

Diagnostic assays developed for the control of foot-and-mouth disease in India

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Abstract

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease of livestock, primarily affecting cattle, buffalo and pigs. FMD virus serotypes O, A and Asia1 are prevalent in India and systematic efforts are on to control and eventually eradicate the disease from the country. FMD epidemiology is complex due to factors like co-circulation, extinction, emergence and re-emergence of genotypes/lineages within the three serotypes, animal movement, diverse farm practices and large number of susceptible livestock in the country. Systematic vaccination, prompt diagnosis, strict biosecurity measures, and regular monitoring of vaccinal immunity and surveillance of virus circulation are indispensable features for the effective implementation of the control measures. Availability of suitable companion diagnostic tests is very important in this endeavour. In this review, the diagnostic assays developed and validated in India and their contribution in FMD control programme is presented.

Key words: Foot-and-mouth disease; Diagnosis; Sero-surveillance; Sero-monitoring; Multiplex polymerase chain reaction; Real-time polymerase chain reaction; Lineage differentiating polymerase chain reaction

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Core tip: To inform scientific community, this short review summarizes existing foot-and-mouth disease diagnostics developed in the recent past and used in India. Immediate and future requirements in the diagnostics are highlighted.

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INTRODUCTION

Foot and mouth disease (FMD) continues to pose threat to the livestock sector in the world. The annual direct loss due to FMD in India is estimated at United States dollar 4.45 billion^[1]. Economic losses due to trade barrier imposed by FMD free countries could be much more. FMD is caused by a single stranded positive sense RNA virus, belonging to the genus *Aphthovirus* of family *Picornaviridae*^[2]. Seven serotypes (O, A, C, Asia1, SAT-1, SAT-2 and SAT-3) and multiple antigenic variants within the serotypes of FMD virus (FMDV) exist because of the variable antigenic nature of its structural proteins. The FMDV genome of approximately 8.5 kb is polyadenylated at 3' terminus and carries a small protein VPg at its 5' end^[3,4]. It encodes four structural proteins (SPs) (VP1-4) and at least 8 non-structural proteins (NSPs). Structural proteins, VP1, VP2, VP3 and VP4 are formed by post-translation cleavage of a precursor coded by 1D, 1B, 1C and 1A genes, respectively^[3]. Non-structural proteins of FMDV consist of L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D. The L gene which encodes L protein is situated at the extreme 5' end of the coding region while all other NSPs are encoded by the P2 and P3 regions, which are situated towards the 3' end of the viral RNA (Figure 1). The P2 region codes for 2A, 2B and 2C while P3 codes for 3A, 3B1, 3B2, 3B3, 3C and 3D.

Most of the developed countries are free from FMD, whereas the disease is present in many developing countries including India. Epidemiology of FMD in India is complex due to prevalence of many variants of FMDV serotypes (O, A, Asia1), mixed farming system, diverse landscape, animal husbandry practices and very large population (about 500 million) of susceptible livestock^[5].

The disease in cattle and buffalo is characterized by high fever, depression, excessive salivation, formation of vesicles on the tongue and oral cavity, epidermis of the coronary band and inter digital space, udder and teats. Formation of vesicles in the oral cavity results in reduced food consumption, weight loss and emaciation. Vesicles may also develop in the epithelium of the pharynx, larynx, trachea, oesophagus and rumen. In young animals, it may lead to death due to myocarditis. Many times it leads to secondary bacterial infection in affected animals. While mortality is generally less than 3%, morbidity is very high and economic losses become unbearable for the farmers on account of decreased productivity and protracted convalescence in affected animals. Though, mortality is notably high in young pigs. Incubation period ranges from 2 to 14 d, but is generally shorter than a week.

FMDV can be transmitted by direct contact, aerosols, mechanical carriage by men or fomites and through animal products such as meat, offal, milk, semen or

embryos. Infected pigs shed large quantities of virus in aerosols^[6] and spread the virus down wind. Under favourable conditions of low temperature, high humidity and moderate winds, virus in aerosols may spread up to 250 km over sea^[7] and 60 km over land^[7]. Virus can remain infective on soil for 3 d in summer and for up to 28 d in winter^[8].

FMD symptoms could be confused with other vesicular diseases like Swine Vesicular Disease (cattle and sheep are resistant), Vesicular Exanthema (cattle and sheep are resistant), Vesicular Stomatitis virus (sheep/goats are resistant). Availability of rapid and sensitive FMD diagnostic assays is essential in order to confirm the initial cases and prevent further spread of the disease. Infected animals may secrete the virus before clinical symptoms develop and the virus could spread rapidly in the susceptible population; hence rapid and early identification of the infected/carrier animals is critical.

Timely identification of serotype of the virus involved in the outbreak is of the utmost importance for disease control. Besides, apparently healthy animal population in endemic settings are to be regularly screened for the presence of antibodies against SPs and NSPs of FMDV and for the presence of the virus in the oro-pharynx to confirm the carrier status. Many diagnostic assays have been developed throughout the world for rapid and specific detection of FMDV and the antibodies against the FMDV proteins. Most of these assays are developed and validated considering the local requirements and prevailing virus pool, whereas some assays have been developed for use in the broad geographical areas. Now a day, molecular methods for FMD diagnosis are playing important role when compared to the conventional methods.

The episodes of FMD outbreaks are to be actively monitored, recorded and investigated in order to support the vaccination based control programme in the country. For all these activities, availability of rapid, sensitive, specific and economical diagnostic assays representing the FMDV pool in circulation is of prime importance and necessity.

A systematic vaccination based control is in operation for control and eventual eradication of FMD from India since 2003-2004 by Government of India (Department of Animal Husbandry, Dairying, and Fisheries)^[5]. The total FMD susceptible livestock population in the country is about 500 million comprising of more than 300 million cattle and buffalo, 71.5 million sheep, 140.5 million goats, and 11 million pigs^[9]. Availability of indigenously developed diagnostic assays is crucial and indispensable to support such a huge control programme. In this review, the role of diagnostic assays developed, validated and used over the last decade in the country (Table 1) along with their contribution in control of FMD in India is being discussed.

FMDV DETECTION IN CLINICAL MATERIALS

FMD is primarily diagnosed by demonstrating FMDV

Table 1 Diagnostic assays for foot-and-mouth disease virus diagnosis with their associated advantages and disadvantage

FMD diagnostic assay	Specimen materials	Target region	Sensitivity	Specificity	Advantages	Disadvantages
Sandwich ELISA	RNA from TE, FL, TE,	VP1 protein	80%	100%	Easy to perform Suitable for handling large number of samples	Less sensitive, not suitable for certain type of clinical samples
Multiplex PCR	RNA from TE, FL, TE, Semen, Milk	1D region	Minimum detection limit of 1×10^{-1} TCID ₅₀ /mL	100% specific for cross serotype detection	Rapid and sensitive Suitable for samples like semen and milk	High risk of generating false positives
Taqman real-time PCR	RNA from TE, FL, TE, Semen, Milk	1D region	Minimum detection limit of $10^{1.0}$ TCID ₅₀ /mL	100% specific for cross serotype detection	More sensitive and specific than gel based assay	high risk of generating false positives
Virus isolation and neutralization	Triturated material of TE, FL, TE,	--	--	--	Gold standard assay for FMD diagnosis	Slow takes 1-4 d for confirmatory results
RNA transfection	RNA from TE, FL, TE, Semen, Milk	--	--	--	FMDV can be isolated from deteriorated clinical materials	--
LAMP	RNA from TE, FL, TE, Semen, Milk	3D region	Minimum detection limit up to 1.1×10^{-4} TCID ₅₀ /mL	--	Require no specialized instruments, can be used as point-of-care diagnosis	High risk of generating false positives
3AB3 I-ELISA	Serum	3AB3 region	96%	99.1% -96.4%	Sensitive and Specific	Only for bovine species
3ABC C-ELISA	Serum	3ABC region			Specific assay	Less sensitive than I-ELISA
2Ct I-ELISA	Serum	2C region			Universal for all species Sensitive and Specific	Only for bovine species

FMD: Foot-and-mouth disease; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; LAMP: Loop-mediated isothermal amplification.

particles or viral genome in the clinical materials viz. tongue epithelium, foot epithelium, saliva, milk and semen, etc. Detection of intact virus particles by sandwich enzyme-linked immunosorbent assay (ELISA) and virus neutralization test provides confirmatory diagnosis, whereas detection of the viral genome by polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) assay is more sensitive method of diagnosis. Samples collected from the FMD suspected animals are processed and routinely analyzed by these assays. The details of the suspected clinical samples tested during the last seven years are presented in the Table 2.

Sandwich ELISA

FMD antigen detection ELISA was shown to be rapid and simpler to perform^[10]. The assay is generally regarded as the primary test for FMD diagnosis especially at the regionally located FMD diagnostic laboratories in the country^[11]. The suspected clinical materials are first submitted to the regionally located FMD diagnostic laboratories in the country working under ICAR-PDFMD, Mukteswar where samples are processed and tested by an in-house sandwich ELISA for identification of FMDV serotype(s). The assay is based on the detection of FMDV structural proteins (Figure 1) and utilizes the serotype specific polyclonal antibodies generated in guinea pig and rabbits^[12]. This antigen-capture sandwich ELISA has 100% specificity for heterologous FMDV and 80% sensitivity for detection of complete virus particles in clinical samples^[12].

Table 2 Details of the clinical materials suspected for Foot-and-mouth disease tested by sandwich enzyme-linked immunosorbent assay and multiplex polymerase chain reaction during the last five years

Year	Sample tested	Serotype O	Serotype A	Serotype Asia 1	Total FMD diagnosed
2009-2010	1155	423	15	7	445
2010-2011	345	83	10	10	171
2011-2012	567	265	4	40	309
2012-2013	701	218	15	52	285
2013-2014	3130	1295	24	10	1329
Total	5898	2284	68	119	2539

FMD: Foot-and-mouth disease.

The assay is easy to perform at regional FMD diagnostic laboratories and large number of samples can be processed without risk of laboratory cross contamination. The assay is being used countrywide since two decades at 23 regionally located laboratories in the country^[11]. As the assay specifically detects the intact virion particles in clinical materials in a serotype specific manner, the lower sensitivity could be attributed to the improper storage and transportation of samples that leads breakdown of the virus particles. The clinical materials are then submitted to the Central Laboratory, Mukteswar for detailed virological and genome analysis. Since 2009-2010, more than 5000 clinical materials have been tested by the sandwich ELISA, both at the regional FMD diagnostic laboratories and the central laboratory.

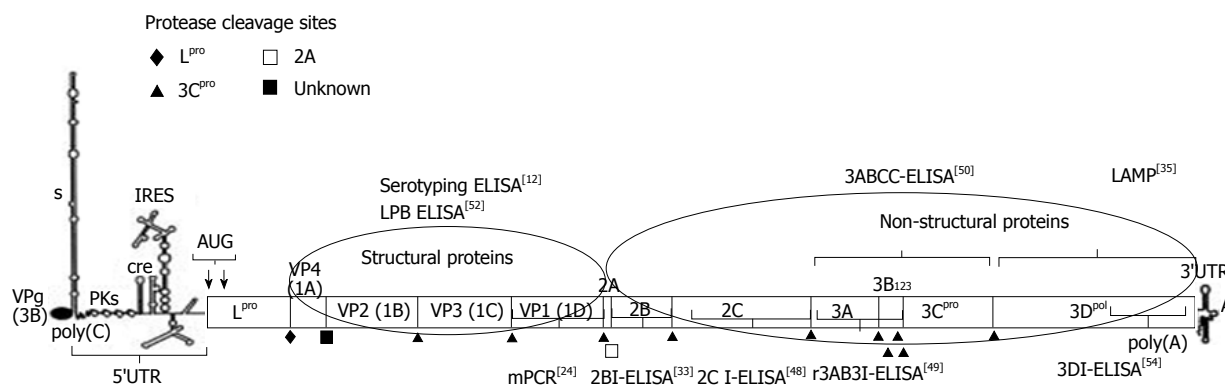


Figure 1 Genome structure of the foot-and-mouth disease virus. Describes in details the different regions of foot-and-mouth disease virus (FMDV) which codes four structural and 8 non-structural proteins. The regions of genome encoding structural proteins are targeted for FMDV serotype determination by various assays, whereas the genome regions coding for non-structural proteins are targeted for serotype independent diagnosis. The SP of FMDV is targeted for serotyping by antigen trapping enzyme-linked immunosorbent assay (ELISA) or for measurement of antibody response by liquid phase blocking ELISA. Antibodies against various non-structural proteins are targeted for differentiation of FMD infected from vaccinated animals.

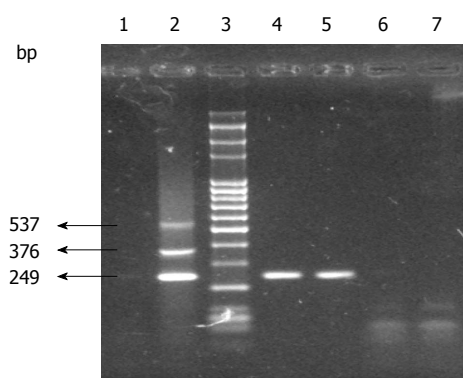


Figure 2 Depicting the foot-and-mouth disease virus serotyping by gel based multiplex polymerase chain reaction assay. Foot-and-mouth disease virus serotype determined by gel based ready-to-use lyophilized one-step realtime-polymerase chain reaction. Lane 1: negative control; Lane 2: Positive control of serotypes O (249 bp), A (376 bp) and serotype Asia1 (537 bp); Lane 3: 100 bp DNA ladder; Lane 4 and 5: Positive sample of serotype O; Lane 6 and 7: Negative samples for FMD.

Virus isolation

Of the established diagnostic approaches, virus isolation (VI) in cell culture is considered as the “gold standard” as described in OIE Terrestrial Manual 2012^[13]. This method can be highly sensitive (depending upon the cell culture system used), although it can be slow, taking between 1 and 4 d to generate the results and require a containment laboratory facility. However, virus isolation from clinical materials is indispensable for antigenic profiling of the virus and vaccine matching. Primary cells, such as bovine thyroid (BTY), are highly susceptible to a wide range of FMDV serotypes^[14], but they are difficult and costly to prepare and lose FMDV susceptibility after multiple passages^[15]. Primary lamb kidney (LK) cells are also very sensitive to FMDV, and unlike BTY cells, LK cells maintain their sensitivity to FMDV infection after cryopreservation^[16]. Immortalized cell lines [e.g., baby hamster kidney (BHK-21) fibroblasts and porcine kidney epithelial cells], are much easier to maintain but are less susceptible to specific animal-derived FMDV

serotypes^[17-20]. Recently LFBK- $\alpha\beta$ 6 stable cell line has been established and was observed to be an excellent cell line for FMDV diagnostic- and research-based cell applications^[21].

In India, all the clinical samples collected/submitted for FMD diagnosis are subjected to virus isolation using the cell lines (BHK21, IBRS, and LFBK cells). Virus isolates after characterization are archived in the National FMDV repository. Currently the repository contains more than 1850 FMDV isolates comprising of serotype O ($n = 1180$), A ($n = 298$), Asia1 (358) and C ($n = 15$). The oldest isolate available in the repository is of the year 1962 (Serotype O). Such a vast pool of virus isolates aids in selection and identification of suitable vaccine candidates through vaccine matching exercise from time to time.

RNA transfection method for FMDV rescue

Isolation of virus from the clinical materials is not always possible due to several factors^[22]. Under such scenarios, the transfection based virus-rescue method as described by Belsham *et al.*^[23], has been optimized in India^[22]. Success rate of RNA transfection for virus isolation was observed to be 62% against 16% in conventional cell culture method that enhances the number and diversity of virus isolates being used in vaccine matching exercise. Till date, 88 serotype O, 24 serotype Asia1 and 09 serotype A viruses have been rescued using RNA transfection method from the samples where conventional method of cell culture passage failed to isolate the virus^[11].

Multiplex PCR

In vitro amplification based detection of genome is more rapid and sensitive than conventional VI^[24]. Initially, assays were developed targeting the conserved 3D region^[25,26] and 5' UTR region^[27]. Subsequently, multiplex PCR for (mPCR) targeting VP1 region were developed for detecting FMDV and differentiating amongst the serotypes^[24,28,29].

On the similar lines, mPCR was also developed in India^[24] and the success rate of FMD diagnosis and serotype detection increased by 8%^[30]. In this assay, the serotype-specific primers targeting 1D region and common reverse primer (NK61) targeting 2B region were used for multiplexing (Figure 1). Figure 2 indicates the mPCR based serotype identification describes the identification of serotype involved by multiplex PCR where product size of 249, 376 and 537 bp are specific for serotypes O, A, and Asia1, respectively. The minimum detection limit of the mPCR has been estimated as 1×10^{-1} TCID₅₀/mL for serotypes O, A, and Asia1^[24].

Although, the mPCR suffered from the disadvantage of generating false positives due to carry-over of PCR amplicons and thus, not considered as an ideal assay for routine testing of large numbers of samples especially at regionally located FMD diagnostic laboratories^[31]. To overcome the chances of cross-contamination and make it more feasible for regional FMD laboratories, a ready-to-use thermo-stable RT-PCR mixture was developed^[30]. All the components of the reaction mixture were mixed together in a vial and lyophilized (Lyodryer, United States). The lyophilized vials are to be reconstituted with nuclease free water before use and supplemented with the extracted RNA from the suspected materials followed by *in-vitro* amplification in a thermal-cycler. This thermostable RT-PCR mix made the assay more user friendly and clinical samples can be now diagnosed by PCR at the field level FMD diagnostic laboratories with uniformity in the results. In addition, the requirement of keeping live FMDV for positive control became obsolete. Since 2005, more than 2037 suspected clinical materials have been successfully tested by the mPCR in the country^[11].

Reverse transcription-LAMP assay

Reverse transcription-LAMP (RT-LAMP) assay is an autocycling and strand displacement DNA synthesis method^[32] which has recently been employed in FMD diagnosis as point-of-care test. The RT-LAMP based targeting 3D and IRES region for detection of FMDV have been reported earlier^[33,34]. In the recent past, LAMP based assay for FMDV detection and serotype differentiation (O, A and Asia 1) has been developed^[35]. RT-LAMP based assay targeting 3D region has also been developed in India and is being used routinely for rapid detection of FMDV (Figure 1)^[36]. LAMP assay requires only a water bath instead of a thermal-cycler as in PCR. In addition, gel documentation system is also non-essential as hydroxynaphthol blue (HNB), an azo dye is used as the indicator. The sensitivity and specificity of the RT-LAMP assay developed were estimated as 4.2×10^{-4} , 2×10^{-4} and 1.1×10^{-4} TCID₅₀/mL for FMDV serotypes O, A and Asia1 respectively. LAMP assay for FMD diagnosis was validated by simultaneous testing of the clinical samples ($n = 139$) by mPCR and LAMP and the results revealed higher sensitivity in case of LAMP.

Real time PCR assay

Reverse-transcription real time PCR (RT-qPCR) assays have been developed and evaluated for the identification of FMDV in different parts of the world using fluorogenic dyes. Both SYBR Green and TaqMan chemistries have been widely utilised in qPCR assays for FMD, however TaqMan provide an additional advantage of multiplexing. In India, a qPCR assay targeting 1D region of FMDV was developed in multiplex format for simultaneous detection and identification of FMDV serotypes in the suspected clinical materials^[37]. The sensitivity of the TaqMan based multiplex qPCR was found to be $10^{-1.7}$ TCID₅₀/mL, $10^{-1.0}$ TCID₅₀/mL, $10^{-1.7}$ TCID₅₀/mL for serotype O, Asia1 and A respectively^[37]. The qPCR assay was found to be more sensitive than gel based assay and provides an estimate through standard curve of viral load in the samples. With high sensitivity and specificity, the qPCR assay has been used as the primary tool for the detection of FMDV in persistently infected carriers among exposed ruminants which is of great importance in disease control^[38].

FMD diagnosis in semen and milk

FMDV can be actively secreted in semen of FMD infected bull before onset of clinical symptoms and up to 5-8 mo post infection^[39]. It has also been reported that FMDV can survive in frozen semen straw, thus artificial insemination can possibly serve as the source of FMDV transmission to wider and farther areas. The extenders used during the production of semen straws provide the conditions conducive to survival of the virus for more than 320 d when stored at -50°C ^[40]. Routinely used FMD diagnostic methods such as VI and antigen ELISA require modifications for detecting FMDV in semen samples^[41,42]. Even mPCR assay was found to be far less sensitive for semen samples. The major reason behind PCR failure was the presence of PCR inhibitors in semen^[28,39]. Hence, existing mPCR assay was improvised for the detection of FMDV genome in semen samples^[43]. The RNA from suspected semen samples (neat or extended) was extracted by a modified method to remove the PCR inhibitors^[43]. This modified mPCR has been used for screening of 980 animals for presence of FMDV genome in semen till now. It was also established that, FMDV could be detected in semen of the infected cattle bull for about 5 mo but not more than 8 mo^[43].

LINEAGE DIFFERENTIATING PCR

There is co-circulation, extinction, and emergence and re-emergence of genotypes/lineages within the serotypes from time to time in India. The emergence or re-emergence of any new lineage warrants rapid and accurate detection to facilitate early warning^[44,45]. Detailed nucleotide sequence of these viruses are analysed to detect emergence of any new group. A rapid multiplex PCR assay was developed for detection of the dominating VP3⁵⁹-deletion group of serotype A

Table 3 Details of the total number of serum samples screened for reactivity to foot-and-mouth disease virus NSP 3AB3 during the last five years in India

Year	Total samples tested	Total positive	% animals 3AB3 reactors in India
2009-2010	29763	8303	27.90
2010-2011	31042	8341	26.87
2011-2012	37467	10410	26.09
2012-2013	40934	10811	26.41
2013-2014	52224	15268	29.20
Total	191430	53133	27.70

virus with 100% sensitivity and specificity^[44]. Genotype differentiating RT-PCR was developed as a fast, cost-effective and user-friendly alternative to 1D region based phylogeny for detection and differentiation of genotypes VI and VII of serotype A^[45]. Similarly, a simple, fast and multi-primer RT-PCR assay has been developed and validated to differentiate genetic lineages of serotype Asia1 viruses^[46]. These assays have been proven as useful tools in preliminary molecular epidemiological investigation of FMD in the country.

SERO-SURVEILLANCE OF FMD IN INDIA

Sero-surveillance is of prime importance in India where FMD control programme is in operation for last 10 years. As per the OIE guidelines, in regions adopting vaccination to control FMD, sero-surveillance should be performed by an assay capable of differentiating infected from vaccinated animals (DIVA)^[47]. Detection of antibodies against various non-structural proteins (NSPs) of FMDV has been successfully utilized for DIVA^[48,49]. Considering the complex epidemiology of the disease in the country, assays for DIVA were developed and validated taking into account the factors such as vaccine quality (in terms of level of NSP contamination in the formulation) and coverage in India. A tool box of one competitive and four indirect ELISAs utilizing 3AB3, 3ABC, and truncated 2C (2C_t) NSPs of FMDV (Figure 1) was developed in India^[50-52]. The performance of these in-house DIVA assays was compared with the two commercially available kits (PrioCheck[®] FMDV-NS and Svanovir FMDV 3ABC-Ab ELISA kit) and indigenously developed assays were found to be equally capable in detecting infected animals among the vaccinated population^[53]. However, the in-house assays performed better than the commercial kits in case of intensively vaccinated samples^[53]. The r3AB3 indirect ELISA is routinely used for countrywide screening of bovines^[51] and results obtained for the serum samples collected at random from the country are presented in the Table 3. The diagnostic sensitivity of this assay is 96% while the diagnostic specificity varied between the naïve and vaccinates as 99.1% and 96.4%, respectively. This assay detects antibodies to 3AB (3AB-Ab) from 10 to as late as 900 d post-infection in experimentally infected cattle. Recently 3B^[54], 2B^[55] and 3D^[56] NSP based

assays have also been developed in India and are under validation.

SERO-MONITORING OF FMD

Post vaccination sero-monitoring is critical to monitor protective antibody level in animals before and after every round of vaccination. Under the Government of India initiated vaccination based FMD control programme (FMDCP) 120 million cattle and buffaloes are routinely vaccinated at 6 mo interval to progressively build herd immunity^[5]. However, vaccines against FMD only protect the animal from clinical disease and not from the super infection by other serotypes of FMDV. Additionally, the vaccine induced protection remains only for about 4-6 mo^[57] and with the decline in herd immunity risk of clinical disease increases due to the creation of infection window. Therefore, quantitative estimation of protective antibody response (titer) in vaccinated animals through sero-monitoring is indispensable for devising appropriate vaccination regime and successful implementation and monitoring of the control programme^[58,59]. With the current sampling policy in the country, village is considered as a herd and from each district covered under FMDCP, 10 villages are randomly selected for sampling, and from each village 20 serum samples (10 cattle and 10 buffalo) are collected at random before (0 d) and 28 d post vaccination (dpv) to have un-biased estimate of vaccination performance and the resulting level of herd immunity. Antibody titers against the serotypes O, A and Asia1 are determined by four fold dilution liquid phase blocking ELISA (LPBE)^[60,61]. With the expansion of FMDCP, there is a considerable rise in the number of serum samples to be tested. Thus, a high throughput LPBE assay was developed recently to fasten the process and save time and labour (Manuscript communicated). This high throughput assay utilizes the linear regression method for extrapolation of titers of test serum samples from the known internal controls^[60]. In addition, the reagents used in the assay are thermo-stable facilitating the transportation to the regional laboratories under high ambient temperature.

CONCLUSION

Considering the fact that India has a large livestock population (about 500 million) susceptible to FMD, the country requires economical companion diagnostic tests tailor-made for the suitability under Indian scenarios to run the progressive control programme for FMD. Though India is now self-sufficient to produce most of the diagnostic kits, but still a lot of improvisation is needed in the current assays. The polyclonal antibodies used in several assays could be replaced with the recombinant antibodies. Some success has been achieved in development of single-chain variable fragment (scFv)^[62] but work is being continued to develop scFv and nanobodies against highly immunogenic epitopes of FMDV and assess their applicability in diagnostics.

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