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CpG-DNA enhancement the immune elicited as adjuvant of foot- and- mouth disease vaccine

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ABSTRACT

In the present study the effect of the locally produced genetic adjuvant of ginea pig specific CpG-motif-containing oligodeoxynucleotide (CpG-ODN) in an inactivated FMD virus vaccine was evaluated. Boosting the ginea pigs with FMD vaccine along with CpG-ODN adjuvant produced relatively higher ratio (5-fold) of FMDV-specific IgG_{2a} / IgG_1 than those vaccinated in the absence of CpG-ODN. The neutralizing antibody (NA) titer induced by FMD vaccine along with CpG-ODN adjuvant was significantly higher (8-fold) than NA titer induced by the classical FMD vaccine in Alum adjuvant. The titer of NA and virus clearance from serum was consistently and significantly higher in animals primed with FMD vaccine and boosted by CpG-ODN than the classical FMD vaccine. The results of this study showed the potential of CpG-ODN as a genetic adjuvant to FMD vaccine in the development of Th1 responses.

Keywords: FMD vaccine, CpG-ODN, Adjuvant, Ginea pig

INTRODUCTION

For years, local and commercial cattle raisers have been incurring considerable economic losses because of disease outbreaks. Foot and Mouth Disease (FMD) is one of the most prevalent and economically important disease affecting livestock, in all provinces of Iran. FMD is extremely contagious, febrile and acute disease of all cloven – hoofed animals caused by Ophthovirus in Picornaviridae family. There are three serotype (A, O, C) of this virus with several numbers of serologically distinct subtypes each with different degrees of virulence (Type C is almost eradicated from the globe). Serotypes SAT1, SAT_2, SAT_3 have been isolated in Africa and one serotype, Asia1 from Asia (Radostits *et al* 2007). A structural feature of the outer capsid surface is a long, conformationally flexible loop of the VP1 protein (Sobrino *et al* 1989, Xie *et al* 1987). This loop, namely G-H loop, forms a major antigenic site on the virus and includes at its apex an Arg – Gly – ASP motif (Strohmaier *et al* 1982, Xie *et al* 1987) amino acids in this loop contain both immune-dominant T and B epitopes that can elicits neutralizing antibody (NA) response (Logan *et al* 1993, Parry *et al* 1989). The abundant efforts has been carried out to determine the potential of using peptides, particularly those containing the amino

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acid sequence of the G-H loop, for the vaccination of naturally susceptible animals. Although this approach has yielded humoral immunity against challenge virus in some experimental animals (Brown 1995, Huang *et al* 1999), its protection has not been satisfactory used as traditional vaccine (Mulcahy *et al* 1990, Taboga *et al* 1997). During the past decade, genetic immunization with naked DNA has been shown to induce long-lived humoral and cellular immune responses in several kinds of animal models (Guranathan *et al* 2000 a, b), suggesting the potential of applying this design for vaccination. Recently, some DNA adjuvant such as oligodeoxynucleotides (ODN) that contain the unmethylated cytosine– phosphate–guanosine (CpG) motif have been applied to induce Th1 responses as distinguished by the secretion of INF–γ, TNF– β, IL –12 and IgG2α (Chu *et al* 1997, Roman *et al* 1997). This kind of DNA adjuvant may thus offer significant advantages in supplementing the deficiency of vaccines that elicit mainly Th2 responses. In the present study, we have evaluated, whether using of CpG ODN followed by traditional FMDV vaccine, has any advantages on enhancement of immune responses and protection against FMDV in ginea pig.

MATERIALS AND METHODS

Animals. A total number of 48 healthy ginea pigs, 6 to 8-month old were allocated to four treatment groups, each consisting of 12 animals. The ginea pigs were raised up to 24 weeks of age at the College of Veterinary Medicine, Department of Immunology, Urmia University, Iran.

Vaccines. Traditional inactivated FMD vaccine (A/Iran/87) (Razi Vaccine Research Institute, Iran) containing 10^8 particles/ml with or without 5% alum (Aluminum hydroxide gel) as adjuvant was used. Each animal received 100µl (10^7 particles) for immunization.

Adjuvant. CpG ODN (5′ – GCT AGA CGT TGA CGT TCA CT – 3′) (Rankin *et al* 2001) was synthesized (CinaGen, Iran) and was used as adjuvant. The molecular weight of CpG was 6043 with concentration of 1253 µg/ml.

Ginea pigs immunization. Ginea pigs were anesthetized and injected subcutaneously in the chest area with either FMD–vaccine/alum alone and or FMD–vaccine along with CpG ODN as follows: group A, received only FMD–vaccine/alum in both priming and boosting stages; group B, primed with FMD–vaccine along with 40 µl (50 µg) CpG/animal and boosted with FMD–vaccine/alum at day 14, 28 and 42 after first injection; group C, primed with FMD–vaccine/alum and boosted with FMD–vaccine along with CpG (50 µg) at day 14, 28 and 42 after the first injection; group D, was remained as naïve control. Blood was taken at one week after the last inoculation and collected sera were kept at –70 °C.

Measurement of anti – FMDV antibodies by ELISA. To determine the titer of IgG1 and IgG2a antibodies, the FMDV antigens coated micro plates (Svanova diagnostics, Sweden) were used. Serum samples in serial dilution (10^{-2} to 10^{-6}) were added to the wells (100 µl/well) and incubated for 1 h at 37 ºC. Plates were then washed 3 times and treated with 100 µl/well of peroxidase conjugated horse anti –ginea pigs IgG1 and IgG2a Abs (Sigma Aldrich, Germany) for 1h at 37°C. The plates were washed and enzyme substrate (100 µl/well) was added and incubated at room temperature for 20 min. Finally, 50 µl of 1M H_2So_4 was added to each well to stop the reaction and the absorbance values were read at 450 nm by an ELISA reader (Denly, well Scan). The OD of highest dilution of each sera that was 2.5 time bigger than OD of negative control serum considered as the end point titer (Shieh *et al* 2001).

Assay of neutralizing Abs titer. Serum samples were diluted (1:4) and inactivated at 56 °C for 30 minutes. 100 µl of each serum sample in duplicate

was put in the wells at the rows A and B of a 96-well tissue culture plate and serial dilutions were made from B to H rows (1:4 to 1:512). Then 50 µl of 100 $TCID_{50}$ FMD virus suspension were added to each well, and the plate was incubated at 37°C for 90 min. After incubation, 100µl of 10^6 BHK-21 Cells/ml suspension in Eagle's MEM containing 5% fetal calf serum (FCS) were added to each well and incubated at 37 °C in a water saturated atmosphere with 5% CO_2 for 48 hours. The reciprocal of the final serum dilution that can induce 80% inhibition from cytopathic effect (CPE) formation considered as NA titer in serum (Shieh *et al* 2001).

Challenging ginea pigs with live virus. All groups of immunized ginea pigs and control group were challenged intra peritoneally with 10^6 $TCID_{50}$ virus A/Iran/87 strain. Blood was collected from animals at 48 and 72 h after challenge. 100 µl of each blood sample was added to BHK-21 cell culture and incubated with gentle rocking at 37°C for 60 min. The cell sheets were washed twice with Hank's medium and incubated with Eagle's medium containing 2% fetal calf serum for 3 days in the presence of 5% CO_2. The presence of virus in the blood of the ginea pigs was determined by the observation of CPE in BHK-21 cell cultures. Virus clearance was determined by the absence of the virus in the blood of the ginea pigs at 48 and 72 hours after challenge.

Assessment of IFN-γ and IL-4 levels in the sera. The amount of IFN-γ and IL-4 cytokines in sera of the ginea pigs was determined by sandwich ELISA using in vivo capture assay. In this assay, normal and experimentally treated ginea pigs were injected intravenously with 10 µg/animal of biotin–conjugated anti-ginea pig IFN-γ and IL-4 Abs (Pharmingen ,USA) in 200 µl PBS, 48 h after the last vaccination. Blood samples were collected 48 h post injection and sera were prepared. The micro plate wells were coated with 50 µl of anti–ginea pig IFN-γ and IL-4 (2 µg/well), separately incubated overnight at 4 °C, and blocked non-specific binding with 1% gelatin in PBS-Tween (200 µl/well). After 1h incubation the plates were washed 3 times. The standard complex was made by mixing 1ng of recombinant ginea pig IFN-γ and IL-4 with 0.4 µg of biotin-anti-ginea pig IFN-γ and IL-4 Abs, in a final volume of 1 ml of diluents solution for preparing 1000 pg/ml standard. Serial dilutions were made from these standards for preparing 500 to 5.6 pg/ml standards, and add 50 µl/well in duplicates to the rows A and B each plate. The diluents solution used as the zero standard. Serum samples from control and treatment groups were diluted (1:5) and added to micro plate wells in duplicates (50 µl/well) and incubated for 2 hour at room temperature. After washing three times, peroxidase-streptavidin (Sigma, Germany), (1:1000) was used as the conjugate and O-phenylen diamine (Pharmingen, USA) as the substrate. The optical density (OD) was read at 450 nm using an ELISA reader and the amounts of cytokines were obtained from the standard curve.

Data analysis. Data was analyzed by using of Minitab statistical package (Version15, Minitab Inc.)

RESULTS

Levels of anti – FMDV, IgG_1 and IgG_{2a} Abs in the sera. The ginea pigs inoculated with FMD–vaccine/alum in both priming and boosting stages (group A) showed the geometric mean IgG1 titer of 70000and IgG2a of 3000, while group B that were primed with FMD–vaccine/CpG and boosted with FMD–vaccine/alum had IgG1 titer of 50000 and IgG2a of 7000. In contrast, when priming was performed with FMD–vaccine/alum followed by boosting with FMD–vaccine/CpG (group C), the titer of IgG1 decreased to 40000 and IgG2a increased to 9000. However, these differences were not statistically significant between the amounts of IgG1 in different groups ($p>0.005$). The amount of

IgG2a in B and C groups increased by about 2.30 and 3-fold (P<0.005) respectively in comparison with group A (Table 1).

while in group B, that received CpG ODN *in* priming stage, only 91% of animals (11/12) were virus free.

Table 1. The titres of ELISA and NA against FMDV in gineapigs immunized with FMD – vaccine along with or without CpG-ODN motif

Gineapigs groups	Gineapigs immunized with:		ELISA titre(Log10)		NA titre*
	Priming	Boosting	IgG1	IgG2a	
A	vaccine / alum	vaccine / alum	70000(4.7)	3000(3.3)	14.3
B	vaccine / CPG-ODN	vaccine / alum	50000(4.5)	7000(3.7)	49.3
C	vaccine / alum	vaccine / CPG-ODN	40000(4.4)	9000(3.9)	117.3
D	None	None	50 (1.5)	20(1.2)	<2

*Each values are mean of data obtained from 12 animals.

Table 2. Clearance of virus from the sera in the gineapigs challenged with live virus.

Gineapigs groups	Gineapigs immunized with:		Virus clearance [a]
	Priming	Boosting	
A	vaccine / alum	Vaccine / alum	10/12 (83.30)
B	vaccine / CPG ODN	Vaccine /alum	11/12 (91.60%)
C	vaccine / alum	Vaccine / CpG ODN	12/12 (100%)
D	None	None	0/12 (0 %)

a. Expressed as the ratio of viremia free gineapigs/ number of challenged gineapigs.

Anti–viral neutralizing Abs (NA) titer. The mean of NA titer in group A, treated solely with FMD–vaccine/alum was 14.30, while group B, treated with FMD–vaccine/CpG priming, followed by FMD–vaccine/alum boosting induced NA titer more than 3-fold higher than group A (P<0.005).

In contrast, primed ginea pigs with FMD – vaccine/alum followed by FMD–vaccine and boosted with CpG ODN induced a titer of 117.30, more than eight fold higher than group A (P < 0.005) and more than three fold higher than group B. These differences were statistically significant.

Virus clearance in ginea pigs challenged with live virus. The data showed that all animals (12/12) immunized with FMD–vaccine along with CpG ODN during boosting stages were apparently virus free 48 hours after challenging with live virus, only 91% of animals (11/12) were virus These animals had no any clinical symptoms, on the plantar pad, till four days after challenge. On the other hand, 17% of animals (2/12) primed and boosted with FMD–vaccine/alum still had detectable levels of virus in their sera as it produced CPE in BHK-21 cells. The naïve ginea pigs exhibited viremia within 48 hrs after challenging with the same dose of live virus (table 2). These animals also showed multiple vesicles on their plantar pad within 2-3 days and vesicle in their mouth one day later after challenging.

Assessment of the cytokine production in ginea pigs treated with CpG ODN. To evaluate the effect of CpG ODN on cytokine production during priming and boosting stages, the serum of ginea pigs were examined for the level of IFN-γ and IL-

4. The IFN-γ level in groups A, B and C was 500, 950 and 1750 pg/ml respectively (Figure 1). The amount of IL-4 in group A was 285 pg/ml, significantly differed from the amount of IL-4 induced in groups B, C and D which were 120, 110 and 20 pg/ml respectively(P<0.005) (Figure 1).

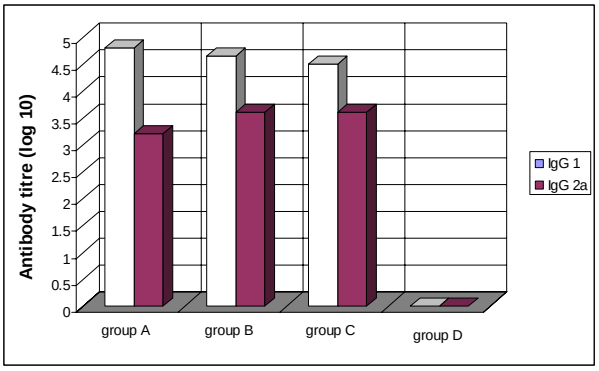

Figure 1. The IFNγ and IL-4 levels in 4 groups of gineapigs correlation with vaccination pattern. Open bars represent levels of IFN-γ and closed bars represent IL-4 levels ±SEM.

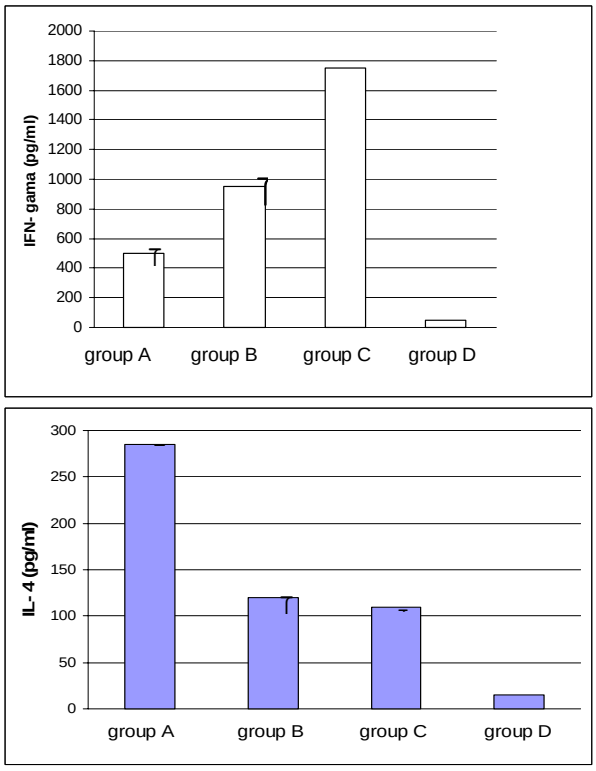

Figure 2. The IFNγ and IL-4 levels in 4 groups of gineapigs correlation with vaccination pattern. Open bars represent levels of IFN-γ and closed bars represent IL-4

DISCUSSION

In this study, we found that the gineapigs immunized with FMD–vaccine/alum (group A) in both priming and boosting stages produced a significant amounts of anti-FMD IgG1 (Mean titer: 70000) and low titers of neutralizing Abs (Mean titre: 14.30) however, 83% of these animals (10/12) only resulted in clearance of virus from the serum in the challenging experiments (Tables 1 and 2). Priming gineapigs with FMD–vaccine/CpG and boosting with FMD–vaccine/alum (group B), on the other hand, showed that although this treatment did not affect significantly the IgG1 titer (mean titer: 50000), but increased the ratio of anti– FMDV IgG2a/IgG1 more than 3-fold (Table 1). In this group, also the titre of neutralizing Ab elevated by about 3-fold (mean titer: 49.30) (Table1), and the virus clearance reached about 91% of the animals (11/12) comparing to group A (Table 2). Interestingly, when CpG was used during boosting stage (Group C), it showed that although the titer of anti-FMD IgG1was decreased (mean titer: 40000), it increased the anti-FMD IgG2a titer by about 3-fold (mean titer: 9000), as the ratio of IgG2a/IgG1, reached about 5-fold. The neutralizing Ab titer in this group (mean titer: 117.30), was elevated to 8-fold (P<0.005) than those vaccinated without using CpG ODN (Table 1). Furthermore the amount of IFN-γ significantly increased (1750 pg/ml) (P<0.005) while IL-4 was at low amount by 110 pg/ml in this group, which significantly differed (p<0.005) from the amountof IL-4 (285 pg /ml) induced in group A (Figure 1). Because, when activated Th1 cells secrete IFN-γ, it stimulates B cell production of IgG2a but lowers production of the other immunoglobulin subclasses. On the other hand, IFN-γ also inhibits the production of IL-4 by Th2 cells (Tizard 2004). This treatment (using of CpG ODN) also caused more cell–mediated immunity as the serum was completely cleared from the virus. In this study, we observed that ginea pigs

treated with CpG ODN during vaccine boosting produced higher titers of neutralizing antibodies than those without CpG ODN. This finding is consistent with the previous report (Shieh *et al* 2001), that presence of CpG ODN along with vaccine boosting produced more than 13-fold higher NA than those primed and boosted without CpG ODN. In another study, it was showed that applying a combination of cholera toxin with the immunostimulatory CpG ODN as adjuvant in a peptide FMD vaccine significantly enhanced the anti–virus neutralization titers and induced both IgG1 and IgG2a in the serum (Beignon *et al* 2005), which it was an indicative of a mixed Th_1 – Th_2 responses. It was concluded that the NA was positively correlate with the ratio of IgG_{2a} / IgG_1 but not the levels of total IgG or IgG_1. Since IgG_{2a} formation is typical for a Th_1 response and IgG1 production is a Th2 response (Mosmann & Coffman, 1989), our findings suggest that using CpG ODN during priming or preferentially boosting stage of immunization may stimulate a selective Th1 immune response that persists upon next challenge with FMD vaccine. CpG ODN has been shown to elicit cell–mediated immune response that is characterized by the secretion of IL-12 from dendritic cell1; and IFN-γ, TNF-β and IL-2 from Th1 after stimulation by Ag. and costimulation by IL-12(Carson & Raz 1997, Constant & Bottomly 1997, Halpern *et al* 1996, Kim *et al* 2000, Klinman *et al* 1999, Klinman *et al* 1997, Klinman *et al* 1996, Lipford *et al* 1997). Moreover, CpG ODNs can serve as an efficient genetic adjuvant in the induction of protective T-cell immunity against infection with lymphocytic choriomeningitis virus recombinant vaccina virus (Oxenius *et al* 1999) and also with VP1 peptide conjugate of FMD virus (Shieh *et al* 2001). In this study, it was revealed that ginea pigs treated with CpG ODN along with FMD–vaccine boosting, produced higher amount of IFN-γ and IgG2a than those did not receive CpG ODN (Figure 1). These findings indicate that CpG ODN can be a potent adjuvant for FMD-vaccine favoring the development of Th1 responses. In conclusion, the results of the present study demonstrates that using CpG ODN as an genetic adjuvant along with FMD–vaccine in boosting immunization can increase FMDV specific IgG2a response and significantly elevate the titers of NA. It also can produce high level of IFN-γ and complete clearance of ginea pigs serum from live virus.

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References

Beignon, A.S., Brown, F., Eftokhari, P., Kramer, E., Briand, J-P., Muller, S., Partidos, C.D. (2005). A peptide vaccine administered transcutaneously together with cholera toxin – elicits potent neutralizing anti-FMDV antibody responses. *Veterinary Immunology And Immunopathology* 104: 273-280.

Brown, F. (1995). Antibody recognition and neutralization of foot-and mouth disease. *Seminar of Virology* 6: 243-8.

Carson, D.A., Raz, E. (1997). Oligonucleotide adjuvants for T helper 1 (Thl) specific vaccination. *Journal of Experimental Medicine* 186: 1621-2.

Chu, R.S., Targoni, O.S., Krieg, A.M., Lehmann, P.V., Harding, C.V.(1997). CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Thl) immunity. *Journal of Experimental Medicine* 186: 1623-31.

Constant, S.L., Bottomly, K. (1997). Induction of Thl and Th2, CD4+ T cell responses: the alternative approaches. *Annual Review of Immunology*15: 297-322.

Guranathan, S., Klinman, D., Seder, R.A.(2000a). DNA vaccines: immunology, application and optimization. *Annual Review of Immunology* 18: 927-74.

Guranathan, S., Wu, C.Y., Freidag, B.L., (2000b). DNA vaccines: a key for inducing long-term cellular immunity. *Current Opinion of Immunology* 12: 442-7.

Halpern, M.D., Kurlander, R.J., Pisetsky, D.S.(1996) Bacterial DNA induces marine interferon-gamma production by stimulation of IL-12 and tumor necrosis factor-alpha. *Cell Immunology* 167: 72-8.

Huang, H., Yang, Z., Xu, Q., Sheng, Z., Xie, Y., Yan, W., You, Y., Sun, L., Zheng, Z.(1999). Recombinant fusion protein and DNA vaccines against foot-and-mouth disease virus infection in guinea pig and swine. *Viral Immunology* 12: 1-8.

Kim, T.S., Kim, K.M., Shin, B.A., Hwang, S.Y. (2000). Efficient induction of an antigen-specific, T helper type 1 immune response by interleukin-l2-secreting fibroblasts. *Immunology*100: 203-8.

Klinman, D.M., Barnhart, K.M., Conover, J. (1999). CpG motifs as immune adjuvants. *Vaccine*17: 19-25.

Klinman, D.M., Yamshchikov, G., lshigatsubo, Y.(1997). Contribution of CpG motifs to the immunogenicity of DNA vaccines. *Journal of Immunology* 158: 3635-9.

Klinman, D.M., Yi, A.K., Beaucage, S.L., Conover, J., Krieg AM. (1996). CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proceeding of National Academic Sciences, USA* 93: 2879-83.

Lipford, G.B., Bauer, M., Blank, C., Reiter, R., Wagner H., Heeg, K. (1997). CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvauts. *European Journal of Immunology* 27: 2340-4.

Logan, D., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Jackson, T., King, A., Lea, S., Lewis, R., Newman, J., Parry, N. (1993). Structure of a major immunogenic site on foot-and-mouth disease virus. *Nature* 362(6420): 566-8.

Mosmann, T.R., Coffman, R.L. (1989). Th_l cell and Th_{-2} cell-different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology* 7: 145-73.

Mulcahy, G., Gale, C., Robertson, P., Iyisan, S., DiMarchi, R.D., Doel, T.R. (1990). Isotype responses of infected, virus-vaccinated and peptide vaccinated cattle to foot-and-mouth disease virus. *Vaccine* 8: 249-56.

Oxenius, A., Martinic, M.M., Hengartuer, H., Klenerman, P. (1999). CpG containing oligonucleotides are efficient adjuvants for induction of protective antiviral immune responses with T-cell peptide vaccines. *Journal of Virology*73: 4120-26

Parry, N.R., Barnett, P.V., Ouldridge, E.J., owlands, D.J., Brown, F. (1989). Neutralizing epitopes of type O foot-and-mouth disease virus. II. Mapping three conformational sites3with synthetic peptide reagents. *Journal of Genetic Virology* 70: 1493-50.

Radostits, O.M., Gay, C.C., Hinchcliff, K.W., Constable, P.D. (2007). Veterinary Medicine, 10th. Edn., Saunders Elsevier, Oxford, Pp:1223-30.

Rankin, R., Pontarolo, R., Ioannou, X., Krieg, A.M., Hacker, R., Babiuk, L.A.(2001). CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense Nucleic Acids Drug Development* 11 (5), 3330- 3340.

Roman, M., Martin-Orozco, E., Goodman, J.S., Nguyen, M.D., Sato, Y. (1997). Immunostimulatory DNA sequences function as T helper- l-promoting adjuvants. *National Medicine* 3: 849-54

Shieh, J.J., Liang, C.M., Chen, C.Y., Lee, F., Jong, M.H., Lai, S.S., Lian, S.M. (2001). Enhancement of the immunity to foot – and – mouth disease virus by DNA priming and protein boosting immunization. *Vaccine* 19: 4002-4010.

Sobrino, F., Martinez, M.A., Carrilo, C., Beck, E. (1989). Antigenic variation of foot-and-mouth disease virus of serotype C during propagation in the field in mainly restricted to only one structural protein (VPI). *Virus Research* 14: 273-280.

Strohmaier, K., Franze, R., dam, K.H. (1982). Location and characterization of the antigenic portion of the FMDV immunizing protein. *Journal of Genetic Virology* 59: 295-306.

Taboga, O., Tami, C., Carrillo, E. (1997). A large-scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of escape mutants. *Journal of Virology* 71(4): 2606-14.

Tizard Ian R.,Schubot Richard M., 2004. Veterinary Immunology: An Introduction, 7th edition, WB Saunders, USA, Pp: 105-116 and 132-134.

Xie, Q.C., McCahon, D., Crowther, J.R., Belsham, G.J., McCullough, K.C. (1987). Neutralization of foot-and-mouth disease virus can be mediated through any of at least three separate antigenic sites. *Journal of General Virology* 68: 1637-47.