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In Process Quality Control Factors Affecting Potency of Foot and Mouth Disease Virus Vaccine

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Abstract.- In process quality control factors affecting potency of Foot and Mouth Disease (FMD) virus vaccine were evaluated. These factors were amount of immunogen (biological titer of FMD "O" virus), inactivants, safety and sterility tests, adjuvant and keeping quality of the vaccine. The biological titer of 7th passage of the serotype on BHK-21 cell line was $10^{6.4}$ mean tissue culture infective dose ($TCID_{50}$) per ml. The dose of immunogen was directly proportional to complement fixing antibody titer in rabbits. The virus in the suspension culture was inactivated at 37°C with 0.12 percent formaldehyde for 12 hours or 0.02 M binary ethylene-imine (BEI) for 48 hours. The sterility of the virus suspension was determined by inoculating the culture on laboratory bacteriological media and safety was monitored on monolayer of BHK-21 cell line. The virus serotype suspension culture qualified for vaccine production. The effect of aluminum hydroxide gel (AHG) and oil base-montanide (OB) in inducing the antibody response in rabbits was the same. Antibody response of rabbits to both of the adjuvant containing vaccines was better than that of lanolin based vaccine. The storage of the vaccine at 6(±2)°C for 6 months did not affect its potency in rabbits. It is concluded that amount of the virus immunogen in the vaccine is directly proportional to the titer of antibody response in vaccinates.

Key words: Immunogen, adjuvant, mean tissue culture infective dose, keeping quality, rabbits.

INTRODUCTION

Foot and Mouth Disease (FMD) is a highly contagious viral disease of cattle, buffaloes, sheep, goats, swine and wild animals like giraffes, deer, blue bulls (nilgai), etc. The disease spreads in a large population of susceptible animals (Rowlands, 2003) and is caused by an Aphthovirus of Picornaviridae (Rucckert, 1996). The virus has seven serotypes such as "A", "O", "C", "Asia-1", "SAT-1", "SAT-2" and "SAT-3" (OIE, 2001). Out of these, only "A", "O" and "Asia-1" types of FMD virus are prevailing in Pakistan (Haq, 1961; Fida *et al*., 1965).

The sick animals demonstrate clinical signs such as weary and rough coat and rise in body temperature. Additional symptoms include dripping of saliva from its mouth due to vesicular and later on ulcerative lesions of tongue, gums, cheeks and hard palate and lameness is because of vesicular lesions on the cleft of feet and coronary bands. The morbidity rate can be up to 100% in susceptible population and the mortality is usually negligible except in young calves (Ajmal *et al.*, 1989). The disease perpetuates in livestock population through out the year. The disease adversely affects milk production, weight gain, working efficiency, progeny production with increased mortality in young calves (Murphy *et al*., 1999; Doel, 2003). Annually, the losses to the farmers caused by FMD in capital investment exceed Rs 2.00 billions (Ajmal *et al.*, 1989).

The disease is controlled through mass vaccination, selective culling and implementing strict bio-security measures. In Pakistan, chemically inactivated monovalent FMD virus vaccine is used for controlling the disease but outbreaks are common even in the vaccinated animals. The failure of immunoprophylaxis might be due to factors such as lack of prevailing serotypes of FMD virus in the vaccine, inappropriate amount of immunogen/ vaccine dose, lack of cold chain, concurrent disease or prevailing biological and chemical immuno-suppressants (Meyer and Knudersen, 2001). These factors along with heavy economic losses caused by

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FMD to the dairy industry, make it imperative to study effect of "*in-process quality control factors"* such as amount of immunogen of "O" serotype of FMD virus per vaccine dose, inactivant, adjuvant and vaccine storage at refrigeration temperature on the potency of inactivated "O" type of FMD virus vaccine.

MATERIALS AND METHODS

Source of virus serotypes

Local serotype "O" of Foot and Mouth Disease (FMD) virus (7^{th} passage) was obtained from WTO-Quality Control Laboratory, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan and cultivated on adherent BHK-21 cell line. The virus serotype was used as seed for production of inactivated FMD virus vaccines.

Cultivation of virus

The virus serotype was propagated on BHK-21 cell monolayer having more than 90 per cent cell confluence in two Roux flasks (Clarke and Spier, 1980). One flask was inoculated with 1 ml of pre-filtered FMD type "O" virus suspension, whereas, the other one served as un-inoculated control. After 3 hours of incubation, medium of both the flasks was replaced with fresh medium containing 1 per cent fetal calf serum (FCS). After every 24 hrs of incubation, each of the flasks was observed for presence of any cytopathic effect (CPE). After 48 hrs of incubation, contents of the virus inoculated flask were harvested and filtered through 0.2 um porosity syringe filter. From this, 1ml of virus suspension was processed for calculation of its biological titer. The remaining virus suspension was stored in duplicate aliquots at 4 ^{0}C till be used for vaccine production.

Biological titration (Mean tissue culture infective dose: $TCID_{50}$)

The biological titer of the virus was determined by inoculating on to monolayer of BHK-21 cell line in 96 well flat bottom cell culture plates. The plate contained 48 hrs incubated BHK-21 cell monolayer in each well. The growth medium was decanted and the monolayer was once washed using sterile phosphate buffered saline (PBS: pH 7.4). Two hundred microliters of first dilution of the 10 fold dilutions of virus, prepared in maintenance medium, was added to the eight wells of 1^{st} column of the plate. This procedure was repeated for every dilution till 11^{-11} dilution. The 12^{th} column was kept as cell control and no virus was inoculated on to it. The plate was incubated at 37°C for 72 hrs. After every 24 hrs the plate was observed under inverted microscope for presence or absence of any CPE in each of eight wells. From these recorded observations, biological titer of the virus was calculated using Reed and Muench method as described by Villegas (1998).

Preparation of virus vaccines

The suspension of the FMD virus was processed for preparation of inactivated FMD type "O" virus vaccine. The further processes involved were inactivation of virus, safety and sterility testing and mixing of stabilizer and adjuvant.

Briefly the FMDV was inactivated at 37°C with 0.001 M conc. of binary ethyleneimine (BEI) for 48 hrs. The residual BEI was neutralized by adding sodium thiosulphate solution to the final conc. of 2 per cent (Bahnemann, 1975). The second aliquot of the virus was inactivated by using 0.12 % formalin (Formaldehyde, 37%: Merck) at 37°C for 24 hrs. Vials containing inactivated virus were separately processed for sterility and safety testing using lab bacteriological culture media and monolayer of BHK-21 cell line plates, respectively (OIE, 2000). Thiomersal sodium at the rate of 0.005 per cent was admixed with the contents of these vials as stabilizer and preservative of the virus vaccines (OIE, 2000).

Four aliquots of BEI inactivated virus were made each having 10^6, 10^5, 10^4 and 10^3 units of $TCID_{50}$. Contents of each of the four vials were admixed with aluminum hydroxide gel (AHG) for preparation of the vaccines: (1) BEI-AHG-FMDV with 10^{-3} units of $TCID_{50}$, F, 4-8°C, (2) BEI- AHG -FMDV with 10^{-4} units of $TCID_{50}$, F, 4-8°C, (3) BEI- AHG -FMDV with 10^{-5} units of $TCID_{50}$ F, 4-8°C, (4) BEI- AHG -FMDV with 10^{-6} units of $TCID_{50}$ F, 4-8 ^{0}C.

The vial containing the virus suspension (10^6 units of $TCID_{50}$) was mixed with either of the

adjuvant such as aluminum hydroxide gel (AHG), OB or lanolin base (LAN) as described by Dalsgaard (1987) for preparation of adjuvant containing FMD vaccines such as (5) BEI-AHG-FMDV with 10^{-6} units of $TCID_{50}$ F, 4-8 ^{0}C, (6) BEI-LAN-FMDV with 10^{-6} units of $TCID_{50}$ F, 4-8°C, (7) BEI-OB-FMDV with 10^{-6} units of $TCID_{50}$ F, 4-8°C.

The vial containing formalin inactivated virus suspension (10^6 units of $TCID_{50}$) was mixed with AHG for preparation of (8) FORM- AHG -FMDV with 10^{-6} units of $TCID_{50}$ F, 4-8°C, vaccine. The BEI- AHG -FMDV with 10^{-6} units of $TCID_{50}$ was stored for 0 (Fresh), 2, 4 and 6 months post preparation to evaluate the effect of storage on its potency. At each storage interval, physical appearance and separation was recorded. These vaccines were (9) BEI- AHG -FMDV with 10^{-6} units of $TCID_{50}$ F, 4-8°C, (10) BEI- AHG -FMDV with 10^{-6} units of $TCID_{50}$ 2M, 4-8 ^{0}C, 11-BEI- AHG -FMDV with 10^{-6} units of $TCID_{50}$ 4M, 4-8 ^{0}C and 12-BEI- AHG -FMDV with 10^{-6} units of $TCID_{50}$ 6M, 4-8°C.

Evaluation of vaccines in rabbits

Each type of the inactivated FMD virus vaccines was evaluated in rabbits that were maintained in the Experimental Animal House, UVAS, Lahore. Rabbits were divided into 12 groups (each group contained 3 rabbits). Each rabbit of each group was injected with either of the FMD vaccines. Blood sample from each of the rabbits of each group was collected on 0, 14, 21 and 42 days post priming from the ear vein. Serum of each blood sample was separated and was given heat treatment at 56°C for 30 minutes. Each serum sample was stored in properly labeled vials at –40°C till required for complement fixation test (CFT) as described by Ferris and Dawson (1988).

Statistical analysis

The data regarding anti-FMDV "O" type CF antibody titer of each rabbit of each group was processed for calculation of geometric mean titer (GMT) as described by Villegas and Purchase, (1989 MISSING AT THE END). Moreover, the GMT was compared amongst groups using ANOVA (Steel *et al*., 1997).

RESULTS AND DISCUSSION

Quality of a viral vaccine is controlled through In Process Quality Control Laboratory that is an integral part of every biologics production unit. Biological titer (mean tissue culture infective dose: $TCID_{50}$) of the virus was $10^{3.0}$ units/ml after its 1st passage on BHK-21 but increased up to $10^{6.4}$ units of $TCID_{50}$ per ml of the biomass suspension on its 7th passage. Amount of immunogen (units of $TCID_{50}$) in FMD virus vaccine was directly proportional to the anti-complement fixing antibody titer in rabbits (Table I). One dose of 0.5 ml adjuvant FMD "O" virus vaccine contained 0.2 ml of the virus with $10^{6.4}$ units of $TCID_{50}$ per ml. There is always a recommended biological titer of a virus per dose of a viral vaccine (OIE, 2001).

Table I.- Effect of immunogen dose on potency of foot and mouth virus disease vaccine.

Sampling time post-priming (days) (n=3)	Amount of Immunogen (units of $TCID_{50}$)*			
	10^3	10^4	10^5	10^6
7	0,2,0 (0.7)	0,0,0 (0)	2,4,2 (3.3)	2,4,2 (3.3)
14	2,2,0 (1.3)	4,8,4 (5.3)	4,4,2 (3.3)	4,8,4 (5.3)
21	4,2,4 (3.3)	2,4,8 (4.7)	8,16,16 (13.3)	32,16,16 (21.3)
28	4,4,4 (4.0)	4,8,4 (5.3)	16,16,16 (16)	32,16,32 (26.7)
35	4,4,4 (4.0)	4,8,4 (6.7)	32,16,16 (21.3)	32,32,64 (42.7)
42	8,4,4 (5.3)	8,16,8 (10.7)	32,64,64, (53.3 (64)	64,64,64 (64)
CGMT± Sd**	3.10^a± 1.76	5.45^a±3.45	18.42^b ±18.52	27.28^b± 23.13

*: Biological titer is expressed in units of mean tissue culture infective dose 50 ($TCID_{50}$)

**Figures having similar superscript are not significantly different ($p<0.5$).

Figures in the parenthesis indicate the geometric mean titer of anti-"O" FMD virus complement fixing antibody titer

The FMD virus was pathogenic even after its 7th passage on BHK-21 cell line. The inactivation of the virus serotype is necessary for production of FMD vaccine. Formaldehyde (37.5 percent: Merck)

when mixed with the virus suspension culture at rate of 0.12 percent inactivated the virus at 37°C for 24 hrs. Binary ethyleneimine (BEI) when mixed with virus suspension culture at rate of 0.001 M concentration inactivated the virus at 37°C for 48 hours. The formaldehyde or BEI treated virus when cultivated on monolayer of BHK-21 cell line, failed to induce any CPE. Sodium thiosulphate when admixed with the BEI treated virus suspension did not exhibit any change in physical appearance of virus suspension such as color, opacity and turbidity. Formaldehyde inactivates the virus proteins and its nucleic acid by cross linkage to confer structural rigidity and modulating the configuration of ligand molecules of the capsid proteins on the virus. In the past, formaldehyde had been an economical and commonly used disinfectant on commercial livestock and poultry farms. It is also used to inactivate the virus infectivity for preparation of antigen and inactivated vaccines (Frenkel, 1947; Barteling and Woortmeyer, 1984; Tosh *et al*., 2002). Presumably, interaction of formaldehyde with virus capsid proteins reduces virus immunogenicity. Its residues in the inactivated virus suspension may act as carcinogen, therefore, binary ethyleneimine (BEI) is preferred to inactivate the virulent virus for vaccine and antigen preparation. BEI cross links nucleic acid chains and thus inactivate viruses. Moreover, its molecules do not bind with the virus surface proteins and thus don't interfere with the virus immunogenicity (Bahneman, 1975; Tekerlekov and Veleva, 1985; Bahneman, 1990; Aarthi *et al.*, 2004).

The inactivated FMD virus suspension, when injected in rabbits, presumably absorbed from the inoculation site, with in hrs of administration without providing a suitable stimulus to immuno-competent cells. These antigens presumably were not processed by the antigen processing cells (APC) hence development of suitable number of plasma cells, memory cells and production of specific antibody titer did not take place. However, inactivated FMD virus suspension when mixed with either aluminum hydroxide gel (AHG), montanide (OB) or lanolin (LAN) induced detectable level of anti-FMDV-CF antibodies in rabbits (Table II). The serum antibodies appeared earlier in the rabbits that were vaccinated with AHG-FMDV vaccine than those vaccinated with either LAN-FMDV or OB-FMDV vaccines. However, the antibody response of each group of rabbits to either of the vaccines was not significantly different up to 42 days post vaccination. Adjuvant when added to virus suspensions increases its antigenicity. The traditional adjuvant such as aluminum hydroxide gel and oils still play a role as a base of vaccine formulations. Various adjuvant such as particulate antigens, oil and emulsifier based adjuvant, controlled antigen delivery, adjuvant based upon specific targeting of antigen and gel type adjuvant are commonly used in veterinary vaccines. The liquid paraffin (oil adjuvant) is used in many veterinary vaccines as a mineral oil. The emulsifiers such as Arlacel-A (Mannide monooleate) and Span-80 (Sorbitan momooleate) are used in 10 percent concentration in vaccines (Dalsgaard, 1987). Addition of these mixtures in aqueous phase of antigen results in milky white product. Addition of the surfactant like tween-80 in oil base reduces the viscosity of the vaccines. The stability of the vaccine depends upon concentration of the emulsifier (Graves, 1971; Kimura *et al.,* 1978; Dalsgaard, 1987: Chowdhery *et al.,* 1996; Jennings *et al.,* 1998). Aluminum hydroxide gel adsorbs the microbial immunogens and can not directly enter into circulation from the inoculation site. The gel is least toxic for animal tissues, hence is commonly used in human, canine, bovine, caprine and equine vaccines (Dalsgaard, 1987). Oil based or non-oil based adjuvants produce a vaccine depot at the inoculation site causing inflammation by irritation, and recruiting immunocompetent cells (Lymphocytes and antigen presenting cells; APC) at the injection site. The APC removes the antigen slowly over a long period of time and hence prolong the duration of immunity. On account of this property, oil based vaccines are effective (Barteling and Vreeswijk, 1991). These cells phagocytose, process and present the antigen on their suface in association with self immune associated (la). The thymus dependent lymphocytes (T-cells) can only recognize the antigen when presented on the surface of APC with la antigen. The antigens stimulated T-cells transform into lymphoblast and populate in different lymph nodes and other lymphoid organs such as spleen, mucus membrane associated

lymphoid tissues such as peyer's patches, etc., (Vanio *et al*., 1988; Tizard, 1998). The cells secrete lymphokines which activate the bursal dependent lymphocytes (B-cell) and resulted into antibody formation. In the primary response, immunoglobulin M (lg M) was released followed with immuno-globulin G (lg G). Both of the immunoglobulins are detectable by complement fixation and virus neutralization tests (Tizard, 1998) which have been replaced by more sophisticated ELISA technique (Ferris and Dawson, 1988). This could be logical explanation for the induction of high titer of anti-FMD virus-CF antibodies in the sera of rabbits vaccinated with adjuvant containing vaccines. Oil based vaccines some time induce local chronic inflammatory response such as granuloma or abscess formation at the inoculation site (Barteling and Vreeswijk, 1991; Tizard, 1998) and may produce adverse anaphylactic reaction if the emulsifier particularly Tween 80 is added in higher quantities (Toman *et al*., 1992).

Table II.- Effect of adjuvant on potency of foot and mouth disease virus vaccine.

Sampling day post-vaccination (n=3)	Antibody response of rabbits to different adjuvant containing FMD vaccines		
	AHG-FMDV*	LAN-FMDV**	OB-FMDV***
0	0, 0, 0 (0)	0, 0, 0 (0)	0, 0, 0 (0)
7	0, 0, 2, (0.7)	0, 0, 0, (0)	0, 0, 0, (0)
14	4, 4, 8, (5.3)	0, 4, 4, (2.7)	4, 4, 8, (5.3)
21	32,16,16,(21.3)	8, 8, 8, (8.0)	16,16,16,(16)
35	8, 32, 32, (24)	2,16,32,(16.7)	32,32,32,(32)
42	32, 32,16, (26.7)	32, 32, 32, (32)	16, 32, 32, (26.7)

Figures in the parenthesis indicate the geometric mean titer of anti-"O" FMD virus CF antibody titer, * AHG: Aluminum hydroxide gel, ** LAN: Lanolin, *** OB: Montanide Oil Base

The FMD virus vaccine when stored at 4±2°C up to six months did not show any significant difference in inducing anti FMDV "O" CF antibody response in the vaccinated rabbits (Table III). The vaccine when stored at 37°C was separated into oil and aqueous fractions which makes it ineffective. Bio-molecules degrade continuously causing failure of immunoprophylaxis. The biodegradation is enhanced if the vaccine is exposed to higher temperature than 4-8°C. To reduce the biodegradation of the immunogens, the inactivated virus suspension is admixed with stabilizer such as thiomrsal sodium, sodium benzoate, potassium meta-bi-sulphite, etc (OIE, 2000) and adjuvant vaccine prepared from virus suspension is stored at 4-8°C. The antigen looses its immunogenic activity at extreme temperatures. It is, therefore, recommended that vaccine may be transported through cold chain from the time of manufacture to doorstep of the end users.

Table III.- Effect of storage of foot and mouth disease virus vaccine at 4°C on its potency in rabbit

Sampling time post-priming (n=3)	Time Post-storage of FMD vaccine at 4°C (in days)			
	0	60	120	180
7	2,2,4 (2.7)	2,2,0 (1.3)	2,2,2 (2)	2,0,0 (0.7)
14	8,8,4 (6.7)	4,4,8 (5.3)	4,4,4 (4)	4,8,4 (5.3)
21	16,8,16 (13.3)	16,16,8 (13.3)	8,8,16 (10.7)	16,8,16 (13.3
28	16,32,8 (18.7)	32,16,16 (21.3)	16, 8, 32 (18.7)	16,16,32 (21.3)
35	32,32,32 (32)	32,16,32 (26.7)	32,16,32 (26.7)	32,16,16 (21.3)
42	32,64,32 (42.7)	64,32,32 (42.7)	32,32,16 (26.7)	64,32,32 (42.8)
CGMT*	19.35 [a]	18.44 [a]	14.80 [a]	17.45 [a]
± Sd	±15.36	±15.22	±10.92	±14.95

*CGMT: Cumulative geometric mean titer. Figures in the parenthesis indicate the geometric mean titer of anti-"O" FMD virus CF antibody titer

It is concluded that amount of immunogen per vaccine dose, nature of adjuvant and vaccine storage temperature are critical factors affecting the potency of FMD virus vaccine

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REFERENCES

AARTHI, D., ANANDA, R.K., ROBINSON, R. AND SRINIVASAN, V.A., 2004. Validation of binary ethyleneimine (BEI) used as an inactivant for foot and mouth disease tissue culture vaccine. *Biologicals*, **32**: 153-156.

AJMAL, M., ARSHAD, M. AND AHMAD, M.D., 1989. Epidemiology of major livestock diseases in Pakistan. *Fifth Annual Report*, 1989-90. Pakistan Agriculture Research Council, Islamabad.

ANONYMOUS, 2000. OIE Manual of standards for diagnostic tests and vaccines, pp. 77-92.

BAHNEMANN, H.G., 1975. Binary ethylenimine as an inactivant for foot and mouth disease virus and its application for vaccine production. *Arch. Virol.*, **47**: 47-56.

BARTELING, S.J. AND WOORTMEYER, R., 1984. Formaldehyde inactivation of foot and mouth disease virus. Conditions for preparation of safe vaccine. *Arch. Virol.*, **80**: 103-117.

CHOWDHURY, S.M., RAHMAN, M.F., RAHMAN M.B. AND RAHMAN M.M., 1996. Strains of foot and mouth disease virus in different districts of Bangladesh. *Austral. J. Anim. Sci*., **9**: 315-317.

CLARKE J.B. AND SPIER R.E., 1980**.** Variation in the susceptibility of BHK-population and cloned cell lines to three strains of foot and mouth disease virus. *Arch. Virol*., **63**: 1-9.

DALSGAARD, K., 1987. Adjuvants. *Vet. Immunol. Immunopathol***., 17**:145-152.

DOEL, T.R., 2003. FMD Vaccines. *Virus Res*., **91**: 81-99.

FERRIS, N.P. AND DAWSON, M., 1988. Routine application of enzyme linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot and mouth and vesicular disease. *Vet. Microbiol*., **16:** 201-209.

FRENKEL H.S., 1947. La culture de la virus fievre aphteuse sur l'epithelium de langue des bovides. *Bull. Off. Int. Epizotol*., **28:** 155-162.

FIDA, H., HAMID, A. AND BHUTTA A.I., 1965. Some epidemiological information of foot and mouth disease in W. Pakistan. Proceedings of 17th Pakistan Science Conference, pp. 17-18.

GRAVES, J. H., 1971. Contact transmission of foot and mouth disease from infected to susceptible cattle. *J. Infect. Dis.*, **123**: 386-391**.**

HAQ, M.M., 1961. Epidemiological survey of foot and mouth disease. Proceedings of 13th Pakistan Science Conference, 19-20.

JENNINGS, R., SIMMS, J.R. AND HEATH, A.W., 1998. Adjuvants and delivery systems for viral vaccines: mechanisms and potentials. *Develop. Biol. Stand.,* **92:** 19-28.

KIMURA, J., NARIUCHI, H., WATANABI, T., MATUHASI, T., OKAYASU, I. AND HATAKEYAMA, S., 1978. Studies on the adjuvant effect of water-in-oil-in-water (w/o/w) emulsion of sesame oil. 1. Enhanced and persistent antibody formation by antigen incorporated into the water-in-oil-in-water emulsion. *Japanese J. exp. Med.*, **48:** 149-54.

MEYER, R.F. AND KNUDERSEN, R.C., 2001. Foot and mouth disease: A review of the virus and the symptoms. *J. environ. Hlth*., **64:** 21-23.

MUHAMMAD, K., 1993. In vitro *studies of chicken macrophages and their role in* Salmonella *related immunization failure*. Ph.D. thesis. School of Biological Sciences, University of Surrey, Surrey, U.K.

MURPHY, F.A., GIBBS E.P.J., HORZINEK, M.C. AND STUDDERT, M.J., 1999. *Veterinary virology*. 3rd Ed. Academic Press, New York, pp. 521-527.

OFFICE INTERNATIONAL DES EPIZOOTIES (OIE), 2001. Foot and mouth disease. In: *Manual of standards for diagnostic tests and vaccines*, 4th Ed., OIE, Paris, pp. 77-92.

ROWLAND, R., 2003. *Foot and mouth disease*. Elsevier Australia, pp. 1

RUCCKERT, R.R., 1996. Picornaviridae: the viruses and their replication, In: *Field's virology*, 3rd Ed. Lippincot-Raven, Philadelphia, pp. 609-659.

STEEL, R. G. D., TORRIE, J. H. AND DICKEY, D. A., 1997. *Principles and procedures of statistics: A biochemical approach* (3rd Edi.). McGraw Hill Book Co. Inc., New York, USA

TEKALEKOV, P. AND VELEVA, E., 1985. Binary etyhleneimine as an inactivator of foot and mouth disease virus. *Vet. Med. (Nauki),* **22:** 3-8.

TIZZARD, I.R., 1998. *Veterinary immunology: An introduction.* 5th Ed. Harcourt Brace and Company Asia Pte Ltd. New Delhi, India

TOMAN, M., KREJCI, J., PINKA, K. AND MANSIK, P., 1992. Causes of anaphylactoid reactions in cattle after administration of lipid preparations. *Vet. Med. (Praha),* **37:** 417-426.

TOSH, C., SANYAL, A., HEMADRI, D. AND VENKATARAMANAN, R., 2002. Phylogenetic analysis of serotype A foot and mouth disease virus in India between 1977 and 2000. *Arch. Virol*., **147:** 493-513.

VANIO, O., VEROMAA T., VEROLA, E., TOIVANEN, P. AND RATCLIFFE, M.J.H., 1988. Antigen presenting cell-T-cell interaction in chicken in MHC-Class II antigen restricted. *J. Immunol***., 140:** 2864-2868.

VILLEGAS, P. AND PURCHASE, H.G., 1998. Titration of biological suspensions. In: *A laboratory manual of isolation and identification of avian pathogens*. Edited by The American Association of Avian Pathologists, USA, pp. 248-253.

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