et al.* 2009. *J. Virol. Meth.***157:** 200–204.

Wang B. *et al.* 2011.*J. Vet. Diagn. Invest.* **23**: 516–518.

Wang Y. *et al.* 2011. *Virol. J.* **8**:108-114.

Wang Y. *et al.* 2011. *Virol. J.* **8**:553-559.

Wongsawat K. *et al.* 2011. *Health* **3**: 447-452.

Yong T. *et al.* (2005). *Vet. Res. Commun.* **29**: 487–497.

Zhao K. *et al.* 2011. *Virol. J.* **8**:126-132.

MANAGEMENT OF HERPES VIRUS INFECTION IN HORSES: DIAGNOSIS AND VACCINATION STRATEGIES

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Equine Herpes Virus (EHV), from the family Herpesviridae, is comprised of five distinct strains. EHV-1 &4 are most common. EHV-1 causes respiratory tract disease, abortion, neonatal mortality, and neurological disease while EHV-4 causes respiratory infection and scattered abortion. EHV-1 in particular is unique in its ability to target and attack three separate organ systems (respiratory, reproductive, and central nervous systems) of the horse. Once a horse contracts Equine Herpes Virus the animal has the virus for life. No method exists which will eradicate the disease because virus remains in latency (viral dormancy) in horses. The subtypes of herpes virus that commonly affect horses are:

1. Equine herpes virus type 1 (EHV-1): The virus formerly called equine viral rhinopneumonitis, causes respiratory disease, abortions, and neurological disease. The first report of EHV-1 induced abortions was confirmed on the basis of histopathology of aborted foetuses. Subsequently, abortion storm due to EHV-1 was reported in an organized equine breeding farm in 1989, where 15% (54/360) mares aborted with no warning signs. Abortions in mares were also recorded during 1996 and 1997.

2. Equine herpes virus type 2 (EHV-2) or equine cytomegalovirus: It is not known to cause clinical disease in horses with a normal immune system.

3. Equine herpes virus type 3 (EHV-3) or coital exanthema is a direct contributor to the development of equine coital exanthema, a disease contracted by way of direct sexual contact. It manifests by way of pustules and sores in the genital region of mares and stallion. EHV-3 has been isolated from Indian horses from Bangalore.

4. Equine herpes virus type 4 (EHV-4) is cited as the primary cause of rhinopneumonitis. In rare cases this form has been known to cause abortions. The neurological form of EHV is not associated with and almost never results from EHV-4. It has been isolated from Indian horses with high seropositivity (82%).

5. Equine herpes virus type 5 (EHV-5) is a respiratory form of the disease which commonly affects foals and yearlings. It is largely isolated in foreign countries such as Switzerland, Germany, Australia and New Zealand. EHV-5 has not been isolated from India.

A. Strains of EHV-1 and their tissue reaction: Three distinct strains of EHV-1 are prevailing in nature. These strains have different tissue's predilection producing different pathogenicity in equines. These strains are as follows:

1.   EHV-1 Abortogenic Strain: Causes spontaneous abortion in a mare that becomes infected while pregnant. The virus also affects the foals, which subsequently become infected. These foals are sick immediately post-birth or become ill one to two days following. Generally, foals succumb quickly to EHV-1 and are largely unresponsive to medical interventions. Sadly, the end result is often death. This strain has been recovered from abortion and foal mortality cases in India.

2.   EHV-1 Respiratory Disease Strain: Leads to respiratory distress, namely upper respiratory tract infections and pneumonia. Contracting the virus can also result in encephalitis (inflammation of the brain). More commonly, infection results in significant destruction of the epithelium in the respiratory tract but is generally non-fatal. This strain is commonly referred to as rhinopneumonitis.

3.   **EHV-1 Myeloencephalitis Strain**: This strain is the neurological form, which is thought to be the result of a genetic mutation within the strain. Horses with this form become uncoordinated, weak, suffer from paralysis, and may have difficulty urinating and/or defecating. This form has an elevated level of related mortalities but should not be regarded as absolutely fatal. There is report of virus isolation from equine suffered from paralytic syndrome due to EHV-1 infection.

B. The Syndromes: Equine herpesvirus causes disease in three distinct syndromes:

1. Respiratory Infection. Also known as "rhinopneumonitis," this syndrome may be mild or inapparent in animals that have had previous infections or vaccination. A common manifestation of the respiratory effects of EHV is in younger horses (weanlings) in horse-dense areas. These outbreaks have generally been attributed to EHV-4. Symptoms include fever, serous nasal discharge, malaise, cough, submandibular and retropharyngeal lymph node swelling. Sometimes a "diphasic" fever is noted; the second phase of fever coincides with the advent of a cell-associated viremia, in which virus travels through the bloodstream inside lymphocytes. Secondary bacterial infections are common following primary infection.

2. Abortion. EHV infection generally is associated with late-gestation abortion. Abortion is sometimes noted several weeks to months after a clinical or subclinical infection with EHV-1, but often occurs with no impending signs.

3. Neurologic Disease. Neurologic signs are the noteworthy component of the recent EHV infections in the news and are significant to the affected individual animals, but are infrequent sequelae of EHV-1 infections. Specific strains of EHV-1 have been implicated when neurologic signs are associated with EHV. There is speculation that this syndrome occurs more frequently in mares that have weathered abortion storms, or in individuals that have experienced respiratory outbreaks. Symptoms may begin as mild incoordination or lameness, progressing to posterior paresis and paralysis, recumbency, and loss of tail and bladder function.

C. Modes of Transmission:

Equine Herpes Virus is transmitted via close proximity with and/or contact with a horse infected with EHV. The virus is shed from an infected horse by way of nasal secretions, commonly referred to as "nasal shedding." Nasal shedding typically occurs during the height of infection: the period where the horse presents elevated temperature. Shedding can continue from fourteen to twenty-one days after the fever has abated.

Generally, in horses that have not previously been infected, shedding of the virus persists for fourteen days. The virus is most communicable within the first few days of the respiratory tract infection. The most common mode of transmission is aerosol transmission, generally through saliva or nasal secretions which are transferred via direct contact or carried in the air and enter into the nasal passage/mouth of another horse. EHV can only be spread from horse to horse over limited distances, i.e. between stalls, paddocks, and fields; it cannot be spread over distances greater than approximately thirty-five feet. The viruses can become airborne by the coughing, snorting, blowing, etc. of an infected horse and can then travel to neighboring horses and infect them. The only way for EHV to infect a horse is through the mucous membranes of the upper respiratory tract.

EHV can also be spread by way of materials/objects used on an infected horse which consequently become contaminated. Individuals caring for and handling infected horses also serve as prime transporters of the virus, which can cling to clothes, shoes, gloves, etc.

Transmission from an EHV-1 infected mare to her foal is also observed. Whether transmission occurs in utero or immediately following birth has not been definitively established. The EHV virus, which is capable of lying dormant in a carrier horse, can become reactivated, thereby causing the virus to become fully activated and infectious.

D. Diagnosis of EHV Infection:

The latency and cell-related nature of EHV infection leads to diagnosis challenging. Generally, PCR or virus isolation is performed on nasopharyngeal swabs to confirm diagnosis. Virus isolation may also be attempted on buffy coat samples, but usually this requires examination during a narrow window of viremia. Neurologic forms of EHV infection are often presumptively diagnosed on clinical signs. Respiratory symptoms of EHV cannot be differentiated from those of influenza or other causes on the basis of clinical signs.

Virus neutralization, complement fixation, ELISA tests are used for paired serology on individuals especially for abortion cases to demonstrate fourfold or more rise in antibody titre. Recently blocking ELISA has also been developed for diagnosis of this disease. ELISA is used for both antibody and antigen detection. Demonstration of virus antigen in infected tissue by IPT, IFAT, etc. may also be used.

Monoclonal antibodies (Mabs) have been used for antigenic differentiation of EHV-1 isolates. ELISA and VN tests detected antigenic (epitope) differences in three EHV-1 isolates, out of six isolates tested with these Mabs. The Indian isolates of EHV-1 have been differentiated by DNA fingerprinting. Likewise, a neutralizing Mabs-based blocking ELISA has been developed for the detection of EHV-1 antibodies. The Scientists of NRCE have developed Equiherpes B-ELISA Kit and compared with conventional micro-VNT. The agreement between the two tests was 85.86% (1224 serum samples).

E. Vaccination:

Current vaccines generally are regarded to produce high levels of circulating antibody, but only provide partial stimulation of the cell-mediated immune system. Inactivated and live attenuated vaccines for EHV-1 and EHV-4 are available. An inactivated viral vaccine (Pneumabort-K®) is available for prevention of equine abortion. Currently there is no vaccine available for aid in prevention of the neurologic signs associated with EHV. In India, killed oil adjuvanted vaccine against EHV-1 has been developed as an alternative to Pneumabort 'K' incorporating indigenous EHV-1 strain (Hisar-90-7). This is and emulsified with monooleate. Efficacy of equine herpes virus-1 (EHV-1) vaccine was evaluated in experimental pregnant BALB/C mice (8 weeks old). None of these vaccinations confer protection from contracting EHV-1 or EHV-4, nor do they confer protection against the establishment of latent carrier state. However, despite their shortcomings, these vaccines diminish the presentation and severity of clinical symptoms.

F. Management Practices:

 EHV becomes latent in a high proportion of infected animals, which may be problematic. However, certain management procedures can limit the chances of animal shedding large amounts of virus.

1. Isolation: When new horses are purchased or added to a facility, or returning from an outside facility, a strict 3-4 week isolation period should be enforced.

2. Disinfection: Equipment, trailers, and other inanimate objects that were in contact with outside horses should be disinfected with the appropriate product. EHV is an enveloped virus, and a disinfectant should be chosen with the appropriate activity. One of the most effective classes of disinfectant against enveloped viruses is in the "aldehyde" family.

3. Stress reduction: Reducing stress-induced reactivation of the virus.

4. Vaccination: The horses should be appropriately vaccinated before exposure.

5. Management Practices: Maintaining horses in the smallest, closed, physically-separated groups as possible.

6. General Hygienic Measures: Enforcing the washing of hands, the use of footbaths to disinfect outer footwear, and insisting on the wearing of newly laundered outer clothing, e.g., coveralls, when entering the stable or barn. Washing of hands with soap and water, or by using an alcohol-based hand disinfectant after handling each horse.

7. Early diagnosis of disease, expedient quarantines, and strict adherence to hygienic practices are paramount.

8. Containment: In the event of an outbreak, authorities versed in quarantine and biosecurity protocol should be contacted to aid in containment efforts. Personnel employed on the premises should also be trained in these areas so as to create the most sterile and secure environment possible. By containing the infected horses effectively, the virus can be isolated, an essential component in preventing epidemics.

BLUETONGUE IN INDIA: MOLECULAR TECHNIQUES FOR EPIDEMIOLOGICAL STUDIES

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Bluetongue (BT) is an Office International Epizooties (OIE) 'Notifiable' (earlier list A), viral disease of domestic and wild ruminants. The virus is infectious but non-contagious, affecting domestic and wild ruminants. BT is an insect-borne viral disease, transmitted by midges of certain species of genus *Culicoides.* The causative agent of the disease is bluetongue virus (BTV), a species of the genus O*rbivirus* within the *Reoviridae* family. The BTV has 10 segmented dsRNA genome prone for easy reassortment and recombination.

The disease is characterized by high fever (42°C), hyperaemia, inflammation of the oral mucosa, tongue, coronary band and occasionally the nasal mucosa, erosions and ulceration of the dermis and laminitis. Severe oedema of the tongue can result in restricted blood flow and cyanosis. The swollen tongue may protrude from oral cavity giving the appearance of blue tongue. Sick animals may exhibit profuse salivation, depression, anorexia, weight loss due to muscle degeneration, stiffness of the limbs, lameness, and excessive nasal and ocular secretion followed by death in 8-10 days (Prasad *et al*., 2008).

Based on neutralization test, 24 serotypes of BTV have been reported worldwide. However, recently, two more serotypes of BTV have been reported i.e. BTV serotype 25 from Switzerland (Toggenburg Orbivirus) and BTV serotype 26 from Kuwait. India being an endemic country for BTV, a total of 22 different BTV serotypes have been reported based on virus isolation and virus neutralization tests. BT disease is spread mainly in monsoon season (hot and humid conditions) when vector activity is more. More than 1400 *Culicoides* species have been reported worldwide. However, *Culicoides* species predominantly identified in Northern parts of India is *Culicoides oxystoma*. The *Culicoides* species such as *C. imicola* and *C. oxystoma* have been reported from Tamil Nadu in southern part of India.

Rapid and reliable detection and confirmation of BTV from clinical material and subsequent characterization of virus into various serotypes is essential on the early onset of an outbreak to control the disease. The BT is primarily diagnosed based on clinical profile, pathological lesions along with the knowledge of differentiation of BTV from FMD, PPRV etc. which exhibit similar type of clinical symptoms. Various biological samples used for diagnosis of BTV include blood, serum, aborted foetus, placenta, necrosed tissue and

exudates from affected lesions and the *Culicoides* vector. The conventional methods such as virus isolation and serum neutralization are though gold standard, but need to be carried out in addition to the rapid molecular tests because of the known limitations of these assays. For confirmative diagnosis and molecular epidemiological studies of BTV, various molecular techniques namely PCR, nested PCR, Real-time PCR (qPCR), multiplex PCR, Isothermal PCR (LAMP test), nucleic acid hybridization and new generation sequencing have been employed. These tests are rapid, highly specific, sensitive, and reproducible for the diagnosis as well as characterization of BTV from wide range of biological samples. Some of these molecular techniques employed for epidemiological studies of BTV are described below:

A. Reverse-Transcription Polymerase Chain Reaction (RT-PCR):

The RT-PCR can detect very small numbers (10-100 TCID^{50}) of BTV nucleic acid molecules in blood, semen and other tissues as well as *Culicoides*. Prasad *et al.* (1999) have reported that RT-PCR is a highly sensitive test, and can detect up to 10 infectious viral particles based on ns1 gene amplification. RT-PCR can be used for 'serogrouping' as well as 'serotyping' of BTV isolates. The diagnostic sensitivity of group-specific PCR of BTV is greater than the virus isolation in embryonated chicken eggs or assays of cytopathic effects on cultured cells. Comparison of the nucleotide sequences of the amplified products, allows better understanding of genetic relationships between the circulating strains of BTV. PCRs can also differentiate between the vaccine and wild BTV strains. Different types of RT-PCR used in BTV diagnosis are:

a. Serogroup specific RT-PCR: Due to huge genomic diversity among different BTV serotypes and within serotypes, there is a need for diagnostic procedure that can detect all the BTV isolates. Primers designed from the highly conserved genes, such as vp3 (Minakshi *et al* 2011), vp7 (Kovi *et al.,* 2006) and ns1 (Prasad *et al.,* 1999) are used for serogrouping of BTV. The vp3 gene provides indications for topotyping of the virus when sequenced.

b. Serotype specific RT-PCR: BTV Segment 2 is the most variable and targeted to determine 24 serotypes of the virus. Along with segment 2, the segment 5 also shows variations and studied along with segment 2 to find the serotype specificity of the BTV in epidemiological studies. Primers designed for various serotypes of BTV are segment 2 specific and provide information on virus serotypes (Dahiya *et al.,* 2004). The vp5 gene based RT-PCR has also be used for serotyping of BTV.

c. Nested RT-PCR: In nested RT-PCR there are two sets of primers used in two successive reactions. In the first PCR, one pair of primers is used to generate DNA products, which may contain products amplified from non-target areas. The products from the first PCR are then used as template in a second PCR, using primers whose binding sites are located (nested) within the first set of primers, thus increasing specificity and sensitivity of the assay. Since ns1 is a conserved gene, it is usually targeted for the development of nested PCR assay.

d. Duplex RT-PCR: It is a RT-PCR reaction which consists of two sets of primers, one specific for target gene (say, group specific or serotype specific gene of BTV) and another primer set for any possible targeting a house keeping gene (β- Actin) of host (vector or cell line, etc.). The amplicon size for the two primer set must be different so that they can be easily distinguished on agarose gel electrophoresis.

e. Multiplex RT-PCR: Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. Since BTV consists of 26 different serotypes therefore, it is difficult to test the biological samples for different serotype specific primers separately. By targeting multiple serotype specific genes (i.e. vp2 & vp5) at once with their different type specific primers, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. However, the sensitivity of multiplex PCR is less than the nested PCR, but is sufficient for the samples collected from fields.

f. Real-time RT-PCR: Real-time RT-PCR is a highly sensitive (10-100 times) method that can be used for the detection of viral RNA from a variety of biological samples. During the real-time RT-PCR reaction, viral RNA is reverse transcribed into cDNA and subsequently amplified in real-time PCR machine. A fluorogenic signal from dual-labelled probe is released during amplification and recorded in real time. The primers and probe combination for each real-time RT-PCR reaction are designed to amplify and detect a targeted region of the BTV genome such as ns1 gene (Vishwaradhya *et al.,* 2011). Various variations in real-time PCR have been used as Duplex Real-Time RT-PCR and Multiplex real-time RT-PCR.

B. Nucleic acid hybridization Assays:

Nucleic acid hybridization assay measures the degree of genetic similarity between pools of nucleic acid sequences from different sources. It is commonly used to determine the genetic similarities or distance between two BTV isolates. Here, the cDNA of one BTV isolate is amplified and PCR amplicon is labeled, and mixed with the unlabeled cDNA of

BTV to be compared. The newly hybridized sequences with a high degree of similarity will bind more firmly and require more energy to separate them. They will separate when heated at a higher temperature than dissimilar sequences. Based on nucleic acid hybridization serotype differentiation of BTV can be done.

C. RNA- Polyacrylamide gel electrophoresis (RNA-PAGE):

RNA-PAGE is a commonly used for analysis of RNA samples because of low cost, ease of use, high sensitivity and gold standard values. The technique is used for separating RNA segments of BTV in polyacrylamide gels. The viral RNA isolated from biological samples can be analyzed in RNA-PAGE followed by silver staining. The separation of 10 RNA segments of BTV in characteristic 3:3:3:1 pattern (electropherotype) confirms the presence of BTV. Based on different electropherotypic migration patterns of RNA segments in the RNA-PAGE genomic diversity has been detected (Ranjan, *et al.,* 2011).

D. DNA Micro arrays:

Microarrays can be used for the detection of unique nucleic acid sequences and disease diagnosis. Compared with traditional nucleic acid hybridization, microarrays offer the additional advantages of high density, high sensitivity, rapid detection, lower cost, automation, and low background levels. With the ability to use a small quantity of sample for a large number of pathogens simultaneously, DNA microarrays represent a potential solution to diagnose multiple pathogens including BTV. Recently, a 'gene chip' or micro-array chip, having short pieces of DNA (ITS1 gene of *Culicoides*) has been developed for species identification of C*ulicoides* vector. The results can be analysed by naked eye as the characteristic pattern of spots for each species of C*ulicoides* vector (Deblauwe *et al*., 2011).

E. PCR-RFLP analysis:

In this technique the cDNA made is amplified by PCR and subsequently the PCR amplicon is broken into pieces (digested) using restriction enzymes and the resulting restriction fragments are separated by gel electrophoresis. The PCR-RFLP technique was the first technique to see the variation between and within serotype of BTV (Dahiya *et al.,* 2005). The PCR-RFLP technique can be performed either wet lab or *in-silico*.

F. Nucleic acid sequencing:

The nucleic acid sequence of BTV has become indispensable for serotyping, epidemiological surveillance and control of BT disease in India (Minakshi *et. el.,* 2012;

Susmitha *et al.,* 2012). Nucleic acid sequencing has provided a unique opportunity to complement studies of BTV epidemiology by providing information on the potential geographical origin of virus isolates, a process termed genotyping or topotyping. Vp3 gene based RT-PCR and its nucleic acid sequences may provide information about whether the virus came from Australia, North America, South Africa or Asia i.e. topotyping of the virus (Minakshi *et al.,* 2011). The nucleic acid sequence of BTV genes may differ with the geographical area of virus isolation. Thus, determination of the nucleic acid sequence of portions of RNA may provide information on migration of the virus. It appears likely that sequencing of BTV isolates from other parts of the world may permit finer discrimination of geographical origin.

(G) Phylogenetic analysis:

The phylogenetic analysis is the study of evolutionary relationship among different groups of organisms (e.g. viruses), through nucleic acid sequencing data. The evolutionary relationship between and within different BT viruses are represented graphically through phylogenetic trees (Fig. 1).
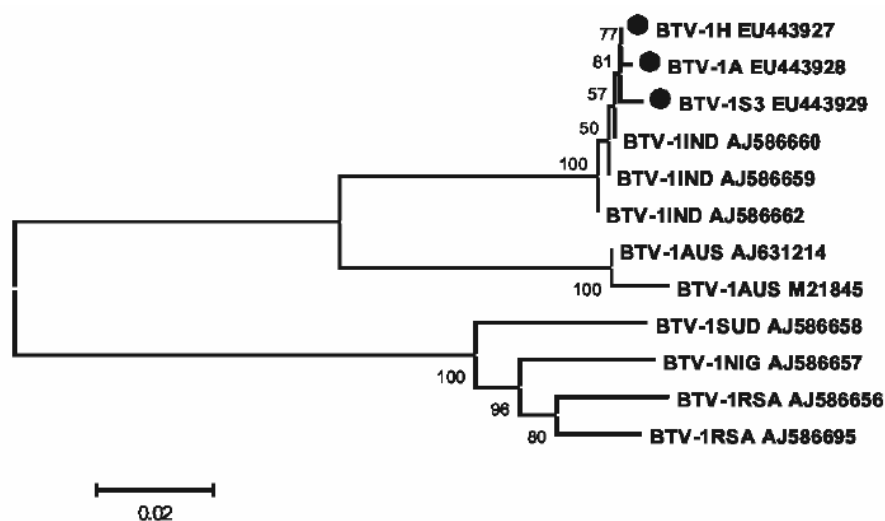

Fig. 1: Phylogenetic tree of BTV1 isolates based on vp5 gene

The vp2 gene of BTV is highly variable, and therefore it is used to establish phylogenetic relationship (Dahiya *et al.*, 2004) along with vp5 gene (next to vp2 gene).

References:

Dahiya, S., Minakshi, Kovi, R. and Prasad, G. 2005. Relationships of bluetongue virus serotype 1 isolates based on restriction analyses of conserved and variable regions of VP2 gene. *Ind. J. Exp. Biol.* **43:** 272-276.

Dahiya, S., Prasad, G. and Kovi, R.C. 2004. VP2 gene based phylogenetic relationship of Indian isolates of Bluetongue virus serotype 1 and other serotypes from different parts of the world. *DNA Seq.* **15:** 351-361.

Deblauwe, J. C. De Witte, G. De Deken, R. De Deken, M. Madder, S. Van Erk, F. A. Hoza, D. Lathouwers, D. Geysen. 2011. A new tool for the molecular identification of Culicoides species of the Obsoletus group: the glass slide microarray approach. *Medic. Vety Entool*; DOI: 10.1111/j.1365-2915.2011.00979.x.

Kovi, R.C., Dahiya, S., Prasad, G. and Minakshi 2006. Nucleotide sequence analysis of vp7 gene of Indian isolates of bluetongue virus vis-a-vis other serotypes from different parts of the world. *DNA Seq.* **177:** 820-823.

Minakshi P, Singh R, Ranjan, K, Kumar P, Joshi CG, YKM Reddy and Prasad, G. 2012. Complete genome sequence of bluetongue virus serotype 16 of goat origin from India*) Journal of Virology*, **86**: 8337-38.

Minakshi, Ranjan, K, Pawan, Rupinder and Prasad, G. 2011. Segment 3 based genetic diversity among Indian isolates of bluetongue virus. *Haryana Vet.* **50:** 68-71.

Prasad, G. Rupinder, Minakshi. 2008. Current status of bluetongue disease in India and its pathogenesis in relation to host and climate change. *Indian J. Pathology* **32**: 111-124.

Prasad, G., Minakshi, Malik, Y. and Maan, S. 1999. RT-PCR and its detection limit for cell culture grown bluetongue virus1 using NSI gene specific primers. *Indian J. Exp. Biol.* **37:** 1255-1258.

Ranjan, K., Minakshi, P**.,** Shafiq, S., Kumar, P. and Prasad, G. 2011. Genetic Diversity of Indian Isolates of Bluetongue Virus 16. *Int. J. Appl. Eng. Res.* **6:** 884-887.

Susmitha B., Rao PP, Uma M ,Prasad G, Minakshi P.,. Hegde NR, Reddy YN. 2012. Evidence of bluetongue virus serotype 21 (BTV-21) divergence. *Virus Genes*, **44**: 466-469.

Vishwaradhya TM, Minakshi P, Ranjan K, Rupinder, Pawan, Kumar A. and G. Prasad. 2011. Taqman Probe based Real Time PCR assay for detection of Indian isolate of BTV-16. VIROCON 2011 at NRCE, Hisar from 29-31 Dec., 2011.

Wilson, W.C., Hindson, B.J., O'Hearn, E.S., Hall, S., Roth, C. T., Torres, C., Arani, P. N., Mecham, J. O. and Lenhoff, R. J. 2009. A multiplex real-time reverse transcription polymerase chain reaction assay for detection and differentiation of Bluetongue virus and Epizootic hemorrhagic disease virus serogroups. *J. Vet. Diagn. Invest.* **21:** 760–770.

PPR IN SMALL RUMINANTS: DIAGNOSIS AND CONTROL

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Sheep and goat rearing is gaining importance due to increased demand for meat and milk, thus contributing to the poor man's economy. Sheep and goat population is threatened by a number of viral diseases including *peste des petits ruminants* (PPR), considered as a major deterrent in the large scale farming and production of these animals. PPR is a highly contagious viral disease of goats and sheep, caused by a virus belonging to *Morbillivirus* genus of family '*Paramyxoviridae*'. Other members of this genus include rinderpest virus affecting cattle, sheep, goat and buffaloes; canine distemper virus of dogs and some wild carnivores; measles virus of humans, phocid distemper viruses affecting seals, morbilliviruses of porpoise and dolphin and equine Morbillivirus.

In India PPR virus was first reported in 1987 in sheep from Arsur village in the Villapurum district of Tamilnadu. Since its first occurrence, PPR was thought to be restricted to southern India up to 1993, after which the epidemics of PPR swept away large numbers of small ruminants from Northern India. Epidemics of PPR in goats have been reported from more than 15 states of India during 1992-1998 and the disease is now considered endemic in our country. The disease is growing with greater magnitude every year causing serious economic losses throughout the country. Approximately, one-third of sheep and goat population of India have been shown to be exposed to PPR infection in India. The extent of the total economic losses in our country due to PPR has been estimated to be about Rs. 1800 million per annum in 2005.

In majority of cases, the disease occurs in an acute form with an incubation period of 3-6 days following exposure to the virus. Following entry through the respiratory tract, the virus localizes and replicates first in the pharyngeal and mandibular lymph nodes and tonsils. Viremia develops 2-3 days after infection and 1-2 days before the appearance of first clinical sign. Subsequently, the viremia helps in dissemination of the virus to spleen, bone marrow and mucosa of gastro intestinal tract and the respiratory system.

Clinically, the disease is characterized by pyrexia up to 41°C lasting for 3-5 days: animal becomes depressed, anorexic and develops a dry muzzle, watery nasal and ocular discharges turning to mucopurulent. If death does not occur, these symptoms may persist for 14 days. Within four days of onset of pyrexia, necrotic lesions develop in the oral cavity

leading to excessive salivation, necrotizing and erosive stomatitis and enteritis. A watery blood stained diarrhoea develops in later stages which is usually foul smelling. Pneumonia, coughing and abnormal breathing is also seen. The morbidity and mortality rate may reach 100% in severe cases but in milder outbreaks these seldom exceed 50%. Goats are more severely affected than sheep. Cattle, buffaloes, camels and pigs could be infected experimentally. Pigs are considered to be the "dead end host" which undergo silent infection by contact with infected goats and do not transmit the virus to in-contact pigs.

Post mortem lesions in PPR are similar to those seen in rinderpest, and include erosive lesions in the mouth including gums, soft and hard palates, tongue and cheeks and in the oesophagus. Lungs exhibit red or purple areas, firm to touch mainly in the anterior and cardiac lobes. Lymph nodes associated with lungs and intestines are soft and swollen. Zebra stripping is seen in large intestine particularly at the caeco-colic junction. Haemorrhagic enteritis is commonly seen. Lymph nodes are enlarged, spleen exhibits necrotic lesions and lungs show apical pneumonia.

A tentative diagnosis of the disease can be made on the basis of signs and symptoms described above but a laboratory confirmation is needed for differential diagnosis with other diseases like contagious caprine pleuropneumonia, rinderpest , foot and mouth disease, blue tongue and pustular dermatitis.

In laboratory isolation of virus can be made in cell culture of ovine origin or in Vero cell line from infected heparinised blood, triturate of lymph nodes, spleen or lungs. Viral isolate is then confirmed by serum neutralization test. Viral nucleic acid can be detected by RT-PCR. Serologically the disease can be diagnosed by detecting presence of PPR specific antibodies by c-ELISA test or by haemagglutination inhibition test.

The control of the disease outbreaks relies on control of movements of animals coupled with the use of focused vaccination and prophylactic immunization in high-risk populations. Vaccination of the susceptible population is done with a homologous tissue culture adapted live attenuated PPR vaccine. All animals above 4 months of age need to be vaccinated annually for five years for eliminating the virus from the small ruminant population. Absence of antigenic variation in virus isolates, mounting of strong immune response both humoral and cellular following vaccination makes it a next suitable candidate disease for eradication from the globe like rinderpest virus infection of cattle.

Further Reading:

Dhar, P., Sreenivasa, B.P., Barret, T., Corteyn, M., Singh, R.P. and Bandyopadhyay, S.K. 2002. Recent epidemiology of peste des petits ruminants virus. *Res. Vet. S*c. **59**: 106-109.

Dhinakar, R., Kumar, A.S.S., Shaila, M.S., Nachimuthu, K. and Palaniswami, K.S. 2003. Molecular epidemiology of peste des petits ruminants viruses from Southern India. *Vet. Rec*. **152**: 264-266.

Karale, P. V. and Batra, S. K. 2011. Comparative evaluation of HI, c-ELISA and SNT for detection of anti peste des petits ruminants virus antibodies in goats. Abstract presented *In: Virocon-2011*, NRCE, Hisar, Dec. 29-31, 2011. pp 58-59.

Manoharan, A., Jayakumar, R. and Govindrajan, R. 2005. HA as a confirmatory test for the diagnosis of PPR. *Small Ruminant Research*. **59**: 75-78.

Peste des petits ruminants. OIE Terrestrial Manual. 2008. pp: 1036-1046.

CONTROL OF HEMORRHAGIC SEPTICAEMIA IN CATTLE AND BUFFALOES

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Abstract

Hemorrhagic septicaemia (HS) is an economically important disease of cattle & buffaloes having well established epidemiology. Buffaloes are more susceptible than cattle and young animals are more prone to the disease than adults. Natural epidemiological cycle operates in enzootic areas. Younger previously unexposed animals continue to die while older animals build natural immunity. In this situation, the disease continues to smoulder without major flashes. Regular vaccination stops this natural epidemiological cycle.

Newer research for vaccine improvement to address the deficiencies of current vaccines, factors for design of well structured vaccination program, measure during HS outbreak and key elements in developing a regional control program for Asia are described.

Haemorrhagic septicaemia (HS) is a highly fatal disease in cattle and buffaloes, caused by specific serotypes of *Pasteurella multocida*. It is a primary pasteurellosis reproducible in susceptible animals with the specific organism alone. The accepted classification of *P. multocida* is based on the identification of its somatic and capsular antigens. On this basis two serotypes designated 6:B (B:2) and 6:E have been found to cause HS. The disease is prevalent in most countries in Asia, the near and Middle East, Southern Europe and in the North, Central and East Africa. In Asia, the disease is caused by serotype 6:B and in Africa 6:E, while a few countries have recorded both. In most Asian countries, it is recognised as a disease of utmost economic importance. Understanding of epidemiology of the disease plays an important role in developing a successful control program.

Seasonal occurrence of the disease: The disease is normally associated with wet and humid conditions prevailing during rainy season. This is because of longer survival of the organism under moist conditions on fomites as wells as herding together of the animals. Therefore, outbreaks occurring during the wet season tend to spread.

Factors influencing the disease outbreak: Buffaloes are more susceptible to the disease than cattle. However, the disease affects both cattle and buffaloes. It is also agreed that greater losses occur in younger animals than in adult animals. The most vulnerable age is between 6 months and 2 years. In areas where regular seasonal outbreaks occur, mortality in individual outbreak is low and is confined to young animals. When outbreaks occur outside the enzootic areas, mortality is high and animals of all ages die. This relationship is based on immunological status of the animals. In an enzootic area, naturally acquired immunity develops in adult animals while only the young animals are susceptible to the disease.

Whenever infection is introduced or outbreak builds up, calf and young adult animals get infected and the older animals acquire immunity. This way the disease continues to smoulder with occasional flashes of outbreaks. The animals lack naturally acquired immunity in non enzootic areas and therefore, outbreaks of higher mortality involving all age group occur.

It is well known that a certain population of healthy cattle and buffaloes harbour *Pasteurella spp.* in their nasopharynx. A correlation between incidence of the disease and carriers is well established. Carrier state develops immediately after an outbreak in clinically unaffected animals. It is also known that the nasopharyngeal carrier state is transient and different animals are carrier at different times in the first few weeks following an outbreak. It is also reported that animals harbour virulent pasteurellae in their retropharyngeal lymph nodes without their being present in the nasopharynx. While carrier state in nasopharynx is transient and can be eliminated by regular vaccination, it is not known as to how long the pasteurellae persist in retropharyngeal lymph node and whether such persistence can occur in any animal or in immune-deficient animals only.

The detection of animals which are nasopharyngeal negative but lymph node positive has totally changed the concept of carrier. Therefore, it seems reasonable to designate the nasopharyngeal positive carrier as active carrier and nasopharyngeal negative but lymph node positive as latent carrier. The outbreak in a closed herd occurs when a latent carrier becomes an active carrier and starts shedding the virulent pasteurellae. Such virulent pasteurellae in sufficient numbers are picked up by susceptible host. Alternatively, introduction of active carrier may also trigger an outbreak.

Wet and moist conditions favour survival of the organism for longer duration on fomites and may also contaminate food and water. The magnitude of the outbreak depends upon the proportion of susceptible animals in the herd.

Disease management: HS is a disease of the countries where animal husbandry practises are poor. The outbreaks are reported very late due to poor disease reporting system and the treatment is of little value in animals showing clinical symptoms.

Vaccination is an accepted method for control of HS in such areas. It is also possible that the disease can be eradicated by adopting effective vaccination program that will not only prevent the occurrence of the disease but may also eradicate the disease by eliminating carriers in the population. It has been stated that regular vaccination eliminates the carriers. A

well designed vaccination program based on judicious and effective use of potent vaccine is a key factor in control program.

HS vaccines: A vaccine giving long lasting immunity is in urgent requirement. Alum precipitated vaccine is most commonly used. However, the duration of immunity is shorter (only 4-5 months). Further improvement on alum precipitated vaccine has been achieved by using alum gel adjuvanted vaccine, but significant improvement on duration of immunity could not be achieved.

Oil adjuvanted vaccines have been reported to improve the duration of immunity (one year duration). In enzootic areas, the immunity of vaccinated animals seems to be prolonged by exposure to infection. However, the high viscosity and the consequent difficulty in administration weigh heavily against oil adjuvanted vaccine. Some reduction has been effected by using 4% lanoline instead of the 8-12% used in some formulation

Double emulsion vaccine has been developed in order to reduce the viscosity of oil adjuvanted vaccine. The original vaccine was re-emulsified with an equal volume of 0.2% polysorbate 80 (Tween-80). The resulting fluid had a free-flowing milky consistency, but the potency of the vaccine was less than that of the standard oil adjuvanted vaccine.

Attempts to identify the antigenic fractions and to use such fractions to immunize cattle were made decades ago in India by Dr. M. R. Dhanda, but this work did not result in a practical vaccine. More recently, capsular extracts have been obtained by solvent precipitation from the supernatant fluid of fermentor grown *P. multocida* type B. The complex technology involved in producing a vaccine of this type may be beyond the scope of many vaccine laboratories in countries affected by HS because of its high viscosity.

Numerous attempts have been made from time to time to produce live vaccines against various forms of pasteurellosis. The main problem has been to achieve stability of the attenuated strain or mutant. Live attenuated vaccines, in general have the advantage of a natural route of entry into the host, which allows targeting of immune-stimulatory factors to the same sites of the immune system that occur in the natural infection. Attenuated *aroA* mutants of *P. multocida* serotypes A and B:2 which cause fowl cholera and HS respectively, have been shown to provide protection against challenge. With the availability of freeze-drying equipment, the opportunity exists for developing a live vaccine in lyophilized form.

Moredun Research Institute near Edinburgh conducted work on developing *aroA* mutants of Indian isolate P-52 of *P. multocida*. The Wellcome Trust awarded one million

pounds over three years to an international consortium of researchers including scientists from Moredun Institute, University of Glassgow and Indian Veterinary Research Institute for development and testing of a new *aroA* mutant vaccine. Current vaccine provides protection for 6-9 months but the new vaccine is anticipated to have a longer duration of immunity and to offer greater cross protection as well as being easier to administer.

Vaccination monitoring: When a vaccination program is implemented in a defined area over a period of time, the important criteria for measuring its success are

- The absence of any adverse reactions in vaccinated herds
- The extent of reductions in occurrence of disease
- Level of antibody following vaccination

Monitoring of antibody/herd immunity following vaccination is an important component of any vaccination program. The vaccination program with a potent and efficacious vaccine might fail to achieve herd immunity due to various factors like administrative factors, host and pathogen factors. There could be logistic factors in transportation, storage, administration of the vaccine adversely affecting the vaccination program. The animals may not adequately respond to vaccinations due to host factors like immunosuppression due to parasitic or other such immunosuppressive diseases, nutritional deficiency, genetics and maternal antibody levels. Pathogen factors like virulence of the pathogen or the immunological mutation of the pathogen resulting into variant strain or involvement of other immunologically different strain or serotypes not covered by the vaccine, may adversely affect the success of vaccination program.

Regular monitoring of antibody levels might help in identifying the factor(s) responsible for failure of vaccination program and this may help policy planners and administrator in cementing the leakages in vaccination program and develop a strong and effective control program.

Measures during an outbreak: Vaccination is a major control measure in the face of a new epidemic. Various vaccine types have been developed viz. the broth bacterin, the oil adjuvanted vaccine, the double emulsion vaccine and a live vaccine. The latter is a deer strain aerosol vaccine developed in Myanmar. Although this vaccine is the sole vaccine used (apparently with success) in Myanmar, it does not seem to have gained popular acceptance outside this country. The reasons advanced to explain this are numerous: the fact that the strain concerned (*Pasteurella multocida* serotype B:3.4) has occasionally been associated with sporadic outbreaks of disease due to hitherto unexplained reasons, its occasional

virulence to young animals when administered by the subcutaneous route, and the fact that antibiotics cannot be administered simultaneously to this live vaccine.

- During an outbreak, one should resort to immediate whole herd vaccination, irrespective of previous vaccination history. The use of either broth bacterin or oil adjuvant vaccine is recommended;
- Sanitary measures include early detection and isolation of new cases and their immediate treatment with antibiotics, deep burial of carcasses or incineration, and the prevention of movements of animals to disease free areas.

Measures in endemic countries/zones: These include:

- Vaccination on a routine prophylactic basis preferably two to three months before the high-risk season (monsoon).
- Awareness of the disease among farmers backed up by a good disease reporting/disease information system.
- Segregation of animals from endemic and non endemic areas to avoid contact with carriers.

Some Key Elements for a HS Regional Control Program in Asia: It must be noted that, unlike FMD and Rinderpest, HS eradication does not seem to be feasible presently, with the limited experience in the Lombok Island in Indonesia With the present information on economic losses and a knowledge of its epidemiology in the countries of the region, the key factors in prevention and control appear to be quick reporting, accurate and rapid diagnosis, strategic use of vaccines with attainment of a high prophylactic vaccination coverage with a high quality vaccine, i.e., potent, economical, and easily administrable. All of these would constitute national level activities within the countries of the region. A regional program aiming at reducing the economic losses associated with the disease should, therefore, focus on strengthening of these activities within the countries and coordinating their efforts.

The proposed elements articulate around the following range of outputs and activities:

- Develop simple, accurate and rapid diagnostic tests that can be carried out even in the modestly equipped laboratories and make them available to all countries of the region.
- Make available, through the Regional HS Reference Laboratory type cultures, reference sera or any other biologicals that may be necessary.
- Produce a Manual of Diagnostic Procedures, for use in all countries of the Region.
- Acquire sufficient knowledge of the epidemiology of the disease in each country so as to formulate a strategic control program.
- Strengthen disease reporting and surveillance systems in general.
- Develop protocols for collection, management and processing of relevant epidemiological data so as to obtain a clear epidemiological picture in each country.
- Develop standard protocols for active surveillance studies and estimating economic losses on HS in particular.
- Assist in the development of a strategic vaccination program for each country based on the above information.

- Make available the technology for the production of a vaccine that is potent, economical, easily administered and with a production technology simple and sustainable in countries of the region.
- Identify vaccines such as the new oil adjuvant vaccines, double emulsion vaccines and live vaccines for further development.
- Encourage and support R&D activities on these vaccines.
- Enlist a range of tested and proven vaccines of acceptable standard which the countries of the region could select from, depending on their resources and level of technology available within the country.
- Publish a 'Manual of Standard Procedures for Production and Quality Control of HS Vaccines, incorporating the range of vaccines identified above.
- Develop training programs for veterinarians and other animal health field officers. Also, enhance awareness among the livestock farmers so as to improve their co-operation and stimulate their participation in the control program. This would require a serious investment geared towards the improvement of the quality of the veterinary extension services.

Concluding Remarks: It is well understood that the HS is a disease of underdeveloped or developing countries. Livestock owners do not show interest or support vaccination despite severe economic losses due to reasons of adverse reactions of loss of milk yield, abortions and wounds at the site of inoculation. Further, shorter duration of immunity forces repeat vaccination twice in year coupled with other vaccination like FMD vaccination; all these put heavy work load on animal husbandry personnel and livestock owners. Continued occurrence of the disease despite vaccination because of low herd immunity status is yet another factor that livestock owners do not have faith in vaccine.

A well designed regional control with all key elements in place may help us control and eradicate HS from India. Availability of live attenuated vaccine, technology to develop combined vaccines with HS, FMD and other agents like BQ, etc. and dividends of implementation of FMD control program in the country may impress animal health authorities to launch similar program for HS control in the whole Asian region.

Suggested Reading:

Benkirane, A. and De-Alwis M.C.L. 2002. Hemorrhagic Septicemia, its significance, prevention and control in Asia. *Vet. Med. – Czech,* **47**: 234–240.

De- Alwis, M.C.L. 1984. Haemorrhagic Septicaemia in cattle and buffaloes. *Rev. Sci. Tech. Off. Int. Epiz.,* **3**: 707-730.

PREREQUISITES FOR THE CONTROL OF INFECTIOUS DISEASES WITH SPECIAL REFERENCE TO HEMORRHAGIC SEPTICAEMIA IN BOVINES

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Just like the other natural disasters such as major fires, floods, hurricanes, earthquakes, radiation incidents and volcanic eruptions, control of disease outbreaks or the trans-boundary diseases need critical levels of **preparedness**. This is particularly crucial with respect to the infectious diseases and is indeed very challenging. In order to control such outbreaks, the preparedness for such emergent situations includes a well worked out plan and the capacity to implement so that there are **minimal loses**.

Control of disease outbreaks involves almost the similar characteristics as other natural disasters *viz.* often with a sudden and unexpected onset, may cause major socio-economic consequences, endanger human lives and requires a rapid national response and therefore should be incorporated into the national disaster plan. Emergency responses present unique challenge which usually cannot be addressed effectively in the same manner as daily work. Therefore, plans need to be documented and agreed in advance so that everyone understands what will be happening and how the response will be implemented.

The basic of such a preparedness protocol which is essentially an ongoing program will minimize the disease spread and the eventual losses. It involves a rapid and accurate disease detection and identification system - **reliable diagnostic tool.** The delay between incursion and detection is one of the most important factors in determining the severity of the outbreak. The components of an effective detection system also include restriction on the entry of disease particularly in case of trans-boundary diseases. Certain times of year may be accompanied by higher risks of the introduction or spread of disease, for example during the cultural or religious festivals which involve large-scale movements of animals.

Prior approval of plans from the civil authorities and sanction of money should be well worked out so that the farmers can understand, cooperate, support and be helpful.

A. **Preparation of animal health emergencies:** As per guidelines/recommendations of FAO, the following aspects must be taken into consideration (FAO, 2011):
  ➢ Establishment of a national disease emergency planning committee;
  ➢ Determine the command structure and responsibilities;
  ➢ Ensure that the required legal powers are in place;

- ➢ Ensure that sources of financing have been identified;
- ➢ Establish compensation policy;
- ➢ Establish sourcing for any required vaccines, diagnostic reagents etc. and other critical supplies;
- ➢ Undertake risk analysis to identify and prioritize potential disease risks;
- ➢ Prepare, practise and refine contingency plans and operations manuals.
- ➢ A well prepared set of preventive measures that will decrease the frequency and possibly the impact of disease incursions.
- ➢ Prevent entry of the disease agent in legal imports and illegal imports;
- ➢ Gathering for early warning of changes in distribution, virulence or epidemiology in affected countries and trading partners;
- ➢ Establishing, strengthening and maintaining cross-border contacts with neighbouring administrations ;
- ➢ Strengthening of routine bio-security measures.
- ➢ Maintaining disease awareness among key staff and stakeholders;
- ➢ Maintaining routine disease intelligence activities and targeted surveillance;
- ➢ Reporting suspicions by the owners, veterinarians etc.;
- ➢ Establishing and following a protocol for investigating suspect cases;
- ➢ Maintaining laboratory facilities to confirm a clinical suspicion.

B. Once the disease has been detected and confirmed, it is essential to:
- ➢ Activate contingency plans;
- ➢ Assess the initial outbreak for its size, geographical spread, epidemiology to judge what control measures may be required;
- ➢ Implement the control measures as quickly and completely as possible;
- ➢ Monitor progress and adjust policies accordingly;
- ➢ Exchange of information and data with neighbouring administrations; and
- ➢ Communicate with the public and all stakeholders, including the OIE.

It may be reemphasized that the amount of time taken between the detection of the disease and full implementation of the control measures is a major determining factor in the overall severity of any outbreak. To prevent entry of the disease agent restrictions on migration and imports need to be enforced through maximizing alertness of the quarantine barrier (for live animals), tightening inspections for legal and illegal imports, increasing bio-security measures be put in place etc.

C. **Four basic components of a disease control program:**

1. **Emergency Preparedness Plan** – It outlines what is required to be done before an outbreak of a disease in order to **be prepared** (i.e. getting ready). This includes things that all of the stakeholders need to do.
2. **Contingency Plan** – It outlines what a government will do in the event of an incursion of a disease, beginning from the point when a suspect case is reported (i.e. responding). Apparently there are some over lapping between the two.
3. **Operations Manual** – It is a comprehensive set of instructions (also called standard operating procedures [SOPs]) that instructs field staff and others how to implement the response.
4. **Recovery Plan** - This is the plan for the safe recovery or restoration of normal activities may also called rehabilitation.

Several aspects of these plans are common for most diseases with some disease specific aspects. These plans need to be updated regularly and hence referred as **live plans.**

Hemorrhagic Septicemia

Hemorrhagic septicemia (HS) caused by *Pasteurella multocida* which mainly affects cattle and buffaloes and is responsible for heavy losses particularly in South-East Asia, Middle-East and Africa. The various sero-groups of *P. multocida* are known for their species specificities *viz.* A for fowl cholera in poultry, D for atrophic rhinitis in pigs while serotypes B:2 and E:2 are responsible for HS in cattle and buffaloes in India and Africa respectively. In India the disease is endemic and the serotype B:2 is mainly responsible. Thus preventing the entry of other than the prevalent sero-groups in India makes it an important aspect of the disease control and surveillance programs for India by critically implementing the strategy as recommended by World Organization for Animal Health.

HS is a per acute and fatal systemic infection associated with a short incubation period varying between 30 hours and 5 days involving dyspnoea, pyrexia, profuse nasal discharge, sub-mandibular edema. The microorganism can be isolated from the naso-pharynx of clinically healthy individuals. The epizootics occur seasonally *viz.* during the months of cold winter and the monsoon. Overcrowding, transportation or concurrent infection particularly with immunosuppressive pathogens may result in precipitation of the disease. Several instances of HS and foot-&-mouth disease out-breaks have been have reported to occur concurrently. A thorough understanding about these factors should contribute significantly for the control of HS.

a. Disease profile in different animal species *viz.* cattle vs. Buffaloes: As the buffaloes are more susceptible than cattle and the duration of illness is shorter in buffaloes than in cattle, it will be more crucial to implement the disease control program at priority in buffalo

dominated areas. As the farmer economy particularly in the northern India is more dependent upon buffaloes in this region, the control program needs to be implemented in total as soon as the first case is detected.

b. Persistence of infection and its endemicity: Countries where animal husbandry practice and disease surveillance systems are inadequate contribute significantly more difficulty in disease control program. There are a number of features and factors about the pathogen's biology which are of immense significance eventually leading to a successful HS control program (De Alwis, 1992)

1. The animals which develop high antibody levels after infection are the ones which become persistent carriers until and unless a very robust immune response is evoked both at mucosal level as well as systemic. Thus every animal affected in an outbreak must be treated as a potential carrier and as a source of infection for making the disease control program successful (Kharb and Charan. 2011).

2. Depending upon the proportion of immune to non-immune animals and the levels of antibodies, the magnitude of the outbreak can possibly be predictable.

3. The role of immune response in carrier state is not clearly understood. However, generating an accurate picture regarding the immune status of the population through regular sero-monitoring would be very important for an efficient control program against HS.

4. In endemic areas the adult animals acquire natural immunity as a result of successive exposures and only a small proportion of unexposed animals that remain susceptible are eventually affected at each outbreak.

5. The organisms harboured in the tonsils (crypts of the tonsils) persist despite treatment with the antibiotic to which the organism might be sensitive in *in vitro* assay.

6. As the carrier animals begin to shed virulent organism with or without the showing the clinical signs, dissemination of infection is further enhanced. Therefore, it will be very crucial to follow the case at the early stage in terms of shedding.

c. Immunogens and Immunity:

The role of an effective vaccine in any disease control program is undisputable. However, a thorough knowledge of its immunogens and their respective contributions need to be well identified. In HS, humoral immune response is generally considered important for protection against the disease but the relative significance of the various immunogens in protection is not well established. *Pasteurella multocida* expresses several antigens that have been identified as potential immunogens viz. capsule, lipopolysaccharides (LPS), outer membrane proteins (OMP) and iron-regulated OMP (IROMP) (Kharb & Charan, 2010).

LPS: LPS is a major immunogen but in mammals vis-a-vis birds its role has been controversial. Immunization with LPS has failed to protect, whereas a monoclonal antibody against LPS has been reported to protect against homologous challenge*,* on the other hand *in vivo* passive transfer of affinity purified anti-LPS serum has failed to protect. In a study,

produced monoclonal antibodies (MAb) against LPS, when tested in mice for their protective capacity these MAb gave only partial protection. Similarly, mice immunized with purified LPS were able to provide only up to 20% protection vis-à-vis 100% protection in the mice immunized with whole killed bacteria. These studies suggest that LPS antigens play a limited role in protection.

OMP: The studies on the immunogenic role of OMP have been studied in rabbits using A:3 serotype of *P. multocida* in which it was demonstrated that immunization with OMP protected against the homologous challenge. MAb against OMP protein has been found to be effective upon challenge in rabbit as well as in mice. Studies on the immunogenicity of OMP vaccinated with OMP emulsified in liquid paraffin and lanolin showed complete protection in buffalo calves. However, the antibody titres in the calves vaccinated with OMP vaccine were not significantly different than the calves inoculated with whole cell killed vaccine. This study along with studies carried out in rabbits and mice conclude that OMP could be used as an immunogen for vaccinating against HS.

IROMP: *P. multocida* when grown under iron limiting conditions expresses several additional OMP ranging from 35 to 109 kDa that are likely to be associated with iron acquisition *in vivo*. These IROMP have been used as immunogen in active protection studies and have been found to be effective not only against homologous challenge but also against the heterologous challenge particularly in birds. In a study to contemplate a correlation between antibody response of cattle against IROMP-enriched fraction of *P. multocida* A:3 strain to experimental challenge, it was observed that vaccination with IROMP-enriched fraction did not offer any significant advantage over vaccination with OMP as there were no significant differences in mean lung lesion scores among OMP or IROMP enriched fraction. Overall, it has been suggested that monitoring humoral antibody immune responses against OMP may be a useful indicator of immune status.

d. Serological methods for detection of HS: Several serological tests have been used for the identification of the HS-causing serotypes of *P. Multocida viz.* rapid slide agglutination test, indirect haemagglutination, agglutination test, agar gel immuno-diffusion, counter immuno-electrophoresis test, mice protection assay and enzyme-linked immunosorbent assay. But the antibody detection based immunoassays are of relatively little significance and rarely undertaken for the routine diagnosis of infection caused by *P. multocida* mainly because of the: a) presence of micro-organisms in the nasopharynx of apparently healthy animals, b) very short incubation period, c) septicemia occurring only at the terminal stage of the disease,

d) endemicity of infection and e) use of several vaccination schedules, etc. These serological assays fail to distinguish between antibody response due to recent infection or as a result of vaccination

i. Indirect haemagglutination test (IHA): The test has been reported to be more efficient when dealing with serotypes B and E. High titres detected by the IHA test are indicative of recent exposure to HS. A high IHA titres from 1/160 up to 1/1280 or higher among in-contact animals surviving in affected herds, are indicative of recent exposure to HS for the purpose of diagnosis (OIE, 2005).

ii. Enzyme-linked immunosorbent assay (ELISA): ELISAs have emerged as an economical, versatile, robust and simple alternative. Because of their combined simplicity, sensitivity and availability of reagents and potentials for automation, ELISAs can be used reliably for screening large numbers of samples in the simplest of laboratory environments and thus has made a great impact in the disease diagnosis. The addition of a polyclonal antibody can greatly increase the breadth of the assay to detect multiple isolates of the species of the pathogens. Therefore use of monoclonal and polyclonal antiserum is likely to have its own advantages and disadvantages. The various versions and modification of this technique are now widely used throughout the world for the diagnosis of infectious diseases including in veterinary medicine for the detection of antigens and antibodies. Although many laboratories have independently developed ELISA techniques for their own purposes, standardization and validation of these techniques is still limited as to draw universally acceptable result and frame establishment of international guidelines and programs for the control, surveillance and eradication of infectious diseases.

iii. Molecular methods:

P. multocida-**specific PCR assay:** The sensitivity and specificity of the *P. multocida* specific PCR offer the most compelling argument for its use in laboratory investigation of suspected HS cases as it allows detection of ≈10 organisms per reaction. Moreover, identification of HS causing type-B-specific *P. multocida* is also possible by PCR assay. Comparative analysis with the *Haemophilus influenzae* genome indicates that DNA regions amplified in both assays reside in close proximity, yet slight differences in specificity are evident. The HS-causing type-B-specific PCR reported as 100% specific for these isolates.

A multiplex PCR for *P. multocida* has been reported to dramatically decrease the time required for *P. multocida* detection and identification. The use of the multiplex *P. multocida* –specific HS-causing PCR on suspected organisms can confirm the identity and provide a

serotype specific diagnosis within 3-4 hours in comparison with biochemical analysis and conventional serotyping which can take up to a few days (OIE, 2005). Once presumptive or definitive identification has been made, further differentiation of isolates can be achieved by genotypic fingerprinting methods. Restriction endonuclease analysis with the enzyme *Hha*I has proved useful for further characterization of type B HS-serotypes. This would not only provide a wide spectrum of options for rapid diagnosis and detecting latent infections, but will also be useful in establishing the clonality of the outbreak strains (Dziva *et al*., 2008).

CONCLUSIONS

Controlling an infectious disease does not require anything technically innovative or difficult principle, but the effective application of logistics at a high level of efficiency by keeping in mind the three pillars:

➤ FIND INFECTION FAST: Through surveillance and public awareness.

➤ ELIMINATE INFECTION QUICKLY: Through culling & disposal, cleansing & disinfection and compensation.

➤ STOP INFECTION SPREADING: Through bio-security, movement restrictions, public awareness and vaccination.

FURTHER READINGS

Charan, S. and Mahajan, V. M. 1981. Comparison of enzyme-linked immunosorbent assay and haemagglutination inhibition test for the detection of Newcastle disease virus antibodies in human sera. *J Clin Pathol* **34: 90-92**.

Charan, S. and Gautam, O. P. 1984. Applications of enzyme-linked immune-sorbent assays in veterinary medicine: a bibliography. *Vet Res Comm* **8: 255-267.**

De Alwis, M. C.L. 1992. Haemorrhagic septicaemia – a general review. *Br Vet J* **148:**99-112.

Dziva, F., Muhairwa, A. P., and Christensen, H. 2008. Diagnostic and typing options for investigating diseases associated with .*P multocida*. *Vet. Microbiol* **128**: 1-22.

FAO 2011. Good Emergency Management Practices: The Essentials. (Eds) N. Honhold, I. Douglas, W. Geering, A. Shimshoni & J. Lubroth. FAO Animal Production and Health Manual No. 11. Rome.

Kharb, S. and Charan, S. 2010. Immunogenicity of iron-regulated outer membrane proteins of *Pasteurella multocida* (B:2) in mice model. *Indian J Expt Biol* **48:**1181- 1187.

Kharb, S. and Charan, S. 2011. Mucosal immunization provides better protection than subcutaneous immunization against *Pasteurella multocida* (B:2) in mice pre-immunized with the outer membrane proteins. *Vet Res Comm*, **35:**457-461

O.I.E. 2005. Haemorrhagic septicaemia. Manual of diagnostic tests and vaccines for terrestrial animals.

STRATEGIES FOR CONTROL OF BRUCELLOSIS IN ANIMALS IN INDIA

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Brucellosis is a contagious bacterial disease of animals, characterized by abortion in late pregnancy, retention of placenta, reduced milk yield and infertility in some percentage of animals. Premature or still births are also seen. At the time of abortion large amounts of bacteria are excreted in the uterine discharge and placenta which results into contamination of farm premises/ shed/ surroundings/ environment, etc. Animal aborts once but subsequent delivery is normal, however, the new born calf is weak and unthrifty. The disease is not self-limiting because only abortion disappears in subsequent pregnancy otherwise animal remains infected. Hence, misunderstanding of self-limiting of brucellosis develops. As a matter of fact, the infected animals become carrier of the disease. They excrete *Brucella* organisms in milk and also in each delivery. The delivery of infected animals is attended causally and the uterine discharge and placenta are not properly disposed off. Because of this the transmission/spread of disease to in-contact animals increases tremendously.

Main route of transmission of brucellosis in bovines is ingestion (oral). The abortion materials such as uterine discharges etc. are mixed with fodder/ water during routine farm management operations and ingested by the in-contact animals. Chances of spread of disease on dairy farms are very high. Under the prevailing animal husbandry practices on dairy farms, animals are kept in limited area/space this seems to be the main reason why incidence of brucellosis is generally high on dairy farms. In rural areas or villages, farmers use to keep few heads of animals where sufficient space/ area is available for animals. Most often animals are tied up in a defined location and chances of abortion materials reaching to nearby house hold are limited which decreases the chances of spread of disease resulting into low incidence of disease in villages/ rural areas.

Brucellosis in bovines (cattle and buffaloes) is caused by *Brucella abortus*. Epidemiology of brucellosis is complex. Hence, incidence/ prevalence of the disease vary according to animal husbandry practices, environmental conditions, antigenic load in the surroundings, etc. Brucellosis is a herd disease and in endemic situation the number of infected dairy farm increases with time if control program is not in place. Systemic study has never been conducted to determine the incidence of brucellosis in India; however, it could be 1 to 3% in animals in rural areas while incidence on infected dairy farms belonging to private

or Govt. sector could be between 15 and 50 per cent. Hence, strategies for control and monitoring of brucellosis in villages/ rural areas and on dairy farms should be different.

STRATEGIES FOR CONTROL

Three general methods for control of brucellosis in animals are: i) test and slaughter, ii) hygienic measures and iii) vaccination. These are most effective when they are combined. Some developed countries have been able to eliminate brucellosis by rigorously adopting the aforesaid principles. However, situation in under developed and developing countries is otherwise due to several factors. The main constraint is socioeconomics which includes burden on Government exchequer to compensate the animal owner for the cost of positive animal if culled and sent to slaughter. In India the situation is further complicated because of religious sentiments and the cow slaughter is banned in several states of the country. It is an acceptable fact that slaughter of infected population of animals is not economically feasible and can only be afforded by developed country. With these facts and circumstances it is necessary to design control strategies accordingly. The practical approach is to follow sanitary measures strictly on dairy farm. The infected animals at the farm should be maintained and managed separately and production from these animals like milk etc. may be obtained. These animals should be given special attention when they deliver. Because when an infected animal aborts or delivers the uterine discharges, placenta etc. contains a huge numbers of *Brucella* up to 10^{13} organisms per gram of abortion materials which highly contaminates the premises surroundings and environment and often reaches to uninfected animals through many ways of transmission. Hence, placenta and any discharge after delivery should be burnt or buried. The area, premises, etc. should be properly cleaned and disinfected. If special care (sanitary measures) is given to infected animals on delivery/ abortion for the first 10 to 15 days, the chances of spread of disease decrease more than 90%.

1. **Strategy when segregation of infected animals is possible on farm:**

In routine the dairymen/ farmers maintain infected and uninfected animals together. As suggested, infected animals should be maintained separately at the farm. Maintaining infected animals separately results in no or minimum spread of the disease to other animals. Secondly, produce form this population could be obtained which will minimally affect the economy of the dairymen. The negative population which includes calves as well as adult animals should be vaccinated. Segregation of infected animals and vaccination of negative animals is the most feasible strategy to control brucellosis in India in the prevailing

socioeconomic scenario. This strategy would be very effective on animal farms and provides rapid control of the disease.

2. Strategy when segregation of infected animals is not possible on farm:

There may not be many takers of segregation strategy for many reasons. Particularly dairymen feel that keeping animals separately is a difficult task for them. Secondly, many dairymen do not want to disclose that brucellosis is present on their farms. Thirdly, when strength of animals is below 10 or 20 on the farm then separate management is difficult. Under these circumstances whole herd vaccination is suggested. This method would take sufficiently long time to control the disease at the farm.

3. Strategy for villages:

At village level separation of infected animals from the uninfected ones is practically impossible because in villages farmers keep few head of animals. Moreover testing animal population in villages requires big infrastructure, huge technical human resource and lot of funds. In view of this, mass vaccination could be done in villages without testing of animals. If above strategies are adapted then brucellosis in bovines could be prevented, losses occurring due to brucellosis could be reduced and incidence of the disease could be brought down slowly.

VACCINATION

The vaccines used to prevent and control brucellosis in large ruminants (cattle and buffaloes) and small ruminants (Sheep and goats) are different. The vaccine for cattle and buffaloes is *Brucella abortus* S19 and for sheep and goats is *Brucella melitensis* Rev.1. Unfortunately, *Brucella melitensis* Rev.1vaccine nor available neither produced in India despite a high prevalence of brucellosis in sheep and goats. However, vaccine for cattle and buffaloes is available from the Govt. organizations or private manufacturer.

Brucella abortus S19 vaccine is a reference vaccine and has been used world over to control brucellosis. This vaccine was developed in USA during 1930 -1934 and thereafter it was successfully used. Many other countries like U.K, France, Germany, etc. used this vaccine. Use of S19 vaccine is prescribed by the OIE. Till today the calf-hood vaccination with S19 is recommended to prevent and control brucellosis in India. Adult vaccination is not practiced in the country. Whereas in India it is necessary to vaccinate calves as well as adult animals together because on infected dairy farms the prevalence of brucellosis is very high and under such conditions calf-hood vaccination alone is not of much help. Adopting the

calf-hood vaccination only is a truncated policy. The situation on infected dairy farms in the country is alarming which requires immediate intervention. The routes of vaccination are:

1. Full dose vaccination of calves (Calf-hood vaccination):

This method of vaccination can be used for calves up to the age between 4 and 8 months. One full dose of vaccine is comprised of $4 - 8\times10^{10}$ live organisms which should be given subcutaneously in the neck region of calf. The vaccinated calf becomes sero positive following vaccination which can be tested by a simple Rose Bengal Test (RBT). However, the vaccinated calf becomes sero negative in RBT after 3 to 4 months or up to the puberty. The method could be used up to one year of age without much interference of sero positivity at the time of puberty and provides life-time immunity. No need to give booster doses.

2. Full dose vaccination of adult animals:

This method could be for animals above one year of age. In vaccinated animals a rise of antibody titre occur and animals remain serologically positive for long periods of time. In addition, the full dose of S19 vaccine may cause abortion in few pregnant animals and some animals in lactation may excrete the vaccine strain in milk. These problems could be avoided using reduced dose of S19 vaccine in adult animals.

3. Reduced dose vaccination of adult animals:

The reduced dose of S19 vaccine could be given to adult animals by subcutaneous route or by conjunctival (Ocular) route. Both the routes are recommended by OIE. The immunity provided with reduced dose of vaccine is as solid as given by full dose.

i. Subcutaneous route of adult vaccination: The reduced dose of S19 vaccine for subcutaneous route is 3×10^{8}-10^{9} organisms for any adult animal. With this dose also animals become serologically positive and some of them may abort and excrete vaccine strain in milk. However, the reduced dose is safer than full dose adult vaccination.

ii Conjunctival route of adult vaccination: The dose of S19 vaccine for conjunctival (ocular) route of vaccination is 5-8×10^{9} organisms. A booster dose is necessary after 4 months. This reduced dose should be in 0.1ml. This route of vaccination solves the problem of abortion and post vaccinal antibody titres (no or very weak antibody response, persists for some weeks). The animals become serologically negative after some time of vaccination. Secondly, vaccinated animal does not excrete vaccine strain in milk. This method is much safer for adult animals including pregnant and lactating ones.

SEROTYPING OF FOOT-AND-MOUTH DISEASE VIRUS BY SANDWICH ELISA

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The double antibody sandwich ELISA for typing of FMD virus being used in our laboratory was developed by Venkataramanan and his colleagues (1995) at Central FMD virus typing laboratory, IVRI Campus, Mukteswar, Nainital. The technique is very specific, sensitive, less time consuming and reproducible. A large number of samples can be analysed in a single day and the results can be obtained within three hours. The principle of the test is that antigen is first bound to the FMD type specific coating antibody (raised in rabbit) already adsorbed on an ELISA plate. This bound antigen is detected again by type specific tracing antibody raised in guinea pigs. This specific serological reaction is further visualized by the anti-guinea pig IgG – Horseradish Peroxidase conjugate followed by colour development by addition of substrate.

Materials Required:

i) Reagents:

1. FMD virus antigens	Inactivated BHK-21 cell culture passaged standard FMDV types O, A, C and Asia 1.
2. Samples to be tested	In the form of 10% suspension in PBS/ BHK-21 passaged material
3. Anti FMDV coating sera	Type specific anti-146S FMDV rabbit sera against types O, A, C and Asia 1.
4. Anti FMDV tracing sera	Type specific anti-146S FMDV guinea pig sera against types O, A, C and Asia 1.
5. Anti guineapig conjugate	Rabbit/goat anti guinea pig Immunoglobulin (IgG)-HRPO conjugate.
6. Chromogen	OPD

ii) Equipments:

1. Multichannel pipettes of variable range (50-200 μl); Single channel pipettes of variable range from 2-20, 20-200 and 200-1000μl and good quality tips.

2. Microplates- 96 wells flat bottomed high binding ELISA plates.

3. Incubator, Refrigerator, Freezer.

4. Pestle-Mortar, Single Pan Balance, Timer and Absorbent Pads

5. ELISA Plate washer and ELISA Reader

Test Proper

1. Before setting the test, optimum dilutions of type specific coating sera and anti-guinea pig IgG-HRPO conjugate are determined by checker board titrations.

2. Mark the wells of ELISA plate as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
O												
A												
C												
AsiaI												
O												
A												
C												
AsiaI												

-ve   +ve   ←--------------------- Clinical Samples --------------------→
Antigen Control

3. Dilute all the four coating sera optimally with coating buffer. Dispense 50μl/well of type 'O' antisera into the wells of A and E row, type 'A' into B and F, type 'C' into C and G and type 'Asia-1' into D and H rows.

4. Cover the plate and incubate at 37ºC for 2 hours. After incubation, keep the plate as such at 4ºC overnight.

5. Wash the plate by filling the wells with the washing buffer using ELISA plate washer. Discard the contents of the plate using an abrupt downward hand motion. Repeat this process 5 times. Blot it forcefully on an absorbent pad of filter paper.

6. Add 50 μl/well of supernatant from uninfected BHK-21 cell culture in all the wells of column 1. The Column 1 serves as negative (-) antigen control.

7. Dispense 50 μl/well of inactivated type 'O' antigen in the A and E well, type 'A' in the B and F well, type 'C' in the C and G well and type 'Asia 1' in the D and H well of the column 2. Column 2 serves as a positive (+) antigen control.

8. Dispense 50 μl/well of test antigen samples in column 3 and onwards.

9. Incubate the plate at 37ºC for 1 hr, discard the contents and wash as described earlier.

10. Optimally dilute the tracing sera in blocking buffer. Dispense 50 μl/well of the optimally diluted type 'O' in all the wells of rows A and E; type 'A' in all the wells of rows B and F; type 'C' in all the wells of rows C and G; and 'Asia 1' tracing antisera in all the wells of rows D and H.

11. Incubate the plate at 37ºC for 1 hr, discard the contents and wash as described earlier.

12. Optimally dilute the anti-guinea pig IgG-HRPO conjugate in blocking buffer and dispense 50 μl/well in all the wells of the microtitre plate.

13. Incubate the plate at 37ºC for 1 hr, discard the contents and wash as described earlier.

14. Add 50μl/well of freshly prepared substrate solution in all the wells of the microtitre plate and incubate the plate in the dark at 37ºC for 5-10 min till the colour develops in positive antigen control wells.

15. Stop the reaction by adding 50μl/well of freshly prepared 1M H_2SO_4 in all the wells of the microtitre plate.

16.   Measure the optical density (OD) of each well at 492 nm wavelength in ELISA reader.

Interpretation of Results:

The interpretation of the results is done on the basis of corrected OD value (OD of test well - OD of negative antigen control well). If the OD value of the test antigen with a particular FMDV type serum is > 1.0, then the antigen in question can be identified as belonging to that type. The OD in other wells of the test sample should be at least three times less.

Bibliography:

Venkataraman, R. 1995. Sandwich ELISA for identification of virus type in field materials. Manual published for the SAARC workshop on foot-and-mouth disease (July 3-7, 1995).

Buffers and Reagents

1. Coating Buffer (100 ml)

Sol. A		**Sol. B**	
Na_2CO_3 0.212 gm		$NaHCO_3$	0.336 gm
D.W.	10 ml	D.W.	20 ml

Sol. A 8 ml + Sol. B 17 ml + D.W. to make 100 ml

2. Blocking Buffer (100 ml)

LAH		3 gm
Rabbit serum		5 ml
New Born Calf Serum	5 ml	
PBS to make		100 ml

3. Washing Buffer (5 Litres)

$NaH_2PO_4 .H_2O$	7.7 gm	
Na_2HPO_4		38.65 gm
NaCl	15.2 gm	
Tween 20		2.5 ml
D.W. (to make)	5 litre	

4. Substrate Buffer (1 litre)

Citric Acid		7.3 gm
Na_2HPO_4		9.468 gm
D.W. to make	1 litre	

5. Substrate solution (15 ml)

OPD	10 mg
Substrate Buffer	15 ml
H_2O_2 (3%)	8 μl

6. Stopper solution 1 M H_2SO_4 (100 ml)

H_2SO_4	5.55 ml
D.W. to make 100 ml	

LIQUID PHASE BLOCKING ELISA FOR DETECTION OF ANTIBODIES AGAINST FMD VIRUS SEROTYPES

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Laboratory confirmation of Foot-and-Mouth Disease Virus (FMDV) antibodies in the serum is usually been undertaken with the complement fixation test (CFT), Serum Neutralisation test (SNT) or Enzyme Linked Immunosorbent Assay (ELISA). At the OIE/ FAO World Reference Laboratory for FMD, the preferred procedure for the detection and identification of antibodies against particular FMD viral serotype is the ELISA. The most commonly used solid phase immunoassays for the measurement of antibodies involve the passive absorption of antigens to a support *i.e.* to well of microtitre PVC plate directly or indirectly via an immunoglobulin which is attached directly to the plate well. Both methods may alter the presentation and / or confirmation of the antigen. A liquid phase blocking ELISA detects antibody/antigen reactions (opsonization; neutralization of pathogen infectivity) which are of direct relevance to the protective immune response *in vivo* and would not alter the structure and reactivity of either antigen or antibody as all the reactions occur in liquid phase.

The liquid phase blocking ELISA (LPB-ELISA) test is routinely used for the quantitation of FMD virus specific protective antibody level in the animals following vaccination or natural infection. The antigen- antibody reactions are carried out in suspension and the homologous antibodies, if present in the test serum, block the antigen from being detected by guinea-pig serum. A series of 2- fold dilutions of test serum are mixed with an equal volume of a predetermined dose of virus and allowed to react overnight at 4^0C. Next day, the free antigen (not blocked by the antibodies in the test serum) is trapped to the wells of the ELISA plates by the pre coated (type-specific) rabbit antibodies. Subsequently, the presence of antigen is traced by adding pre-titrated guinea-pig (type-specific) serum and anti-guinea-pig-HRPO conjugate which can be detected by adding substrate with extensive washing after each step. Substrate (color) reaction is stopped and the plate is read at 492 nm. Antigen control is kept for comparison with the test wells. Antigen control contains antigen without test serum. Back-ground is kept to find out if there is any nonspecific rabbit (coating) and guinea-pig (tracing) antibody binding in the test.

Materials Required: As mentioned in double antibody sandwich ELISA

Test Procedure:

1. ELISA plates are coated with 50μl/well optimally diluted rabbit anti FMDV type specific sera in coating buffer. Plates are covered and incubated at 37ºC for 1h and left at 4ºC overnight.

2. In U-bottomed multiwell plates (carrier plates) 40μl/well in duplicate, two fold serial dilution of each test serum is prepared starting at 1:16. To each plate, 40μl/well of optimally diluted homologous viral antigen is added and the mixture is left overnight at 4ºC. The addition of the antigen increases the starting serum dilution to 1:32.

3. Next day wash the coated plate by filling the wells with the washing buffer. Discard the contents of the plate using an abrupt downward hand motion. Repeat this process 3 times. Blot it forcefully on an absorbent pad of filter papers.

4. Then 50μl of serum-antigen mixtures are transferred from the carrier plate to the ELISA plates already coated with anti-FMDV type specific rabbit serum as shown in table below. Incubate at 37ºC for 1 hr.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ag control											
B	+ve serum											
C	-ve serum											
D	blank											
E	+ve serum											
F	in serial											
G	two fold											
H	dilutions											

←---------------------- **Test serum samples** --------------------→

5. The plates are again washed as described. The anti-FMDV type specific tracing sera raised in guinea pig and optimally diluted in blocking buffer is dispensed @ 50 μl/well to the respectively coated plate.

6. Incubate the plate at 37ºC for 1 hr, discard the contents and wash as described earlier.

7. Optimally dilute the anti-guinea pig IgG-HRPO conjugate in blocking buffer and dispense 50 μl/well in all the wells of the microtitre plate.

8. Incubate the plate at 37ºC for 1 hr, discard the contents and wash as described earlier.

9. Add 50μl/well of freshly prepared substrate solution in all the wells of the microtitre plate and incubate the plate in the dark at 37ºC for 5-10 min till the colour develops in positive antigen control wells.

10. Stop the reaction by adding 50μl/well of freshly prepared 1M H_2SO_4 in all the wells of the microtitre plate.

11. Measure the optical density (OD) of each well at 492 nm wavelength (λ) using an ELISA reader.

Interpretation of Results

Mean OD of background wells is subtracted from OD of each well for calculation. Percent reactivity against each serum dilution is calculated as follows:

$$\% \text{ reactivity} = \frac{\text{Mean OD of the test wells}}{\text{Mean OD of the antigen control wells}} \times 100$$

Titre of serum samples is expressed as the reciprocal of the serum dilution giving 50% OD as compared to the antigen control, or in other words, reciprocal of the serum dilution, which inhibits 50% of the binding of guinea-pig serum to the homologous virus.

Bibliography

Liquid Phase Blocking ELISA: Bench Protocol. Project Directorate on FMD, IVRI Campus, Mukteswar-Kumaon, Nainital.

MONOCLONAL ANTIBODY BASED ELISA FOR THE DETECTION OF FOOT AND MOUTH DISEASE CARRIER ANIMALS

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Foot-and-Mouth disease (FMD) is one of the most feared viral diseases of cloven hoofed animals: most prevalent in cattle and buffaloes followed by sheep, goats and pigs. The disease is highly contagious, spreads very fast and listed under list A diseases of Office International des Epizooties. The disease has debilitating effects, including weight loss, decrease in milk production, loss of draught power, resulting in a loss of productivity for a considerable time.

Foot-and-mouth disease virus (FMDV; which belongs to genus *Apthovirus* of family *Picornavirideae*) may lead to establishment of in-apparent persistent infection in both vaccinated and naïve animals, mechanism of which is still unknown (Sutmoller et al., 2003). These animals from which the FMDV is recovered at or beyond 28 days post-infection from oropharyngeal fluid (OPF) are called carrier animals and these may be responsible for outbreaks of the disease among in-contact susceptible animals (Sutmoller *et al.,* 2003). The virus is recovered intermittently from OPF during persistence up to several months (Haas, 1997) or even up to years (Salt, 1993). The identification of FMD carrier animals is of paramount importance to participate in international trade of animals and their products (OIE, 2004).

The current FMD vaccine available commercially for FMD prevention and control is an inactivated whole FMDV particle mixed with an oil adjuvant. When animals are immunised against such a vaccine, they mount antibody response only against outer coat (structural) proteins of the virus. On the other hand, when an animal becomes infected with FMDV, antibodies also develop against non-structural proteins (NSPs: viral polymerases and proteases) of the virus as the virus replication takes place inside host. The conventional diagnostic tests (e.g. ELISA) detect antibodies only against structural proteins and so are unsuitable for differentiation of infected and vaccinated animals (DIVA strategy). Detection of antibodies against non-structural proteins might be a tool for DIVA strategy. Immunised animals that are infected and subsequently become carriers will also develop antibodies against NSPs, allowing carriers to be identified in vaccinated stock.

In this chapter, laboratory protocol for monoclonal antibody based NSP-ELISA has been described for differentiation of FMDV infected and vaccinated animals.

Non-Structural Proteins based ELISA:

The wells of the ELISA plates are coated with the anti-FMDV NSP monoclonal antibodies followed by incubation with FMDV-NSP antigens. The FMDV-NSP antibodies in the field serum samples, if any, are detected by bound NSP antigens coated on the plate. Subsequently, the reaction is visualized by the anti-bovine IgG-horseradish peroxidase conjugate and color development by adding suitable substrate (OPD). The materials required and protocol of the test is as described below:

Materials Required:
1. Purified NSP
2. Monoclonal antibodies against NSP
3. Anti-bovine peroxidase conjugate
4. Substrate (OPD)
5. Ninty six well ELISA plate
6. Carbonate-Bicarbonate buffer pH 9.6
7. Washing buffer
8. Blocking buffer (PBS containing 10% horse serum, 5% Nonfat dry milk and 0.1% Tween20)
9. 4M H_2SO_4
10. ELISA reader (492 nm)

Test procedure:

1. Coat the 96 well ELISA plate with monoclonal antibodies (50μl per well) in Carbonate-bicarbonate buffer at 4°C overnight.

2. Wash the plates 3 times with washing buffer

3. Dilute the purified antigen (NSP) in PBS (1:200) and add 50 μl into each well. Incubate the plates for 1 hr at 37°C

4. Wash the plates 3 times.

5. Mark the wells as test serum wells, positive serum control wells, negative serum control wells, and antigen control wells (Keep for each control at least two wells).

6. Dilute 1:16 of each of test serum, positive and negative control serum in blocking buffer and give 50 μl to the corresponding well. Incubate the plate for 1hr at 37°C.

7. Wash the plates 3 times.

8. Add 50 μl of the anti-bovine peroxidase conjugate (1:2000, diluted in blocking buffer) into each well. Incubate the plates at 37°C for 1 hr.

9. Wash the plates 5 times.

10. Add 50 μl of the substrate containing 0.05% H_2O_2 into each well. Incubate the plates for 15 minutes at room temperature in dark.

11. Stop the reaction by adding 50 μl of 4M H_2SO_4.

12. Read the OD at 492 nm.

Interpretation of the results:

OD (corrected) = [OD of sample] – [OD of antigen control (without serum)]

% Positivity = [OD (corrected) of test serum] / [OD (corrected) of positive serum] X 100

% Positivity ≥ 20%: (Particular sample is positive in antibodies against NSP)

% Positivity ≤20%: (Particular sample is negative in antibodies against NSP)

Test is invalid if the percent positivity of the negative control serum is more than 20%

Bibliography:

Haas, B. 1997: Report of a session of the research group of the standing technical committee of the European Commission for the control of Foot and Mouth Disease, Brasov, Romania, 23-27 September 1997, 52-54.

Kumar, N. 2006. Molecular and immunological studies on persistence of FMD virus. Ph.D. thesis submitted to CCS Haryana Agricultural University, Hisar, Haryana.

OIE. 2004: Manual of Standards for Diagnostic tests and Vaccines, Fifth ed. OIE, Rue de Prony, 75017 Paris, France.

Salt, J. S. 1993: The carrier state in foot and mouth disease: an immunological review. *Br. Vet. J.* **149:** 207-223.

Sutmoller, P., Barteling, S. S., Olascoaga, R. C., Sumption, K. J. 2003. Control and eradication of foot-and-mouth disease. *Virus. Res*., **91:**101-44.

RECOMBINANT 3AB3 NSP ELISA FOR DIFFERENTIATION OF FOOT-AND-MOUTH DISEASE INFECTED AND VACCINATED ANIMALS

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Foot-and-mouth disease (FMD) is a highly contagious and economically important disease affecting all the ruminants and pigs including many wild life species. The disease has serious impact on international trade of animals and their products including germ-plasm. Besides, there are indirect losses in terms of severe trade restrictions on account of WTO/GATT agreement. In India, where world's largest livestock population exists, FMD is endemic, occurs in all parts of the country throughout the year and a leading cause of loss of livestock economy.

The FMD vaccine consists of inactivated virus particles and therefore induces antibodies to the structural proteins of the virus provided the vaccine is free from non structural proteins (NSPs). Replication of virus during infection results in the production of a number of NSPs that can be exploited as markers for detection of FMD virus infection. Hence while antibodies to the capsid proteins are induced by both vaccination and infection, antibodies towards NSPs are elicited only during FMD virus infection.

The conventional serological tests for FMD detect antibodies to the structural proteins of the virus induced by vaccination as well as infection. These tests do not differentiate between the infected animals from those that have been vaccinated. **D**ifferentiation of **I**nfected and **V**accinated **A**nimals (DIVA) is important during serological surveys to detect evidence of infection, as a follow up to intensive vaccination campaign in FMD endemic countries and determination of subclinical carrier status (where the virus is actively replicating but without conspicuous signs) for import/export serology.

Recently, in FMD the DIVA strategy has been based on the detection of the antibodies to NSPs, in particular 3ABC or 3AB as a reliable indicator of FMD virus infection or exposure. Anti-3AB3 antibodies have been detected in animals where no virus or occasional virus isolation from oropharyngeal fluid could be done. The 3AB3 NSP based indirect ELISA for DIVA is designed, developed and evaluated completely using in-house produced and standardized reagents at the Central Laboratory, Project Directorate on FMD, Mukteswar, very much in toe with the OIE approved test as a companion test for inactivated vaccines.

PRINCIPLE:

Seroconversion against NSPs (3AB3) is observed 10-14 days after FMD virus infection. On the other hand, if the animal is vaccinated with inactivated polyvalent FMD vaccine, no anti-NSP immune response is elicited. The differential induction of anti-NSP antibody is exploited to distinguish between FMD virus infected and vaccinated animals. In this DIVA test reactivity of anti-3AB3 antibodies in the serum of an infected animal (bovine species only) is assessed against purified recombinant 3AB3 (~38 kD) NSP in an indirect ELISA format. A sample producing OD value of more than the fixed cut-off ratio {(test serum sample mean OD/positive control serum mean OD) x 100 i.e., percent positivity value or PP value $\geq$ 40%} is diagnosed as positive for FMD virus infection.

PROTOCOL:

1. Dissolve the freeze dried recombinant protein in the vial with 1 ml of coating buffer and add another 10 ml of coating buffer to it (One single-use vial is sufficient for coating two 96-well ELISA plates). The reconstituted protein vial should be used for coating immediately without storing. Reconstitute the freeze dried vials of positive (one month postinfection serum) and negative (one month post vaccination serum) control serum with distilled water (as appears on the sticker) and store at -20^0C after reconstitution in single use aliquots. Dissolve the content of freeze dried vial carrying *E. coli* lysate in 70 µl of PBS and store at -20^0C.

2. Coat 96-well polystyrene (Nunc Maxisorp) Immuno plates with the diluted recombinant protein @ 50 µl per well (40 ng of purified recombinant protein per well). Tap the plate gently from all sides and incubate (refrigerate) the plate at 4^0C for overnight.

3. Remove the plates from the refrigerator and thaw them at 37^0C for 15 minutes.

4. In a low protein binding Perspex plate dilute the test and the supplied negative and the positive control serum @ 1:20 in diluent buffer. Prepare a total volume of 220 µl of diluted serum so that 100 µl of the mixture can be transferred to the coated ELISA plates in duplicate. On a coated ELISA plate a total of 45 test sera samples, one positive and one negative control serum and two background controls can be accommodated. For background controls only 100 µl of diluent buffer is dispensed without any serum.

5. Give three continuous wash (no hold time) with wash buffer.

6. Transfer 100 µl of the serum and diluent buffer mixture from Perspex plate to the ELISA plate in duplicate wells. Incubate for 1 hour at 37^0C in a plate shaker with 20-30 rpm or tap the plate gently from all sides at every 15 minute intervals.

7. Give three washes of 3 minute soak period each.

8. Dispense anti-bovine-HRP conjugate diluted in the diluent buffer (1:2000) @ 50 µl per well and incubate for 30 min at 37^0C in a plate shaker or 1 hour at 37^0C and tap the plate gently from all sides at every 15 minute intervals. 6ml of diluted conjugate solution is sufficient for an ELISA plate.

LAY OUT OF THE ELISA PLATE FOR INDIRECT DIVA ASSAY

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1	1	2	2	3	3	4	4	5	5	6	6
B	7	7	8	8	9	9	10	10	11	11	12	12
C	13	13	14	14	15	15	16	16	17	17	18	18
D	19	19	20	20	21	21	22	22	23	23	24	24
E	25	25	26	26	27	27	28	28	29	29	30	30
F	31	31	32	32	33	33	34	34	35	35	36	36
G	37	37	38	38	39	39	40	40	41	41	42	42
H	43	43	44	44	45	45	PC	PC	NC	NC	BG	BG

TS: Test Sample; PC: Positive Control; NC: Negative Control; BG; Background

9. Give three washes of 5 minute soak period each.

10. Add freshly prepared substrate solution @ 50 µl per well and incubate for 15 minutes at 37^0C without shaking. Then stop the colour reaction by adding 1M H_2SO_4 @ 50 µl per well.

11. Measure the optical density of each well at the wave length of 492nm (Reference 620nm).

C. Performance and Interpretation:

The test is to be considered valid provided the mean absorbance of the positive control wells is not less than 0.8. Likewise the plate has to be rejected if the mean absorbance of the supplied negative control serum is > 0.3. The O.D. in back ground control wells should be< 0.01.

To reduce inter-run variation due to differences in absolute absorbance between runs, final results for each test serum needs to be expressed as the PP value {(test serum sample mean OD/positive control serum mean OD) x 100 i.e., percent positivity value or PP value ≥ 40%},), calculated by dividing the reaction of the test serum by that of the positive control serum and then multiplying with 100. The results should be interpreted based on the following cut-off:

1. 3AB3 NSP reactivity positive: If PP value is more than 40%

2. 3AB3 NSP reactivity negative: If PP value is less than 40%

Composition and Preparation of Various ELISA Buffers

Washing Buffer (1X): 1 Lit

$NaH_2PO_4.2H_2O$	0.39g (0.0025 M)
$Na_2HPO_4.2H_2O$	1.336g (0.0075M)
NaCl	29.325g (2.93%w/v)
Tween- 20	500 µl (0.05% v/v)
Distilled Water to make	1 litre

Check pH. If required adjust pH with 1 N NaOH as 7.2-7.4

1. Coating (Carbonate-bicarbonate) Buffer: 100 ml

One Carbonate-bicarbonate capsule (Sigma) dissolved in 100 ml distilled water.

Check the pH as 9.6. Store at 4^0C.

2. Diluent Buffer: 20 ml

Skimmed Milk Powder	1g (5% w/v)
Chicken Serum	2 ml (10 % v/v)
Healthy *E.coli* lysate (BL 21 DE3 pLysS strain)	2.5 µl (0.01% v/v)
Washing Buffer	Upto 20 ml

OR

10X casein based blocking buffer (Sigma)	2ml (1X)
Chicken Serum	2 ml (10 % v/v)
Healthy *E.coli* lysate (BL 21 DE3 pLysS strain)	2.5 µl (0.01% v/v)
Washing Buffer	Upto 20 ml

3. Substrate (phosphate- citrate) Buffer: 100 ml

One phosphate- citrate tablet (Sigma) dissolved in 100 ml distilled water. Check pH as 5.0. Store at 4^0C.

4. Substrate Solution: 7.5 ml Prepare immediately before use)

Substrate (phosphate- citrate) Buffer	7.5 ml
OPD	5 mg
H_2O_2 (30 %)	4 µl

5. Stopper Solution (1 M H_2SO_4): 100 ml

H_2SO_4 : 95-97%	5.55 ml
Distilled Water	94.45ml

SERUM NEUTRALIZATION TEST FOR DETECTION OF ANTIBODIES AGAINST FOOT- AND- MOUTH DISEASE VIRUS

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Neutralisation test was the first technique to be used for detecting antibodies against viruses and is still the gold coin for measurement of antiviral antibodies. This technique is very specific and sensitive and is based on the principle of neutralisation of viruses by the antibody molecules by stereo-chemically inhibiting the combination of virus with the receptor site at cell surface, thereby, preventing penetration and subsequently intra-cellular multiplication.

A number of techniques have been employed for the estimation of serum antibody titres in FMD vaccinated animals. Martin and Chapman (1961) used tissue culture colour test (a modification of SNT) for assaying virus-neutralizing antibody to FMD in cattle. Serum neutralisation test (SNT) has been used to evaluate the immunity status of animals against FMDV as well as for typing and sub-typing of FMDV. Although, SNT is quite cumbersome, time consuming and requires cell culture facilities, still it has got an upper edge over ELISA, because SNT antibody titres can be well correlated with protection as compared to ELISA in case of FMD because most sensitive LPB-ELISA detect both immunoglobulin and non-immnuoglobulin competitions. Also SNT is of utmost importance where serum neutralization titres are quite low (in pigs may range from 1:4 to 1:32) because SNT is more sensitive than ELISA. The present communication describes the SNT for demonstration of FMDV antibodies.

Materials Required:

Standard FMDV types O, A, and Asia1
Baby Hamster Kidney (BHK-21) cell line
Sera sample to be tested
Millipore filters (0.22µm), Micropipettes
Fetal bovine serum (FBS)
Tissue culture medium (M-199 containing 20mM HEPES buffer, 1mM Glutamine, 0.5% Tryptose phosphate broth, antibiotics and 2%FBS)
Hank's Balanced Salt Solution (HBSS)
Flat bottom 96 wells tissue culture plates

Preparation of antigen: Antigens of FMDV serotypes are prepared in BHK-21 cell monolayer. The cells are infected at a MOI of 1:100 and harvested when they show 90% virus-specific CPE. The virus is extracted from the cells by freezing and thawing. Cell debris

is removed by centrifugation at 10,000 g for 20 min at 4°C. The preparation is used as an antigen for a particular serotype in SNT after virus titration.

Virus titration:

Different virus serotypes are titrated in 96-well flat bottomed tissue culture plates using BHK-21 cells.

1. Ten fold dilutions of virus serotype are prepared in maintenance medium and 50μl of each dilution was dispensed in triplicates.

2. Then, 50 μl of growth medium is added to each well and plates are kept in CO_2 chamber at 37°C for 1 h.

3. 100μl of freshly prepared trypsinized BHK-21 cell suspension (1×10^6 cells/ml) is then added to each well.

4. Appropriate cell controls without virus are kept in each plate.

5. The plates are sealed with pressure tolerant cellophane tape and again incubated in CO_2 chamber at 37°C for 24-72 hrs.

6. The plates are examined for presence of CPE daily under inverted microscope.

7. The cells are stained with 0.5% crystal violet.

8. The virus titres are calculated in term of $TCID_{50}$/ml according to the method of Reed and Muench (1938). The proportionate distance (PD) between the two dilutions, where the 50% end Point lies is calculated by the formula.

Table 1: Number of wells showing CPE in microtitre plate and calculation of TCID50

Log dilution of virus	Number of wells		Accumulated totals		% of wells with CPE
	With CPE	Without CPE	With CPE	Without CPE	
2	5	0	25	0	100
3	5	0	20	0	100
4	5	0	15	0	100
5	4	1	10	1	91
6	3	2	6	3	67
7	2	3	3	6	33
8	1	4	1	10	10
9	0	5	0	15	0

Proportionate distance =
(Interpolation formula)

$$\frac{\% \text{ CPE above } 50\% \ - \ 50\%}{\% \text{ CPE above } 50\% \ - \ \% \text{ CPE below } 50\%}$$

Substituting : $\frac{67 - 50}{67 - 33} = 0.5$

Corrected Interpolative value: 0.5 X dilution ratio = 0.5X (-1) = -0.5

The endpoint dilution associated with 50% CPE is located between 10^{-6} and 10^{-7} dilution. The Log_{10} of the 50% endpoint dilution is estimated by adding corrected interpolative value to the dilution above 50%. The 50% end point titre in the present case will be (-6 + (-0.5)) $10^{6.5}$.

Serum neutralization test: The test is performed by constant virus and varying serum technique as per recommendation of OIE (O.I.E. Manual, 2000).

1. Before testing, serum samples are filtered and heat in-activated at $56^{\circ}C$ for 30 min.
2. Serum samples so treated are tested in triplicates in flat bottom 96-well tissue culture plates by making serial two fold dilutions from 1:4 to 1:32.
3. From each well 50 µl is transferred to three different tissue culture plates meant to be used for three different serotypes (O, A and Asia-I).
4. To these plates, 50 µl of a particular virus serotype diluted to 100 $TCID_{50}$ in MEM maintenance medium is added.
5. The cell controls are kept without virus in each plate. Appropriate virus controls were also kept.
6. The plates are sealed with pressure tolerant tape and incubated at $37^{\circ}C$ for 1h.
7. After 1 h, 100 µl of BHK-21 cell suspension is added to each well having cells @ 1 x 10^6 cells/ml.
8. The plates are again sealed and incubated at $37^{\circ}C$ for 2-3 days.
9. The plates are examined daily for appearance of CPE. When 90% CPE have reached, the plates are unsealed; wells media discarded with a jerk and the cells are stained with 0.5% crystal violet dye in 10% formal saline for 1/2 hr. After this, the plates are washed with distilled water and kept inverted on filter paper.

Interpretation of Results: The results are noted macroscopically. Positive wells, where the virus has been neutralized and the cells remained intact are seen to contain blue-stained cell sheet. The negative wells, where virus has not been neutralized, remained unstained.

Estimation of titre
The SN Ab titres are expressed as log_{10} SN_{50} by Karber method (1931).

Log_{10} SN_{50} = L-d (s-0.5)

L : log_{10} of the most concentrated serum dilution tested
d : log dilution factor
s : Sum of proportions of culture protected.

VIRUS NEUTRALIZATION TEST FOR EQUINE HERPES VIRUS-1 DIAGNOSIS

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Principle:

Antibody with specificity for certain antigens in or on virus particles can neutralize the infectivity of the virus. Virus neutralizing (VN) antibody is highly specific and is the antibody responsible for the protective effect of immune serum. The measurement of the antibody on the herd level gives the immune status of the herd against a given virus. Hence, it is also used to measure the effectiveness of a vaccine. The VN antibody against equine herpes virus-1(EHV-1) infection in serum samples are assessed by two methods, namely i) constant virus-variable serum (CV-VS) and ii) constant serum variable virus (CS-VV) method.

Materials Required:

Equipments: Sterile flat bottomed 96 wells microtitre plates with lids, single- and multi-channel micropipettes (5-50 μl and 50-200 μl) with sterile tips, 37^0C CO_2 incubator, metal storage racks for vials, 56^0C water bath, $–70^0C$ deep freezer, Fridge, sterile media bottle, sterile reagent troughs, inverted microscope, marker pen and timer.

Reagents: viral antigen, test sera, known positive serum sample, 10% Growth medium for cell culture, RK 13 Cells Or cells of equine origin at $1.8x10^5$/ml

Protocol for Virus neutralization test (VNT):

Having determined the $TCID_{50,}$ VNT is performed by either of the two methods mentioned above (CV-VS or CS-VV) . Each test has its own merit. The result of first method is expressed in term of antibody titre while the result of the second method is expressed in term of virus neutralization index. **In our routine practice we are following the CV-VS method for EHV-1 sero surveillence programme.**

Procedure:

1. For each serum to be tested take 100μl of the serum and dilute it 1:4 in the serum free tissue culture medium. Inactivate the diluted serum at 56^0C for 30 min. in the water bath.

2. In a 96 well tissue culture plate, add 25 μl of serum free tissue culture medium (SF-MEM) to all the wells except all 12 wells of row no A.

3. For one serum 2 rows are used if the serum is tested for sero-surveillence. Put the heat inactivated and 1:4 diluted serum no 1 in column 1 and 2 of row A and B. For second serum sample put 1:4 diluted serum in column 3 and 4 of row A-B and similarly for serum sample no 6 put 1:4 dilution of serum in the column 11 and 12 row A-B. In one plate six field serum sample will be tested.

4. Make double fold dilution of the serum as shown in Table 3 from row B. In every step of dilution change the new tips of the multi channel pipette.

5. When diluting the last row i.e. H row then discard 25 μl of the diluted serum from each well of this row.

6. Add 100 $TCID_{50}$/ 25 μl of the virus in all the wells of rows B to H.

7. On the second tissue culture plate titer this virus suspension inoculating 2-fold dilution on to 5 wells per dilution and check the actual virus titre used in this test.

8. In row A add 25 μl of serum. It will work as serum control to ensure that the serum samples are not toxic.

9. Separate control wells should include titration of both a negative and positive horse serum of known titre, cell control(no virus), virus control (no horse serum), and a virus titration to calculate the actual amount of virus used in the test.

10. Incubate the serum –virus mixture at 37^0C for 60 min.

11. Add 100 μl of a cell suspension susceptible to the viruses under study (generally 10^5 to $1.8x10^5$ cells/ml) in tissue culture growth medium containing 15% FCS.

12. Incubate at 37^0C in 5% CO_2 tension for 4 to 8 days depending on the virus used in the test system.

13. Examine the plates microscopically for CPE and record the results on a worksheet.

14. The absence of CPE in the wells indicates the neutralization of the virus.

15. Calculate the neutralization titre for each test serum, and compare acute and convalescent phase serum titre from each animal for four- fold or greater increase.

16. For the test which has been performed in the paired row then the highest dilution of the serum giving 100% protection in both wells are considered as end VN antibody titre of the serum tested

Precautions:

1. Conduct the test in sterile condition in the laminar flow.

2. Serum free MEM is used throughout as a diluent.

3. Virus stocks of known titre are diluted just before use to contain 100 $TCID_{50.}$

4. Put the serum, virus and cell control for each test.

5. Add counted required cells in the tissue culture plate.

6. Some time one has to express virus titre/ antibody titre in per ml, which is forty fold of 25 μl. So this factor should be added in the $TCID_{50}$ dilution to compensate.

SEROLOGICAL DIAGNOSIS OF EQUINE INFLUENZA BY HEMAGGLUTINATION

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Diagnosis of influenza virus infections is usually only accomplished by tests on paired sera; the first sample should be taken as soon as possible after the onset of clinical signs and the second approximately 2 weeks later. Antibody levels are determined by Haemagglutination Inhibition (HI) test.

The antigen is first treated with Tween 80/ether in order to increase the sensitivity of the test, particularly for H3N8 viruses. The test is best done in microtitre plates using the appropriate dilution equipment. A macrotest may be used, for which antigen is diluted to a final HA titre of 1/8 per well and the volumes for PBS, sera and antigen are 0.5 ml. Sera are pretreated to remove nonspecific haemagglutinins, and inactivated at 56°C for 30 minutes. Pretreatments include the use of one potassium periodate. The treated sera are diluted in PBS, a standard dose of antigen is added (HA titre of 1/4 per well for microtitration assay), and these are kept at room temperature (23°C ±2°C) for 30 minutes. After gentle mixing, RBCs are added and the test is read 30 minutes later. The HI titres are read as the highest dilution of serum giving complete inhibition of agglutination. Either chicken RBCs (1% [v/v] packed cells) in V-bottomed microtitre plates or guinea-pig RBCs (0.5% [v/v] packed cells) in V- or U-bottomed plates may be used. If chicken RBCs are used, the plates may read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Nonagglutinated guinea-pig cells appear as a 'button' in the bottom of the well and may take longer to settle. Titre increases of fourfold or more between paired sera indicate recent infection.

Tween 80/ether treatment of the virus:

i) To 39.5 ml of infective allantoic fluid, add 0.5 ml of a 10% (v/v) suspension of Tween 80 in PBS to give a 0.125% (v/v) concentration of Tween 80.

ii) After mixing gently at room temperature for 5 minutes, add 20 ml of diethyl ether to give a final concentration of 33.3% by volume, and mix the suspension well at 4°C for 15 minutes.

iii) After allowing the layers to separate by standing, remove the aqueous layer containing the disrupted virus particles to a glass bottle with a loose lid and allow the excess ether to evaporate off overnight.

iv) Store treated virus in aliquots at –70°C.

Titration of haemagglutination

i) Add 25 μl of PBS to all wells in a row of a microtitre plate.

ii) Add 25 μl of virus to first well (dilution = 1/2) and titrate through, leaving the last well as a control.

iii) Add an extra 25 μl of PBS to all wells.

iv) Add 50 μl of RBCs to all wells. Leave at 22°C (±2°C) for 30 minutes. The HA titre is taken as the last virus dilution giving partial HA.

Potassium periodate pretreatment of sera

i) Mix one volume (150 μl) of serum with two volumes (300 μl) of freshly prepared 0.016 M potassium periodate (0.38 g in 100 ml PBS), and leave at 22°C (±2°C) for 15 minutes.

ii) Add a further one volume of 3% glycerol in PBS to neutralise any excess periodate solution, mix and leave at room temperature (23°C ±2°C) for 15 minutes.

iii) Inactivate in a 56°C water bath for 30 minutes.

Test procedure

i) Dispense 25 μl of PBS to all wells of a microtitre plate.

ii) Add serum (25 μl) to the first well of a row of 12, and titrate through, leaving the last well as a control (1/8 to 1/512, allowing for dilution of 1/4 from treatment of serum).

iii) Dilute the antigen to give a dose of 4 HA units (4 × minimum agglutinating dose, i.e. titre/4).

iv) Add 25 μl to each well, and incubate at 22°C (±2°C) for 30 minutes.

v) Add 50 μl of RBCs to each well. Leave at 22°C (±2°C) for 30 minutes.

vi) The plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. No agglutination is recorded as a positive result.

ESTIMATION OF ANTIBODY TITRES TO HAEMORRHAGIC SEPTICAEMIA IN SERUM SAMPLES OF CATTLE AND BUFFALO - USE OF HS ELISA KIT

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ELISA is a test of choice because it has high specificity and sensitivity. In addition to these important characteristics, the test needs various bioreagents in very little amounts and thereby substantial savings of the bioreagents. Cost of testing samples becomes important consideration when the test is required to be applied on very large sample size. Not only the cost of testing samples but also speed is important factor in large sample size. By converting the test in to a single dilution ELISA, the cost of sample testing is considerably reduced and also the samples are tested faster. In single dilution ELISA, the samples are tested in only one dilution and from the intensity of the colour developed, the antibody titres are calculated. Making only one dilution of the samples allows testing of more number of samples in one plate and thus speed and economy in sample testing are achieved.

Haemorrhagic septicaemia (H.S.) is an important disease of cattle and buffaloes. *Pasteurella multocida* (B:2 type) is causative agent of the disease in India. It causes heavy mortality especially in buffaloes. A potent vaccine prepared with P-52 strain of the bacteria is used to vaccinate the animals and the immunity to the disease is primarily conferred by antibodies to the bacteria.

Principle of the assay:

The kit is based on indirect ELISA using outer membrane proteins of the P-52 strain of the bacteria as antigen. Outer membrane protein of the bacteria has been shown to be inducing protective antibodies in animals. The binding of the H.S.antibodies in cattle/ buffalo sera samples with plate surface bound antigen, is detected by a combination of a mouse monoclonal antibody reacting with both cattle and buffalo immunoglobulins (IgG) and anti-mouse horse radish peroxidase conjugate because it has also been reported that anti-HS antibodies in cattle and buffalo sera samples are mainly present in IgG class of immunoglobulins.Using an equation of regression, antibody titres are calculated from the intensity of colour developed in a chosen single dilution of the test serum sample.

The Kit

The kit is suitably developed so as to make it user friendly in such a manner that a skilled technician can easily test the samples. Positive and negative controls are in built in the ELISA plates with antigen already coated on the surface of the plate wells. All other reagents are either in ready to use state or can be easily prepared.

Eighty eight samples are tested in one plate. Lay out of ELISA plate given below:

Control wells←Wells for test serum sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	Conjugate Control											
B	Negative											
C	Control											
D	Wells											
E	(Wells B1											
F	To F-1)											
G	Positive control well											
H	(G1 & H1)											

Contents of the Kit:
1   ELISA plates- 4 nos.
2   Washing buffer (25X)- 100ml
3   Monoclonal antibody (Mab)- 25ml
4   Conjugate (Stabilized)-25ml
5   Chromogen (100X) - 250µl
6   Substrate Buffer-25ml
7   Stopping solution - 25ml

Materials required but not supplied:

Micropipette, Multichannel micropipette, Disposable tips, Reagent troughs, Pipettes, Glass vials, Blotting paper pad and Wash bottle.

The Test Procedure:

1.   Add 20ml of 25X washing buffer to 480ml of distilled water so as to make 500ml of the 1X working washing buffer (Enough for one plate)

2.   Take out the test sera samples, and thaw.

3.   Peel off the cover of the ELISA plate

4.   With washing buffer in a wash bottle flood wells with washing buffer and decant immediately by flick of wrist, do it three times

5. Tap the inverted plate on pad of blotting paper to blot it dry

6. Add 50μl of the diluent to well A1 of col.1 of the ELISA plate and 45μl to all other well of the ELISA plate .Add 5μl of a test serum sample to a well of the ELISA plate. This way, test sera samples are diluted to 1:10 in 50 μl volume. Add 5μl known negative cattle serum to wells B1 to F1 of col.1 and 5μl of known positive serum to wells G1 and H1

7. Cover the ELISA plate with the peeled off cover and incubate at 35-37^{0}C for 1hour

8. Wash and dry the ELISA plate as done in step 4 & 5

9. Using a multichannel micropipette, aliquot 50μl of the diluted Mab prepared in all wells of the ELISA plate and incubate as done in step-7

10. Wash and dry the ELISA plate as done in step-4 & 5

11. Add 50μl of the ready to use conjugate provided in the kit to all wells of the ELISA plate and incubate as done above in step-7

12. Wash and dry the ELISA plate as done in step-4 & 5

13. Prepare chromogen substrate by adding 60μl Chromogen (100X) to 6.0ml of substrate buffer, shake to dissolve precipitate. It disappears immediately. Add 50μl of the ready to use chromogen –substrate to all wells and keep the plate in incubator at 35-37^{0}C for 5min. to facilitate colour development (Blue colour develops in positive reaction)

14. Stop the colour developing reaction by adding 50μl of the stopper solution to all wells. The blue colour turns to yellow.

15. For test to be successful, blue colour should develop in positive control wells (G-1 & H-1) and no or very little in negative control wells (B-1 to F-1) and similarly no or faint colour in the conjugate control well (A-1).

16. Read optical density (O.D.) of the colour developed in ELISA Reader at 450 nm. The well A-1 be blanked

17. Calculate ratio by dividing O.D. value of the test well by mean of O.D. values of negative control wells + 3 Standard deviation of the values in negative control wells (3 S.D).

$$\text{Ratio (X)} = \frac{\textbf{O.D.Value of a test well}}{\textbf{Mean+ 3 S.D. value of negative control wells}}$$

18. Place the value in the equation

` $$Y = a + bX$$

Where Y= Antibody titre in $\log_{10}$, Constant a = 1.35

Constant b =0.05X= *Value of ratio found in step 18*

Antibody titre in Log $_{10}$ (Y) = 1.35 +0.05 X

The standard error of the Y estimate (antibody titre) is $\pm 0.19 \log_{10}$

Samples with ratio <2.5 should be interpreted as Negative

Take value 0.05 if calculated mean O.D. value of negative control wells + 3S.D is <0.05

Interpretation

Antibody titre < 1.5 $\log_{10}$- Not protected

Antibody titre > 1.5 $\log_{10}$ and < 2.0 $\log_{10}$- Partially protected

Antibody titre > 2.0 $\log_{10}$ Protected

Advice:

Some turbidity precipitation/ sedimentation may be seen due to precipitation / sedimentation of salts in solutions at refrigeration temperature. Mix well and use.

Storage and stability:

Store as recommended. Use before the expiry date.

Warnings and precautions:

1. The kit should be used *in vitro* for diagnostic purpose only.

2. The components of kit should be stored under recommended conditions

3. The test should be performed as per the instructions given in this leaflet

4. Good quality test sera samples, stored at -20^0C should be tested and testing of bacterial or fungal contaminated samples may be avoided

5. Proper care in preparation of dilution of test sera may be taken

6. Care has been taken that reagents provided in the kit are not spoiled by bacterial contamination, however due cleanliness may be observed. The test can be performed on bench without any particular aseptic technique.

7. The steps in the test are performed swiftly so as to avoid over drying of the wells of the plate especially during hot summer months. Else use a cool place for performing the test

8. Due care be taken to avoid intermixing of various reagents provided in the kit. Use separate pipette or tip for taking out various reagents, bio reagents of the kit

9. Use whole plate at a time and not part of the plate.

10. The jet of the washing buffer should be aimed towards the wall of the well and not towards the surface to avoid leaching off the antigen/antigen-antibody complexes and also the plate be held little tilted with control wells column kept upward to avoid overflow from test wells to control wells

MOUSE MODEL OF HEMORRHAGIC SEPTICEMIA: *in vivo* TITRATION OF *Pasteurella multocida* (B:2) IN DIFFERENT ORGANS OF MICE

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Hemorrhagic septicemia (HS) is septicemic pasteurellosis of cattle and buffaloes caused by *Pasteurella multocida* serotypes B:2. After the onset of symptoms of disease, chances of mortality in the affected animal are very high. For the control of the disease in the endemic areas killed vaccines are used. The limitations of the killed vaccines currently in use are that their immunity is of short duration and their role in prevention of carrier status is not established. Therefore research work on the aspect of development of more effective vaccines against the disease is an area of priority.

Objective:

The basic parameters like the determination of multiplication of the bacteria in the different organs and the measurement of antibodies in the sera of the immunized animals *vis-a-vis* the control animals upon challenge with the pathogen of immunized mice are of paramount importance for development and testing the efficacy of candidate vaccines.

Upon the successful evaluation of the candidate vaccine in the laboratory animal model, the candidate vaccine is tested in a small cohort of natural host of the pathogen followed by its efficacy study in bigger population under field conditions. Thus the development of an appropriate animal model can serve as an important tool in this direction.

Materials:

Mice, anesthetic agent (chloroform), normal saline solution or PBS, 2.0% brain heart infusion (BHI) agar, 24-well plates, pestle and mortar, dissection kit, pipettes, micro-pipette etc, electronic balance.

Procedure:

Bacterial multiplication in different organs of mice: To study the efficacy/ability of a vaccine in preventing the multiplication of bacteria, bacterial titration in different organs of mice pre-immunized is performed after challenge. Various steps of bacterial titration are described as:

1. Mice are anesthetized under mild anesthesia using ether in a suitable glass or plastic container using the cotton swab soaked in the anesthetic reagent.

2. As soon as the mouse stops the circling moments, the mouse can be secured gently between the fingers and does of inoculums is laid ranging 10-20ul over both the nostrils of the mouse and let the same be inhaled by the mouse gently before the anesthetic effect is over.

3. After the desired time interval mice are sacrificed by deep anesthesia for collection of tissue/organs or their samples.

4. Various organs (lung, liver, spleen) are collected individually from each mouse aseptically and weighted on the electronic balance and triturated using sterile pestle and mortar.

5. 10% suspension of triturated suspension is prepared and further ten-fold dilutions are made in cold PBS/NSS.

6. A 20 μl suspension from each dilution is cultured on 2.0% BHI agar plates in 24-wells.

7. The plates are then incubated at 37^0C for 24 hrs. and colonies in the highest dilution well are counted.

8. The titer of bacteria is expressed as cfu per gram of tissue of individual mouse as follows:

Calculation of bacterial titers in the organs:

Titer per gram of tissue (CFU) = No. of colonies per well x 50 x dilution factor

DIAGNOSIS OF BRUCELLOSIS IN ANIMALS THROUGH CONVENTIONAL AND FLUORESCENCE POLARIZATION ASSAYS

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The diagnosis of bovine brucellosis may be initiated using the ring test on the milk of dairy animals, serological tests or assays based on Polymerase Chain Reaction (PCR). However, isolation of *Brucella* spp. is still the gold standard for diagnosis. While cultivation of *Brucella* bacteria is the most conclusive method, facilities and expertise for these procedures are often not available. Therefore, serological tests are usually the only diagnostic methods that are performed.

The standard agglutination test (SAT) and Rose Bengal test (RBT) are often the first tests on sera and followed by the complement fixation test (CFT) as a confirmatory test on positive sera with agglutination reactions. The CFT is a complex test hence most countries have replaced this test with ELISA which can be used on sera or milk samples. Milk samples cannot be tested by CFT. Workers world over are active on the development of other procedures with special emphasis on simplicity and the differentiation of antibodies from previous vaccination and those of infection. Indirect ELISA tests, competitive ELISA and Fluorescence Polarization Assay (FPA) have a high performance value because of high sensitivity and high specificity. The conventional and FPA for the diagnosis of brucellosis in animals is described.

The FPA for diagnosing brucellosis in cattle was first described by Nielsen *et al.* (1996). Since then the assay has been used and also applied in other animal species including human beings. Currently the test is prescribed by OIE for testing bovine brucellosis in animals for international trade.

The FPA makes use of direct binding principle when seeking detection of antibody. It measures tracer binding directly, without the need of a separation procedure. The specificity of the assay is thereby combined with the speed and the convenience of a homogeneous method, providing a precise and reliable procedure for determining the concentrations of biologically interesting substances in serum, plasma, and other environments. This method is based on the principle that an antigen bound in an immunocomplex will have a higher polarization value than free antigen. The principal advantage is that no separation of bound

from free antigen is required. The entire assay is performed in solution, in a single tube, with no precipitation or washing steps.

In a typical direct binding assay to detect presence of antibody, fluorescent antigen or "tracer" is added to a tube already containing the sample being tested. If the target antibody is present in the sample, then the antigen tracer binds with it producing a larger fluorescent molecule. Polarization values are measured before and after tracer addition; the change in polarization is proportional to the antibody concentration.

Most other forms of immunoassays are not homogeneous and require at least two steps which include the formation of an immunocomplex and the physical separation of the bound from the free antigen. In the case of ELISA, one or more steps are required to remove the liquid phase. The retained immunocomplex is repeatedly washed to remove the unbound and non-specifically bound molecules. With each washing step, some of the desired bound molecules are also released from the solid phase. This amount of loss depends on several factors, some of which cannot be controlled. A few examples of these variables are: the type of solid matrix used, the equilibrium constants of the immunocomplexes and the duration, temperature, and solvent conditions of the washing. These uncontrollable losses cause both systematic and tube-to-tube or well to well variability.

In comparison, FPA provides a faster, more precise and lower cost alternative since the assay is homogeneous; it requires no separation step. Additionally, the homogeneous format offers a substantial time and cost savings but most importantly it avoids the loss of bound material during washing and artifact created by solid phase immunoassays. The reagent and disposal costs related to FPA are significantly lower than those associated with other immunoassay methods. FPA does not require the use of hazardous materials such as radioactivity or carcinogenic enzyme substrates and can be carried out in disposable borosilicate glass tubes. The only specialized reagent required is a fluorescently labeled antigen in a direct binding assay.

For the diagnosis of brucellosis, a small molecular weight fragment O-polysacchride (OPS) (molecular weight 22kD) of the *B. abortus* smooth lipopolysacchride (sLPS) is labelled with fluorescein isothiocyanate (FITC) and used as the antigen. This antigen is added to diluted serum or whole blood and a measure of the antibody content is obtained in about 2 minutes for serum or 15 seconds for blood using a fluorescence polarization analyzer.

The FPA can be performed in glass tubes or a 96-well plate format. The bovine serum is diluted 1 to100 or, if EDTA-treated blood is used, the dilution should be 1 to 50 (heparin-treated blood tends to increase assay variability). The diluent used is 0.01 M Tris (1.21 g), containing 0.15 M sodium chloride (8.5 g), 0.05% Igepal CA630 (500 μl) (formerly NP40), 10 mM EDTA (3.73 g) per litre of distilled water, pH 7.2 (Tris buffer). An initial reading to assess light scatter is obtained with the fluorescence polarization analyzer (FPM) after mixing. Suitably labelled and titrated antigen (usually giving fluorescence intensity (FI) of 250,000-300,000) is added, mixed and a second reading is obtained in the FPM about 2 minutes later for serum and 15 seconds for blood. A reading (in millipolarisation units, mP) over the established threshold level is indicative of a positive reaction. A typical threshold level is 90-100 mP units, however, the test should be calibrated locally against International Standard reference sera. Control sera of strong positive, weak positive and negative, as well as S19 vaccinate serum, should be included.

The production of antigen and its labeling with a fluorescent dye can be performed in a laboratory having moderate facilities. Only two equipments i.e. freeze drying machine and fluorescence polarization analyzer are a bit costlier otherwise production of fluorescent dye labeled antigen is easy.

Test Procedure:

1. One ml of Tris buffer is added to a 10 x 75 mm borosilicate glass tube followed by 10 μl of serum or 20 μl of EDTA-treated blood. It is important to mix well. A reading is obtained on the FPM to determine light scatter.

2. A volume of antigen, which results in a total fluorescence intensity of 2, 50,000- 3, 00,000 is added to the tube and mixed well. This volume will vary from batch to batch, but is generally in the range of about 10 μl.

3. A second reading is obtained on the FPM after incubation at ambient temperature for approximately 2 minutes for serum and 15 seconds for EDTA-treated blood.

4. A reading above the predetermined threshold is indicative of a positive reaction.

5. The following are included in each batch of tests: a strong positive, a weak positive, a negative working standard serum (calibrated against the OIE ELISA Standard Sera).

The diagnostic sensitivity and specificity of the FPA for bovine brucellosis are almost identical to those of the competitive ELISA. The test is effective in differentiating infected animals from vaccinated ones. The diagnostic specificity for cattle recently vaccinated with *Brucella abortus* S-19 vaccine is over 99% (Nielsen *et al*., 1996).

Reference:

Nielsen K., Gall D., Jolley M., Leishman G., Balsevicius S., Smith P., Nicoletti P. and Thomas F. 1996. A homogenous fluorescence polarisation assay for detection of antibody to *Brucella abortus. Journal Immunological Methods* **195**: 161-168.
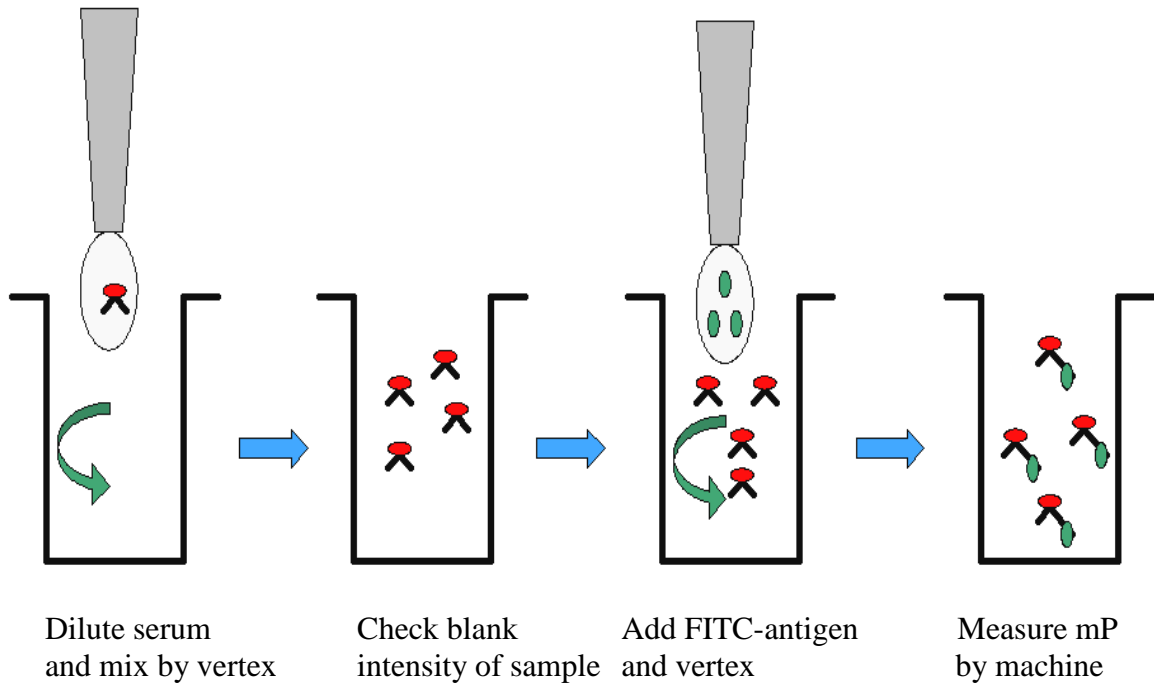
| Dilute serum and mix by vertex | Check blank intensity of sample | Add FITC-antigen and vertex | Measure mP by machine |

Fig.1: Diagrammatic representation of Fluorescence Polarization Assay (FPA)

ANALYSIS OF THE BoCD4$^+$ T AND BoCD8$^+$ CELLS OF BOVINE MILK BY FLOW CYTOMETERY

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Mastitis, inflammation of the mammary gland, is a common and economically important disease in dairy animals. Mammary pathogenic organisms invade the teat canal, milk ducts, and mammary alveolar space, replicate in mammary secretions, and elicit a local inflammatory response characterized by massive recruitment of blood polymorphonuclear neutrophil leukocytes (PMN) into the alveoli and milk ducts. Functional properties of these cells influence the integrity of milk synthesizing tissues. Generation of effective specific immunity involves both antigen presenting cells and lymphocytes. There are two distinct subsets of lymphocytes, which differ in function and protein products i.e. T and B lymphocytes. The T lymphocytes can be further subdivided into αβ T lymphocytes which include CD4$^+$ (T helper) lymphocytes and CD8$^+$ (T cytotoxic or T suppressor) lymphocytes, and γδ T lymphocytes. Depending on stage of lactation and tissue location, the percentages of these cells can vary significantly. Flow cytometric analysis of somatic milk cells is useful for the detection of mastitis in its initial phase. In this practical population of different T cell markers viz. BoCD4$^+$, and BoCD8$^+$ cells will be analyzed using monoclonal antibodies (MAb) against these T cell markers by flow cytometry

Principle of Flow Cytometry:

Flow cytometry is a powerful and fast technique where fluorescent and light scattering properties of cells or particles are analyzed. Particles are lead through an illuminating beam of coherent light, usually originating from a laser. The scattered laser light and emitted fluorescence is translated to information about the cell. Flow cytometry can be used to analyse intracellular and surface properties of cells and particles, distinction between live and dead cells can be made and physical sorting of cells with interesting properties can be performed.

Procedure:

A- Isolation of Leucocyte from milk :

1. Aseptically collect milk into sterile flasks and then centrifuge at 3000 rpm for 20 min, 4^{0}C.

2. Remove the creamy layer with sterile spatula and gently decant milk. Resuspend the cell pellet in 20 ml of PBS. Layer the cell suspension on to histopaque gradient (Sigma Chemicals Company, U.S.A.) and centrifuged at 1200 rpm for 30 min at 4^0C.

3. Separate Mononuclear cells at interface by sterile pasture pippette.

4. Resuspend the cell pellet in 10 ml PBS and centrifuged at 1200 rpm for 20 min at 4^0C in refrigerated centrifuge. Discarded the supernatant and finally resuspend cells in 5 ml of PBS.

B- Immunofluorescant staining and flow cytometry :

1. Incubate approximately 1×10^6 mammary mononuclear cells with lineage - specific monoclonal antibodies against T lymphocyte antigens (Table 1) for one hr at 4^0C.

2. After incubation, wash cells thrice with PBS containing 0.1 per cent sodium azide and incubated with 1: 80 diluted fluorescein isothiocyanate (FITC) - conjugated goat antimouse immunoglobulin G for one hr at 4^0C.

3. Following incubation wash the cells thrice with PBS containing 0.1 per cent sodium azide and resuspend in PBS-sodium azide containing 3 per cent BSA and 2 per cent formaldehyde.

C- Data acquisition:

A Becton-Dickinson C6 Accuri flow cytometer and CFlow Plus Software will be used `for data acquisition. Calculate the percentage of positive cells out of 10,000 by substracting background staining with flourescent conjugate control.

Table 1: Details of specific monoclonal antibodies against T lymphocyte

S No.	Clone*	Specificity	Ig isotype
1	CACT80C	CD8	IgG1
2.	CACT138A	CD4	IgG1