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GENE EXPRESSION IN YEAST SYSTEM

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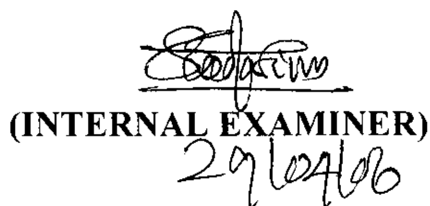

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ABSTRACT

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Foot-and-mouth disease virus (FMDV) is an aphthovirus of the family Picornaviridae and the etiological agent of the economically most important animal disease. As a typical picornavirus, FMD virions are nonenveloped particles of icosahedral symmetry and its genome is a single stranded RNA of about 8500 nucleotides and of positive polarity. FMDV RNA is infectious and it replicates via a complementary, minus strand RNA. FMDV RNA replication is error-prone so that viral populations consist of mutant spectra (quasispecies) rather than a defined genomic sequence. Therefore FMDV in nature is genetically and antigenically diverse. This poses important challenges for the diagnosis, prevention and control of FMD. A deeper understanding of FMDV population complexity and evolution has suggested requirements for a new generation of anti-FMD vaccines. This is relevant to the current debate on the adequacy of non-vaccination versus vaccination policies for the control of FMD. With these facts in view, I have done a part of work entitled: Gene (FMDV) expression in yeast system.

The integrated gene sequence was expressed in the yeast cell by inducing with 0.5% methanol. The optimal quantity of protein was secreted out in to the medium after 96 hours of methanol induction. Proteins secreted were analyzed by SDS-PAGE and Western blot to detect the expression. The molecular weight of the expressed protein was found to be 26kDa. The expressed protein was quantitated and found to be 2g\l of culture.

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ABBREVIATIONS

aa	amino acid
AOX	Alcohol oxidase
A+ T	Adenine and Thymine base content
BHK- 21 $clone_{13}$	Baby Hamster Kidney Cell Line
bp	Base pair
CBB	Coomassie brilliant Blue
cDNA	Complementary deoxyribonucleic acid
CPE	Cytopathic effect
Cre	Cis- acting replication element
CsCl	Cesium chloride
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	2' – deoxyribonucleoside- 5' triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FMD	Foot and mouth disease
FMDV	FMD virus
G+ C	Guanine and cytosine base content
GMEM	Glasgow modified minimal essential medium
GPI_{50}	Fifty percent Guinea pig infective dose
H_2O_2	Hydrogen peroxide
HRPO	Horse radish peroxidase
HS	Heparin Sulphate
IPTG	Isopropyl- β- D- thiogalactoside
Kbp	Kilo base pairs
kDa	Kilo Dalton
LB	Luria- Bertani
Log	Logarithm
MCS	Multiple cloning sites
MMLV- RT	Moloney murine leukemia virus
mRNA	Messenger RNA
NA	Nucleic acid
NCM	Nitro cellulose membrane
OD	Optical density
ODD	Ortho dianasidine dihydrochloride
ORF	Open reading frame
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PolyA	Poly adenylation
P. pastoris	*Pichia pastoris*
RNA	Ribonucleic acid
RNase	Ribonuclease

rpm	Revolutions per minute
RGD	Arginine- Glycine- Aspartic acid
RT	Reverse transcriptase
RT- PCR	Reverse transcription- Polymerase chain reaction
S	Svedberg units
S. D.	Standard deviation
SDS- PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
Sec	Seconds
ssRNA	Single stranded RNA
TBE	Tris borate ethylene diamine tetra acetic acid
$TCID_{50}$	Fifty percent tissue culture infective dose
TE	Tris ethylene diamine tetra acetic acid
TEMED	N. N. N' N' – Tetra methyl ethylene diamine
Tris	Tris (hydroxyl methyl) amino methane
UTR	Untranslated region
WT	Wild type
X- gal	5- bromo- 4 chloro- 3- indolyl- β- D- galactose

UNITS OF MEASUREMENT-SYMBOLS

%	Percentage
Ω	Omega
μg	Micrograms
μl	Microlitres
ρM	Picomole
p.mol	Picomole
cm	Centimetres
°C	Degrees Celsius
g	Gram(s)
h	Hour(s)
IU	International units
KDa	Kilo Daltons
M	Molar
mA	Milli Amperes
mg	Milli grams
min	Minutes
ml	Milli litres
mm	Milli metres
mM	Milli molar
MW	Molecular weight
Ng	Nano grams
Nm	Nano metres
nM	Nao moles
U	Unit
V	Volts
v/ v	Volume/ volume
w/ v	Weight/ volume
x- g	Centrifugal force equal to gravitational force

Chapter 1

INTRODUCTION

INTRODUCTION

History proclaims that the earliest recorded account describing Foot and Mouth Disease was made by Fracastorius in 1546 A.D. However, even after a century lapse of celebrating the momentous observation by Loffler and Frosch that an infectious animal disease was caused by a filterable agent, 'Fievre Aftosa' manifests itself as a perennial global menace. Foot and Mouth Disease is a highly contagious and economical devastating viral disease of wild and cloven-hooved animals (Deigo, et al., 1970, and constitutes one of the main animal health concerns. The case fatality rate among youngstock is below 5% but morbidity is very high. In typical cases, Apart from the sudden onset of acute illness, illustrative by vesicle formation and erosions of the epithelium in and around the mouth and the inter-digital space and pyrexia (up to 40 °C), the sequelae of the disease such as loss of milk yield, primary and secondary Mastitis. repeat breeding, abortions and chronic sub-fertility ensue in milch animals, growing retardation in beef cattle, unthriftness and 'panting syndrome' due to pituitary involvement are exhibited by draught animals.

The productivity losses inflicted by FMD are estimated at 25% in terms of milk and meat production and impaired locomotive potential of the traction animals.The rapidity in the mode of spread among the susceptible animal population with a 100% decrease in the productive performance of the affected and recovered animals can easily cripple a nation's livestock industry and close export markets for meat and other agricultural goods (Lubroth and Brown, 1995). By way of their geographical isolation and application of quarantine measures New Zealand, Australia, and North America are privileged to be FMD free zones. It is enzootic in Asia, Africa, and South America and incidence is very low in many of the European countries (Domingo, et al., 1990).

The causative agent, a single stranded positive sense RNA virus is a type member of the genus *Apthovirus* (Pringle, 1999) in the family *Picornaviridae* (Franki, et al., 1991) existing as 7 antigenically and genetically distinct serotypes viz., O,A, C Asia I, SAT-1, 2, 3 (Cooper, et al.,1978) with numerous strains in each serotype. The genome is linear, RNA molecule of about 8500 nucleotides encapsulated within a capsid composed of 60

copies (Fross, et al., 1984), of each of the four structural polypeptides viz VPI-VP4 (Acharya, et al., 1989). The RNA is infectious and act as the messenger RNA, (mRNA) for the translation of viral proteins. Upon infection, viral genome is released in the cytoplasm and translation of the single ORF produces a polyprotein that is proteolytically processed by two viral proteases, producing equimolar amounts of structural and non-structural proteins (Belsam, 1993).

The PI region of the genomes encodes viral proteins L, IA, IB, IC AND ID coding for the structural proteins. The P2 and P3 regions encodes for the non-structural proteins and their intermediate precursors. Non-structural proteins of FMDV comprises of L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D (Ryan, et al., 1989). The cleavage products are L, PI-2A, P2 and P3 (Reukert and wimmer, 1984).The PI-2Aprecursor yields the structural proteins, while P2 and P3 contain proteins required for genome replication.

The P2 region encodes the non-structural proteins 2A, 2B and 2C of which 2C is probably involved in virion RNA synthesis. The 2A region is highly conserved in all the serotypes (Vakharia, et al., 1987). The P3 region encodes the non-Structural proteins 3A, 3B, 3C, 3ABC and 3D. The non-structural protein 3ABI, is a precursor of 3A and 3B (VPg) which is involved in viral RNA replication, and processed in to 3A and 3B by the 3C protease. 3A contains a hydrophobic domain that might serve as the membrane anchoring region (Porter, 1993). 3B encodes VPg (Fross, et al., 1982). 3C catalyses most of the cleavages of PI, P2 and P3 (Vakharia et al.,1987 ; Clarke and Sanger, 1988). 3D functions as RNA polymerase, also called VIAA (virus infection associated antigen) (Polatnick, 1980), and diagnostic of viral infection.

FMDV deals with unpredictable shifts and drifts of the genomic distribution contribution to the multitudes of antigenic variants that often co-circulate in a given geographical area(Mateu, et al., 1988), leading to a persistence status in the animals, by mechanism still obscure. A relevant property of the virus is its ability to establish sub-clinical infection and these silent infections harbored by the persistently infected cattle pose the danger of competitive selection of new variants against the immune pressure by escaping neutralization by the antibody concerned in these carrier animals. The Carrier

animals are thus expected to be potential source of new epidemic strains (Brooksby. 1982) putting the susceptible and vaccinated populated at risk.

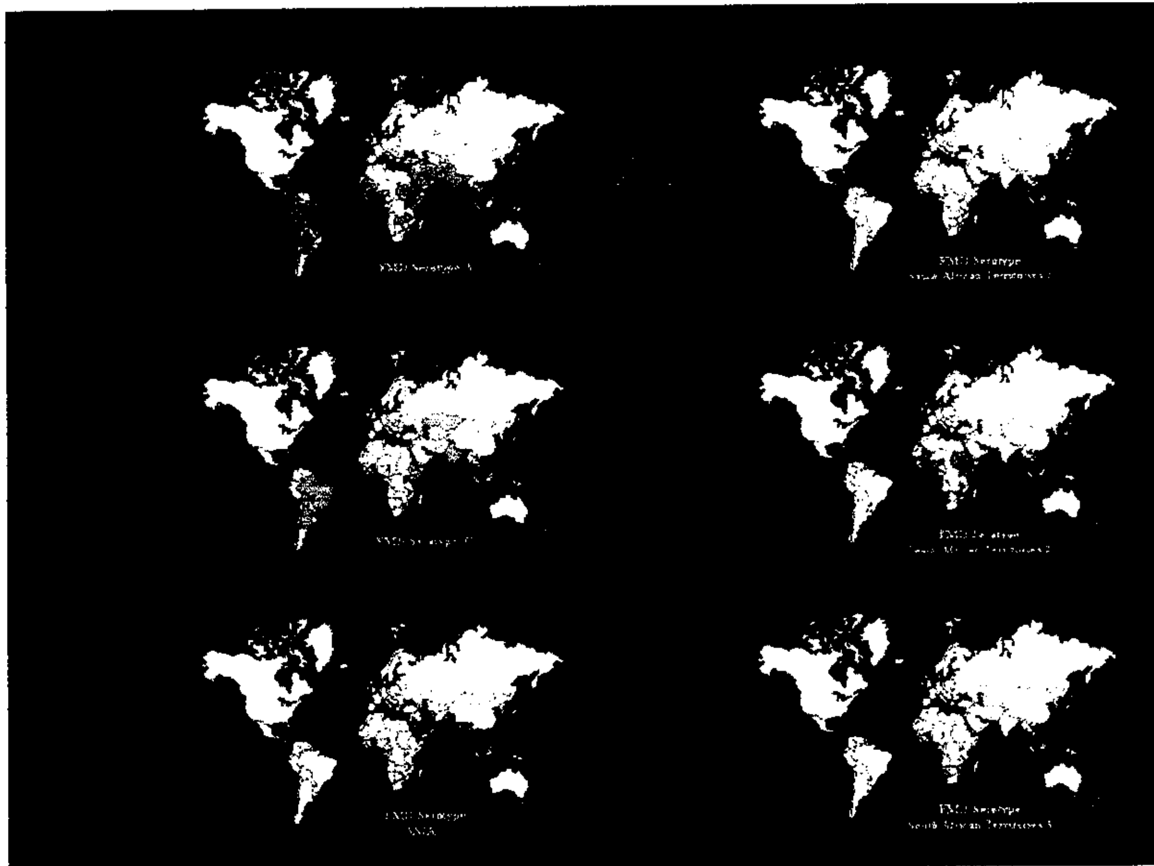
FMD is controlled worldwide by effective and systemic vaccination through sero-surveillance and vigorous stamping out wherever possible. The antigenic plurality arising due to the quasi-species nature of the virus (Domingo et al., 1985), its extreme contagion, wide geographical distribution, prevalence of large numbers of strains (Bachrach, 1985) within the seven serotypes, virus persistence and carrier status (Gebauer, et al., 1988) and short duration of the immunity makes control of the disease far from easy and FMDV an intractable and deprecatory agent.

Annually around 5000 outbreaks of FMD are reported in the Indian subcontinent and pilot study estimates the losses incurred to be the tune of 40 billion rupees with an astronomical impact on the Indian economy. Slaughter is not conducive due to socio-economic and religious factors, therefore regular ring / Barrier vaccination of the 470 million plus domestic livestock population implemented by periodic updating of the circulating vaccine strains is the only the alternative for an effective disease control. The effective control of the disease in endemic countries needs both better vaccines and the strong support of effective diagnostic tests, which can detect both type and strain variation. Alternatively, identification of already existing disease free population and regions and vigilantly monitoring virus activity around such zones by reliable diagnostic procedures with restriction of locomotion and regular vaccination will enlarge these disease free zones. Making the country in to such smaller zones would make the goal of FMD eradication appear less formidable.

Chapter 2

REVIEW OF LITERATURE

Figure 1.1 Different types of FMDV around the world



REVIEW OF LITERATURE

The foremost claim in medical literature of the immunological and antigenic differences in an animal virus predates that of a human virus is an eminent historical record and elative to any veterinarian. Celebrating the momentous discovery of the causative agent as a filterable particle by Loeffler and Frosh in 1897, the importance of FMDV has pioneered several aspects in the field of virology and development of modern methods for disease control. But still now, FMD is one amongst the threatening and acute contagious viral diseases of the wild and domesticated cloven hooved animals of paramount economic importance (Pereira, 1981). It is the major constraint to free international trade of livestock and animal by products (Rweyemamu and Leforban 1999).

2.1. Virion Structure and its Genomic organisation

The order of the gene products on the genomic segment sets apart the Apthoviruses uniquely. The mature virion consists of a single molecule of single stranded, positive sense RNA that is infectious and about 8.5 Kb long, encapsidated within a non-enveloped icosahedron of 30nm in diameter. The purified whole virus particle has a sedimentation constant of 146S whereas the empty particle has 75S. Its buoyant density in CsC1 is 1.43-1.45 g/cm^2. The virus is unstable at acidic pH.

Apthoviral RNA is covalently linked to a small virual coded protein VPg at the 5' end. The genome is flanked at the 5' end by an exceptionally long untranslated region (UTR) of about 1300 bases which is composed ofa (small) fragment of 400 bases long, a polycytidylic acid (poly-C) tract of 80-200 nucleotides (De la Torree et al., 1988), and a750 nucleotide L (large) fragment. The large fragment (L) of 5' UTR important in translation initiation of the picornaviruses in a cap-independent mechanism., has an Internal Ribosome Entry Site (IRES) which is of Type II in the Apthoviruses. The single open reading frame of 6996 nucleotides follows the 5' UTR and codes for a polyprotein of about 2332 amino acid residues (Belshanm, 1993). The leader (L) protein is the first comonent and produced in two distinct forms termed Lab and Lb both possessing similar proteloytic activities Functional L proteinase is not essential for replication. Except for the leader (L) protein that undergoes co-translational cleavage the remaining polyprotein undergoes primary cleavage mediated by virus proteinase mediated by virus proteinases

giving rise to P1, P2 and P3 polyproteins, which are rapid intra molecular (cis) events. Secondary cleavage of P1 gives rise to four structural proteins (VP1, VP2, VP3 and VP4) and the P2 and P3 regions give rise to non-structural proteins (2A, 2B, 2C, 3A, 3B1, 3B2, 3B2, 3C and 3D) all of which are a combination of a series of intra and inter molecular (trans) event. Fig1.

The capsid typically comprises of 60 copies of each of the 4 structural proteins VP4 [85aa], VP2 [218aa], VP3 [220aa] and VP1 [213aa] (Acharya, et al., 1989) encoded by 1A, 1B, 1C and 1D gene respectively. The VP1, VP2 and VP3 proteins are partly exposed to capsid surface while, VP4 is buried internally and is myristylated at the N – terminus (Madshus et al., 1984), all of which constitute structural proteins involved in capsid formation. Empty viral particle is made up of 60 copies each of VP1-VP4, linked covalently, Another UTR of 92 bases found at the 3' end is believed to contain major cis-acting (specific sequences) signal required for negative strand RNA synthesis and is essential to complete a full replication cycle.

FMDV encodes 3 distinct versions of VPg molecule that are used with equal efficiency. This triplicate copy of the 3B coding sequence and the virus isolate together determine the length of the poly-C tract from the 5' end whose function is presumably related to viral virulence. The high degree of secondary structure with the core stem loop D-L between the 5' end and the poly (C) tract folds into a complete hairpin. The repeat domains between the 3' end and the poly (C) tract folds as pseudoknot structure. The 3' end is poly adenylated derived from the polyprotein after processing and is involved in capsid formation (Rueckert and Wimmer, 1984). Ribosomes recognize the RNA for translation initiation in the immediate vicinity of the initiation codon (AUG 11) of IRES within 450 bases upstream the first initiation codon having a major role in initiation of viral translation in a cap independent manner (Belsham, 1992). RNA also recognizes a second initiation site 84 bases further downstream.

It is presumed that the FMDV receptor belongs to an integrin family of proteins to which a conserved Arg-Gly-Asp (R-G-D) motif present in the major antigenic domain (145 to 147 aa) of VP1 of all serotypes binds (Fox et al., 1989). Formation of the mature virion is a ramified process requiring multiple proteolytic steps. Following adsorption, penetration and intracellular uncoating, the single monocistronic open reading frame

2.2.2 Proteins of the P2 region

The P2 region encodes proteins 2A, 2B and 2C. The length of the genes and the proteins they encode are given in Table.1. Protein 2A is functionally a serine proteinase and is the smallest identified viral proteinase with just 16 amino acids (Ryan et al., 1991). It is involved in the cleavage of the P1-2A\2B junction which is independent of the L and the 3C proteinase activity (Ryan et al., 1989). The exact functions of the other proteins are still obscure, though sequence similarity has been shown between protein 2C and cellular NTP ases (nucleotide triphosphatases) in other picornaviruses.

2.2.3 Proteins of the P3 region

Proteins encoded by the P3 regions are 3A, 3B the trimer of VPg (found in three copies in all FMDV), 3C and protein 3D (Table1.). Of these, the proteins 3A, 3B1-3 and 3D are involved in the replication of the viral RNA genome. The actual role of 3A is not clear, but it is thought to function in binding the viral RNA replication machinery to the cellular membranes (Xiang et al., 1998). The interaction of 3A with the cellular membranes to causes a cytopathic effect, prevents surface expression of proteins and to inhibit protein secretion from 3A-expressing cells (Doedens and Kirkegaard, 1995; Doedens et al., 1997). Protein 3B is otherwise known as viral genome linked protein" or VPg, since it is attached to the 5' end of the viral RNA and has important roles in priming viral RNA synthesis. Protein 3C is a serine proteinase and is responsible for all the cleavages in the P1, P2 and P3 regions to yield individual viral protein from the translated polyprotein (Ryan et al., 1989).

2.3. Molecular basis of Pathogenicity

FMDV is an explosive cytolytic virus, which has a short replication cycle of about 2-3 hours from the stage of virus entry to release. The host machinery is paralyzed by numerous ways most of which are unknown. FMDV coded proteases are involved in most of the changes (Porter 1993). Direct Ribosomal entry at the 'Starting Window' infected cells results in rapid inhibition of cellular translation accompanied by autocatalytic cleavage of the L protein in cis or in trans from the aa terminus of the P1-2A capsid protein precussor. It also directly initiates the cleavage of the cap-binding complex (eIF-4F) component p220 and indirectly by the P2A protease.

Predominant among the known activities that follow virus entry are the rapid inhibition of host cell protein synthesis by cleavage of elF 4G, a component of cap binding complex elF 4G by L protease activity. (Devaney, et al., 1988). Recent studies indicated that eIF 4G is also cleaved by 3C protease at a site that is different from that of L protease site. 3C was also shown to cleave at the N terminus of elF 4A of the elF 4F complex. (Belsham, et al., 2000). The consensus sequences of the cleavage sites indicated the least conserved nature of the sites of cleavage for 3C suggesting the involvement of extensive processing activity on the host system by this protease. (Tesar and Marquardt, 1990). 3C has been shown to affect transcription and Histone H3 processing. (Tesar and Marquardt, 1990, Falk, et al., 1990). A single amino acid substation in nsp 3A Q(44) R either alone or in combination with the replacement I(248) T in 2C, was sufficient to give FMDV the ability to produce lesions in guinea pigs (Nunez, et al., 2001).

2.4. Carrier or Persistent infection

Carrier cattle are defined as animals from which FMDV is intermittently recovered in probang fluid after 28 days infection and maintain higher neutralizing antibody titres in serum, mostly Ig G, to FMDV than the convalescent non-carriers. (McVicar and Sutmoller, 1972).

The role of the carrier animal in FMDV transmission is still obscure and the mechanism persistence is not known. The virus persistence and carrier status in convalescent and recovered animals makes them main reservoirs and demonstration of transmission from one animal to other under controlled conditions has been elusive. Only circumstantial evidence suggests involvement in the spread of the disease by persistently infected animals (Salt, 1993). Waldmann, et al., (1926) by demonstrating the virus in the urine of the affected animals, gave the first evidenced to refute the non-persistence status of FMDV. The virus persists and multiplies to a low titre in the secretory epithelial cells of the dorsal soft palate and pharynx (Brooksby, 1982) which constitute an immunologically privileged site due to restricted accessibility for elements of the immune system in carrier animals and can be recovered from cattle for over two years.

Neitzert, et al., (1991) suggested that the serum antibody response to non-structural FMDV proteins is also prolonged in convalescent carrier cattle compared to

cattle groups to test its detection value for FMD and concluded that antibodies to VIA antigen persist longer than the virus in the pharynx indicating previous experience with FMDV. Antibodies to VIA antigen were absent in uninfected cattle and those immunized with inactivated vaccine, but were detectable in serum of FMD recovered cattle (Sobko, et al., 1976).

Studies by Restrepo, et al., (1978) for presence of antibodies to VIA antigen from sera of Colombian cattle concluded that the incidence of reactors were attributed either to prior contact with natural disease or to repeated vaccinations with imperfectly inactivated vaccines. Presence of antibodies to VIA antigen could either be due to its presence in the vaccines or the sub-clinical infection of the animals by small amounts of live virus which remained un inactivated in the vaccines (Dawe and Pinto 1978).

Pinto and Hedger (1978) applied the DID to detect antibody to VIA antigen in sera from various species of African wild ungulates for its use in epizootiological studies of FMD in wildlife and for the assessment of the safety of animals prior to movement either internationally or within the country.

Odend'hal, (179) tested sera from small rodents trapped on premises where the cattle sera demonstrated previous evidence of infection by the VIA test and the results indicated that the rodents had a strong resistance to natural infection with FMDV.

Estupinan, et al., (1979a, b) reported that the antibody titers to VIA antigen were absent or low in vaccinated animals and high in naturally infected animals during the first month after infection and suggested its utility to distinguish infected from the vaccinated cattle.

Centeno, et al., (1979) adopted counted immunoelectrophoresis (CIE) for the study of antibodies to VIA antigen and found it to be more sensitive, economic and less time consuming than the AGID test.

Antibody response to VIA antigen and 146S particles in cattle, guineapigs and mice by indirect immunofluorescence (IIF) was found to be comparable to the immuno-diffusion test, but the speed of IIF and the possibility of handling many samples made it more practical according to studies done by Schudel, et al., (1985).

AGID test would be helpful in determining the prevalence and species distribution of the disease in the un-vaccinated sectors are to preliminary serological survey done by Dawe and Durojaiye (1986).

Villinger, et al., (1986), performed an indirect ELISA using a bio engineered antigen and found it to be sensitive, safe and rapid than the Immuno diffusion test in agarose gel (IDAG) which used purified antigen from infected cells to detect antibodies to FMD VIAA.

Comparison of the results of a liquid-phase ELISA and agar gel diffusion test to identify and quantify antibodies against FMD VIA antigen by Alonso et al., 1990 inv accinated and re-vaccinated cattle was shown to be dependant on the non-purified antigen concentration in the vaccine. They concluded that the ELISA technique could be a more satisfactory test in prevention, control and eradication of FMD.A similar test conducted by Espinoza and Ameghiuno (1993) indicated that ELISA is more efficient and useful in supporting the prevention and control programme of FMD.

However, presence of antibodies to VIA antigen in both infected and vaccinated animals have been demonstrated (Bahnemann, 1990; Berger, et al., 1990). Berger, et al 1990) could identify abortive FMDV infections in mice upto one year by detecting the presence of antibodies to VIA antigen by radio immunoprecipitation test.RIP.). Further. Lubroth and Brown (1995) demonstrated the presence of antibodies to VIA antigen detectable up to 365 DPI in cattle and 301 DPI in Swine, both in convalescent and vaccinated cattle by RPI.

Since the development and application of an indirect ELISA to detect antibodies to FMD virus in sera of cattle by Abu Elzein and Crowther (1978) several forms of ELISA have been tried for the detection and quantification of antibodies or antigens to diagnose FMD. This and the low sensitivity of AGID test have led to the wide spread application of ELISA in detecting antibodies to VIA antigen in animals.

2.5.2. Studies using other non-structural proteins

Due to problems associated with the use of protein 3D as an antigen to detect infected animals, researchers turned their attention to other non-structural proteins which are also produced in equimolar amounts in infected cells and animals are also exposed to them at the time of cytolysis. Berger et al., (1990) used sera from infected, vaccinated and convalescent animals to radio immuno-precipitate viral proteins from infected cells to identify antibodies in bovine sera which specifically indicate a post infectional state. Only the convalescent sera precipitated non-structural proteins2C, 3AB and 3ABC.

successful and all post infection sera were tested positive while 99% of vaccinated sera were negative.

Foster, et al., (1998), carried out studies on serological and cellular immune response to nsp's in FMD infected animals in order to provide a rational base for nsp's as diagnostic probes. Antibody titres were highly variable and of short duration and discrimination between infected and vaccinated cattle were possible only at the herd level. 2C and 3D along with 3A provided a more sensitive test. Delayed type hypersensitivity (DTH) tests, to measure Th1 type cellular responses were directed towards them.

Sorensen, et al., (1998), used baculovirus expressed 3D, 3AB and 3ABC nsp's of FMDV, as antigens to recognized immune sera in ELISA for cattle and sheep against any of the serotypes of FMDV. All three were specific and precise. FMDV infected and those reinfected even after vaccination, gave positive results for both 3AB and 3ABC tests.

Mackay, et al., 1998 developed a monoclonal antibody (Mab) based indirect FLISA to detect antibody to nsp's L, 2C, 3A, 3D and polyprotein 3ABC. Their work also showed that antibodies to 3ABC and one or more nsp's were detected in animals reported to have shown clinical signs of FMD, but sera from vaccinated groups were reactive only to protein 3D.

Brocchi, et al., (1998) compared different monoclonal antibody (Mab) based immunoassays measuring the antibodies to nsp's to differentiate infection from vaccination and found that of the three nsp's 2C, 3C and 3ABC evaluated in the study the polypeptide 3ABC was the most immunogenic. Of the ELISA's tried, 3ABC Mat-ELISA based on the direct binding of antibodies to the 3ABC trapped by a specific Mab provided the best combination of sensitivity and specificity.

Fondevilla, et al., (1999), carried out a seroepidemiological study to obtain information about the prevalence of FMD in the border areas of Argentina, using serological methods based on the detection of antibodies to structural and non-structural proteins 3A, 3ABC and 2C of FMDV. Serum samples obtained from different species of animals were negative to at least one FMD protein. Thus it was concluded that carriers of FMD are not present in the population studied.

infection. n non-vaccinating countries this is done by measuring antibody to the structural proteins. In countries wishing to obtain the status of 'free with vaccination' or those nations that wish to cease vaccination, measurement of antibody to NS proteins has been approved as the test of choice by the O.I.E. to be used as an indirect estimate of viral activity in the vaccinated population. The objective of performing FMD serology is to identify whether or not an animal constitutes a risk of introducing FMD virus, and absence of antibodies against non-structural proteins might be taken as an indication of the absence of viral activity in the animal. This paves a pathway for free animal Movement in context of international trade.

Chapter 3

OBJECTIVES

OBJECTIVES

"GENE EXPRESSION IN YEAST SYSTEM"

1. To express the FMDV genes in yeast system, and
2. To check the level of expression.

Chapter 4

MATERIALS AND METHODS

4.1.5. Biochemicals

The following molecular biologicals and bio-chemicals either molecular biology grade or analytical grade were used in this study.

Acrylamide, N, N'- Methylene bisacrylamide, Bromophenol blue, Ethidium bromide, Sodium dodecyl sulphate (SDS), Sucrose, Tris base, etc., were from Sigma Chemicals Co., St. Louis, USA. Yeast Nitrogen Base (YNB) without amino acids, tryptose phosphate broth (TPB) were from Himedia [India]. Protein molecular weight markers and DNA molecular size markers were from Bangalore Genei or Gibco BRL or Fermentas. Agarose, low melting agarose, peptone, and yeast extract were from Bethesda Research Laboratories, USA. Positively charged nylon membranes were from Boehringer Mannheim.

4.1.6. Glass and Plastic ware

All the glassware used in the study was from Borosil India Ltd. They were thoroughly washed and sterilized as per standard procedure. Micro centrifuge tubes and micropipette tips were either from Tarsons or Axygen and microlitre plates for tissue culture were from NUNC Denmark or other internationally reputed firms.

4.1.7 Other Chemicals and Consumables

Other chemicals of analytical grade were either from Glaxo Laboratories India Ltd., or E. Merck (India) Ltd.

4.1.8 Media and Buffers

Composition of media, buffers and solutions used in this study are given in the appendix or at appropriate places.

4.1.9 Sera and Conjugate

Rabbit hyper immune sera raised against, either whole virus (146S particles) or C-terminal region of VP1 of FMDV and convalescent serum from FMDV Type C infected cattle were used in the studies. These were available in M. V. Lab. Goat anti-rabbit

BglII digest was purified from LMP agarose as described earlier. After purification, concentrations of the digested DNAs were measured and stored at -20°C until used for transformation.

4.2.4. Preparation of host cells

4.2.4.1. Revival of glycerol stock

The glycerol stock of Pichia pastoris cells was streaked on Yeast extract- Peptone-Dextrose (YPD) agar plates and incubated at 30°C for 3 days. After incubation plates were stored at 4°C for further use.

4.2.4.2. Preparation of electrocompetent yeast cells

YPD broth (5 ml) in a 50 ml conical flask was inoculated with single colony from the YPD agar plates and incubated at 30°C for 16 h with shaking (250-280 rpm). This over night culture (0.5 ml) was used for inoculating 500ml fresh YPD broth in a 2L flask and allowed to grow at 30°C to reach an OD_{600} of 1.3-1.5 (overnight incubation). The cells were pelleted at 4000-x g for 5 min at 4°C and suspended in 500 ml ice-cold sterile distilled water. The cells were pelleted at 4°C as above and suspended in 250 ml sterile ice-cold distilled water. The cells were again pelleted at 4°C as above and suspended in 20 ml sterile ice-cold 1M Sorbitol. The cells were again pelleted as above and resuspended finally in sterile ice-cold 1M Sorbitol to a final volume of 1.5 ml and aliquoted as 80 μl in sterile 1.5 ml micro centrifuge tubes and used immediately.

4.2.4.3. Transformation by electroporation

Freshly prepared competent cells (80 μl) were mixed with ~12 μg of linearized plasmid, transferred to an ice-cold electroporation cuvette (0.2 cm) (Bio-Rad, USA). The cells were incubated for 5 min. on ice and subjected to electric field as described by the manufacturer of the electroporator, Gene Pulser (Bio-Rad inc. USA). The charging voltage was 1500V with a capacitance of 25μF at 200 Ω resistance which gives a field strength of ~7500V/cm. Immediately after giving the electric pulse (within 8 seconds), 1 ml of ice-cold 1M Sorbitol was added to the cuvette and the contents were transferred to a sterile microfuge tube. From this about 200-300 μl of the cells were plated on

as per standard procedures (Sambrook et al., 1989; Kumar, 2000). Clone without insert was used as a negative control. Methodology, in brief is described below.

4.2.6.1. SDS-PAGE Analysis

SDS-PAGE was performed for analyzing the proteins secreted into the medium by the yeast cells. Proteins from the fresh or stored culture supernatants (500 µl) of yeast cells with and without FMDV genes were precipitated separately with ammonium sulfate (50% saturation) and pelleted at 15,000-x g for 10min. The pellet in each case was suspended in 20 µl of TE (pH 8.0), mixed with 5 µl of 5x SDS-PAGE sample buffer (Laemmli, 1970) (Appendix) and heated at 95°C for 5 min. The samples were analyzed by 12% SDS-PAGE using protein molecular weight markers (Genei, Bangalore- Medium range) as a standard (Sharpiro et al., 1967).

The separating gel of 10% pH 8.8 was prepared in between the glass plates using the Bio-Rad Gel casting assembly spacers of 1mm thickness were used so as to get a thickness of 1mm.. Stacking gel (5% in Tris buffer of pH 6.8) was poured over it and comb was placed. The gel after polymerizing was transferred into an electrophoretic apparatus (Bio-Rad) and buffer chambers were filled with running buffer (Appendix) and subjected to electrophoresis at 80V till the tracking dye reached the separating gel. The voltage was increased to 100V till the dye reached the bottom of the separating gel.

After completion of electrophoresis a single gel was stained with 0.25% W/V Coomassie Brilliant Blue (CBB-R 250) in 45: 10: 45: methanol / Acetic acid/ water for 10-12 h and destained with regular changing of the destaining solution (10% V/V acetic acid solution in water for 5-6 h). Gel after visualization on X-ray illuminator was stored in 5% acetic acid solution in water.

4.2.6.2. Concentration and purification of the secreted protein

In order to achieve the purification and concentration of the secreted proteins for further use, different methods as followed in the laboratory (Renji, 2001) were tried.

i) Concentration by sucrose

The culture supernatant was kept in a dialysis bag. and subjected to reverse osmosis using sucrose crystals in a Petri dish at 4°C for 8-12 h. The concentrated (25 fold) supernatant was dialyzed against PBS at 4°C for 12 h aliquoted into 1 ml aliquot and stored at -70°C till used.

ii) Ammonium sulphate Precipitation

The proteins secreted into the media were precipitated at room temperature (25°C) by ammonium sulfate at 50% (v/v) saturation using saturated ammonium sulfate solution pH 7.2 as per the standard method described in Methods in Enzymology (vol. 182). The precipitated proteins were pelleted at 15.000-x g at room temperature. The pellets were suspended in 1/10 of the initial volume in TE (pH 8.0) dialyzed and stored at -70°C as described above.

4.2.7. Protein Estimation (Bradford method)

The amount of the proteins, produced by the clones either before or after purification was assayed by Dye binding assay. (Bradford. 1976). Aliquots of 10 µl, 20 µl, 40 µl, & 60 µl of protein solution from each sample was diluted to 100 µl with TE and to this 1 ml of Bradford reagent (Appendix) was added and mixed. The A_{595} nm of the colour development was read spectrophotometrically. The concentration was deduced from the standard curve drawn using BSA at various concentrations as standard.

4.2.7.1. Determination of the Molecular weight of the protein

Molecular weight of the proteins was determined as described by Sharpiro, et al., (1967). The migration distance of the different protein band and the standard molecular weight markers were measured with a ruler. the Relative mobility (R_f values) of the protein was calculated as suit follows:

$$R_f = \frac{\text{Distance migrated by the protein}}{\text{Distance migrated by the dye front}}$$

A graph was polltted with the log of known MW against their relative mobilities. MW of the recombinant proteins and the yeast proteins were determined by reading the log MW against the R_f values.

Chapter 5

RESULTS AND DISCUSSION

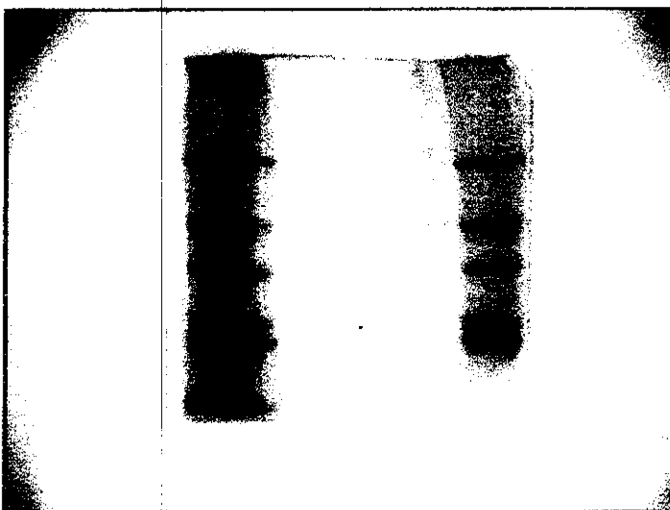
5.1. Transformation of yeast cells produced His+ transformants

Though no difference is shown in the level of expression between Mut+ \ Muts phenotypes (Clare, et al., 1991) for expression studies as Muts it grows faster than the Mut+ with methanol induction and reduces contamination of culture media and handling. Culture was maintained by methanol induction at every 24 hours to sustain protein expression upto 96 hours at 28 –300C when it reached a peak level (Renji, 2001). Culture supernatants from recombinant vector with or without insert were used for further characterization of the secreted recombinant protein. The proteins secreted into the medium was concentrated by reverse osmosis using sucrose crystals or precipitated by ammonium sulphate and purified by dialysis. The quantity of protein was found to be 2 g. per litre of culture supernatant (i.e. 0.5g per 250ml. culture). Varying amount of protein secretion (ranging from 6.3 mg to 12g / l of culture) was observed by various group of workers when genes coding for different proteins were used in Pichia expression system (Romanos, et al., 1992, Clare, et al., 1991: Vozza et.al., 1996).

Analysis of secreted proteins by SDS-PAGE has shown a protein band of 26kDa corresponding to size of the expressed 3AB protein. It is comparable to the molecular weight calculated from the size of the insert.

Hence, elaborate work using recombinant 3AB proteins should be taken up for correct interpretation of surveys simulated at the field level.

Figure 5.1 SDS PAGE



Chapter 6

CONCLUSION

CONCLUSION

The findings are also in agreement with the results published earlier by other workers. The cut-off values selected also allowed distinguishing the test sample results as positive or negative. Differences in protein conformation obtained from that of E.coli antigen and Yeast expressed antigen could account for the discrepancies observed. Also factors like animal's age, vaccine interference, methods of antigen preparation could have an influence on the results. Hence, elaborate work using recombinant 3AB proteins should be taken up for correct interpretation of surveys simulated at the field level.

Chapter 7

FUTURE PROPECTS

FUTURE PROSPECTS

In recent years, worldwide attention is focused on detecting antibodies to non-structural proteins of FMDV for differentiation of infected and uninfected \ Vaccinated animals. Antibodies to non-structural proteins are not type specific like the structural proteins (Graves, et al., 1978). These antibodies as compared to those against structural proteins persist for longer times in the animal body after infection (Bachrach, 1968, Lubroth and Brown, 1995). So a simple test employing these proteins can easily and reliably differentiate these animals.

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